Quality Control and Evaluation of Herbal Drugs

Evaluating Natural Products and Traditional Medicine

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Preface

The spectacular growth in the market for herbal medicinal products is one of the most interesting aspects of healthcare throughout the world. The rediscovery of natural substances with therapeutic potential has raised questions of quality, safety, and efficacy on the part of the consumer and also from health professionals. The majority of natural substances used in this way consist of plant parts or simple extracts. Such materials have been used in medical practices in most parts of the world for millennia, and this has resulted in a long and rich heritage in many cultures, which is now being shared worldwide as a part of globalization.

The development of herbal drugs requires integration in various fields of science and technology with a major emphasis on quality control and standardization. Medicinal plants and extracts have been utilized in the ancient practices of traditional medicine in many countries, including China (TCM), India (Ayurveda, Siddha, Unani, Homeopathy), Korea, Japan, Tibet, South America, the Middle East, and Russia, among others. These codified traditional medicines and uncodified folk medicine practices have immense importance in healthcare worldwide for the prevention and treatment of various acute and chronic ailments.

The standardization and quality control of herbal drugs are crucial for the production, evaluation, regulation, and safety surveillance of plant-made pharmaceuticals. This book intends to bring together current thinking and practices in these areas and also to highlight several facets of the research, which will facilitate improvement in the topics under consideration.

In the light of increasing legislation to enforce better standards for herbal products and the public demand for assurance of their safe and effective use, this publication seeks to provide state-of-the-art reviews that present information and guidance for the promotion and development of natural products.

This book elucidates various challenges and opportunities for quality evaluation and quantitative analysis of herbal drugs, including marker analysis and stability testing. Some of the topics highlighted include traditional systems of medicine, harmonization, ethnopharmacology- and ethnomedicine-inspired drug development, morphological and microscopic evaluations, and bioactive phytocomponents and their analysis. Dedicated chapters cover classical pharmacognosy along with other topics such as DNA bar-coding and chemometric methods for identification involving HPLC, TLC, HPTLC, and LC-MS/MS.

This comprehensive volume has specific chapters on the methods for screening pharmacological activities, on safety, and on anti-microbiological and antiviral activities along with the mechanisms of action useful for the evaluation of herbal medicines. Plant metabolomics and the safety-related quality issues of phytopharmaceuticals and nutraceuticals also find detailed discussion in this book.

I hope it will be a very useful treatise, which will not only serve as a handy tool for students and researchers in this area but will also be useful to technocrats in industry and to legislators who want to know more about the methodologies developed for the quality evaluation of herbal drugs in metabolomics, chromatographic studies, and the evaluation of safety and efficacy.

In issues of emerging interest, this book covers the regulatory harmonization of nutraceuticals and traditional medicine, as well as the role of ethnopharmacology in global healthcare generally. This volume also covers the trends in quality assurance and good practices in agriculture, collection, manufacturing, and clinical aspects, which are very crucial for the quality standardization of herbal drugs. This book provides articulate enumeration of various aspects of the quality assurance of herbal medicines, which will help chemists and manufacturers to know more about the validation strategies for evaluating traditional medicines, particularly for their safety and efficacy.

The main aim of this book is to elevate the level of understanding of the quality evaluation of natural products so that they can be practiced with improved quality, raising a scientifically sound evidence base for herbal medicine. The text is intended to present both the technical and theoretical backgrounds of different aspects of the evaluation of herbal medicinal products. It will also be an imperative essential reference for students and professionals, and for those involved in the fields of herbal medicine, traditional remedies, pharmaceutical sciences, and natural product research.

I hope this book, aimed at a global readership, will provide updated guidance on the required structured approach for quality control and evaluation of herbal drugs to all the interested stakeholders in this field.

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Foreword

GOOD AFFORDABLE EVIDENCE-BASED (HERBAL) MEDICINES FOR EVERYONE

The past century was the era of drug development based on synthesis of organic molecules, often based on natural products as a model. This resulted in a wide range of medicines mostly for the symptomatic treatment of diseases, although only a few medicines really cure the patient. It has been said that "The vast majority of drugs—more than 90%—only work in 30% or 50% of the people" (Allen Roses, vice-president of genetics at GlaxoSmithKline). These are just some of the factors that have led to the abandoning of drug development based on the "single compound–single target" approach. Instead, a more systemic approach is imminent. Twenty-first century science has introduced systems biology as a novel approach, in which observing the whole system is the key. This approach dates back to our ancestors who found all medicinal plants without any of the present-day tools: curare, opium, Artemisia, and many others—no pharmacology, no pharmacognosy, no medicinal chemistry; they were possibly found by just trial and error. This means that observation-based approaches have led to the discovery of approximately 30,000–70,000 plant species for various medical uses.

Now we have all kind of tools to measure (observe) things that our ancestors could not observe. These new tools help us study the whole system using "omics" and physiological and pharmacological tools. By analyzing these results using novel algorithms, new hypotheses can be generated and subsequently be tested in the classical hypothesis-driven science. This will provide evidence for the safe use of traditional medicines and will also be a source of novel leads for drug development. In particular the polypharmacy aspects of traditional medicines, including synergy, will be of great interest for developing novel therapies. Obviously, these techniques will also result in the identification of a number of plants whose risk–benefit ratio does not favor any further medical applications. A major driver in this process of changing paradigms of drug development is the increasing interest in the well-documented Asian medical systems. With thousands of years of written information, there is a wealth of knowledge that may be turned into evidence-based traditional uses, and may even result in new leads for drug development. The 2015 Medicine and Physiology Nobel Prize for the discovery of artemisinin and an antibiotic against river blindness is a recognition of biodiversity and traditional knowledge as important resources for drug development. Interestingly this recognition comes from western medical sciences, which "traditionally" were quite reluctant to accept traditional medicine as an alternative to global medicine, possibly owing to the lack of awareness that the roots of all medical systems lie in the knowledge of our ancestors.

The changing global economy is a further driver for studying traditional medicinal (herbal) treatments. The exponential growth in the quality and quantity of the research in this field is now setting the scene for studies of medicinal plants. The present book deals with all these historical aspects, in which the same problems have to be solved to assess and learn from all the different traditional medical practices. It brings together current thinking and practices in the area of quality control and standardization of herbal drugs. The major problem is to develop evidence-based traditional medicines whose toxicity is known and efficacy is proven. Once this problem has been overcome, the next step is to ascertain the production and distribution of good quality traditional medicines. With proven activity, at least the chemical profiles of the extract and/or plant needs to be correlated with activity to ensure proper quality management and quality control. This is a major challenge, as crude extracts might be contaminated with many things like adulterants, substituents, wrongly identified plants, mycotoxins, agrochemicals, and heavy metals. The methods for ensuring quality are many, and the costs for quality control can be enormous, which could become a major bottleneck in the development of evidence-based traditional medicines. Quality management, which refers to the identification of the critical steps in the chain from seed to plant, could contribute to keep these costs low, that is, the introduction of good practices in the whole chain from seed to patient. It requires quality control using simple techniques that do not necessarily depend on expensive advanced equipment. The equipment will be useful to define quality, but should eventually lead to the development of simple methods for quality control.

This book provides an overview of all these aspects of quality control for herbal medicinal plants (right from simple physicochemical tests, morphology, phytochemical tests to chemo-profiling and marker analysis for quality evaluation of herbal drugs, HPTLC, HPLC, LC-MS/MS to high-end hyphenated techniques such as chemometrics for validation of traditional medicines), presents avenues for traditional medicine-inspired drug discovery, and will be an important guide for further studies on evidence-based traditional medicines. I appreciate the work done by Prof. Pulok Kumar Mukherjee, a prolific author in this field and a major contributor in the area of ethnopharmacology and ethnomedicine, which will help students understand the complete picture of all the pharmaceutical aspects of developing herbal medicines. This book will also be helpful for experts to learn from other experts about the multidisciplinary approach required to achieve the final goal of "Good affordable evidence-based medicines for everyone."

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Acknowledgments

The resurgence of interest in herbal medicine has augmented its role in global healthcare. There has been an enormous growth in herbal medicinal products worldwide, creating a paradigm shift in healthcare. The quality of herbal medicine, including its safety, efficacy, validation, and regulation, can be best explored through interdisciplinary sciences.

The main aim of this book is to describe and discuss different approaches and techniques for evaluating the quality and efficacy of herbal medicines, particularly methods to assess their activity and to understand the metabolomic aspects and their underlying mechanisms of action.

My entire research group at the School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India owe a special thanks for their enthusiasm, creativity, and efforts toward this book. I gratefully acknowledge the support rendered by my research scholars, particularly Mr. Subhadip Banerjee, Mr. Amit Kar, Mr. Shiv Bahadur, Mr. Joydeb Chanda, Mr. Debayan Goswami, Mr. Sayan Biswas, Mr. Bhaskar Das, Ms. Akanksha Sharma, Mrs. Kasturi Basu, Ms. Seha Singha, Mr. Shibu Narayan Jana, Mr. Milan Ahmed, and Mr. Prasanta Kumar Maitra, and others for their active cooperation and assistance.

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My father, late Harihar Mukherjee, a man I intensely admire, has always inspired me in my work and he continues to live in my heart.

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Chapter 1

Traditional Systems of Medicine and Harmonization

Chapter Outline

1.1 TRADITIONAL MEDICINE IN HEALTHCARE

Traditional medicine (TM) refers to the knowledge, skills, and practices that are nurtured from the beliefs and age-old experiences of indigenous people from different cultures that are used to maintain health and to prevent and treat different ailments. Other terms for TM are complementary, alternative, and/or nonconventional medicine. The World Health Organization (WHO) notes that traditional and complementary medicine (T&CM) is a component of the worldwide socio-global healthcare framework. Considering its importance, WHO has prescribed legislative frameworks, which should be incorporated to promote health and wellness. WHO also recommends the development of regulation, research, education, marketing and training to promote health and wellness. In October 2013, WHO published the "WHO Traditional Medicine Strategy 2014–2023" to oversee the needs, controls, and administration of traditional medicine inside their own country (WHO, 2013a, b).

Archaeological evidence reveals that the consumption of medicine has a long history (Randive, 2010). By necessity, the drugs used in ancient civilizations were extracts of plants or animal products, with a few inorganic salts. In India, the Ayurvedic system of medicine developed an extensive use of medicines from plants dating from at least 1000 BC. The earliest Chinese records give descriptions of diseases but not medicines; illnesses were thought to be godly punishments and they were treated by prayers and offerings. The earliest recorded Chinese prescription after about 500 BC shows the beginning of the use of natural products as drugs. The first classic texts in Chinese medicine appeared in AD 25–220, and some of their formulae remain in use today (Huang, 1984; Hikino, 1989). Similarly, the Egyptian Ebers papyrus (around 1550 BC) contains descriptions of several active ingredients (notably purgatives) that are still used today (Panda, 2010).

TM alludes to health practices, methodologies, wisdom, and principles, incorporating plant-, animal-, and mineral-based medications, spiritual treatments, manual strategies and activities, connected independently or in combination, with the aim to treat, analyze, and counteract ailments or to promote wellness. Nations in Africa, Asia, and Latin America utilize TM to meet some of their essential health needs. In Africa, up to 80% of the population utilizes T&CM for essential medicinal services. In industrialized nations, the practice of TM is called "Complementary" or "Alternative" Medicine (CAM).

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The use of TM has rapidly spread across industrialized nations. In China, Traditional Chinese Medicine (TCM) represents 30%–50% of the total healthcare arena. In Ghana, Mali, Nigeria, and Zambia, the principal line of treatment for 60% of children with ailments, such as high fever, is the use of herbal medications as home remedies. In Europe, North America, and other industrialized areas, more than half of the population has utilized T&CM at least once. In San Francisco, London, and South Africa, 75% of individuals living with HIV/AIDS utilize TM and/or CAM. In Canada, 70% of the populace has utilized integrative medicine at least once, while in Germany 90% of the population has utilized a complimentary cure at some point in their life (WHO, 2002). The worldwide market for herbal medicines presently remains at over US $60 billion yearly and is growing consistently (WHO, 2013a, b). Scientific evidence from randomized clinical trials is strong for the use of medicinal plants in several therapies, including acupuncture. Further research is needed to ascertain the safety and efficacy of several other practices and uses of medicinal plants.

Unregulated or incorrect utilization of TM and practices can have negative, even perilous impacts. For example, the herb "Mama Huang" (*Ephedra*) is customarily used to treat respiratory problems. In the United States, the herb was advertised as a dietary supplement, but it caused deaths, heart attacks, and stroke. In Belgium, individuals required renal transplants or dialysis for interstitial fibrosis of the kidney after taking an herbal medication for weight loss treatment, which was produced using the wrong type of plant (WHO, 2013a, b). In spite of persistent safety issues, the herbal market is developing and its business growth may represent a risk to biodiversity through the over collection of crude material for herbal pharmaceuticals. These practices, if not controlled, may prompt the annihilation of jeopardized species and the destruction of the environment and ecological assets. Another related issue is the requirement for insurance in international standards for patent law, which does not consider biodiversity.

While countries, including India, China, Korea, Thailand, and Vietnam, have incorporated TM into their medicinal service frameworks, several nations have yet to gather and incorporate institutionalized proof of this kind of healthcare system. Traditional systems of medicine in different countries are presented in Fig. 1.1. In spite of the fact that about 70 nations have a national policy on herbal medicine, the administrative control of medicinal plants has not been developed in an organized way. This is because therapeutic items or herbs are characterized differently in various nations and assorted methodologies are used to license, dispense, manufacture, and trade. Hence, it is imperative for governments of all countries to have a national policy and to ensure the correct utilization of TM/CAM into their national health service frameworks in accordance with the arrangements of the WHO methodologies for TMs. Consideration should be given to administrative components to control the safety and nature of products and improve understanding of protected and successful TM/CAM treatments among the general society and consumers (WHO, 2013a, b).

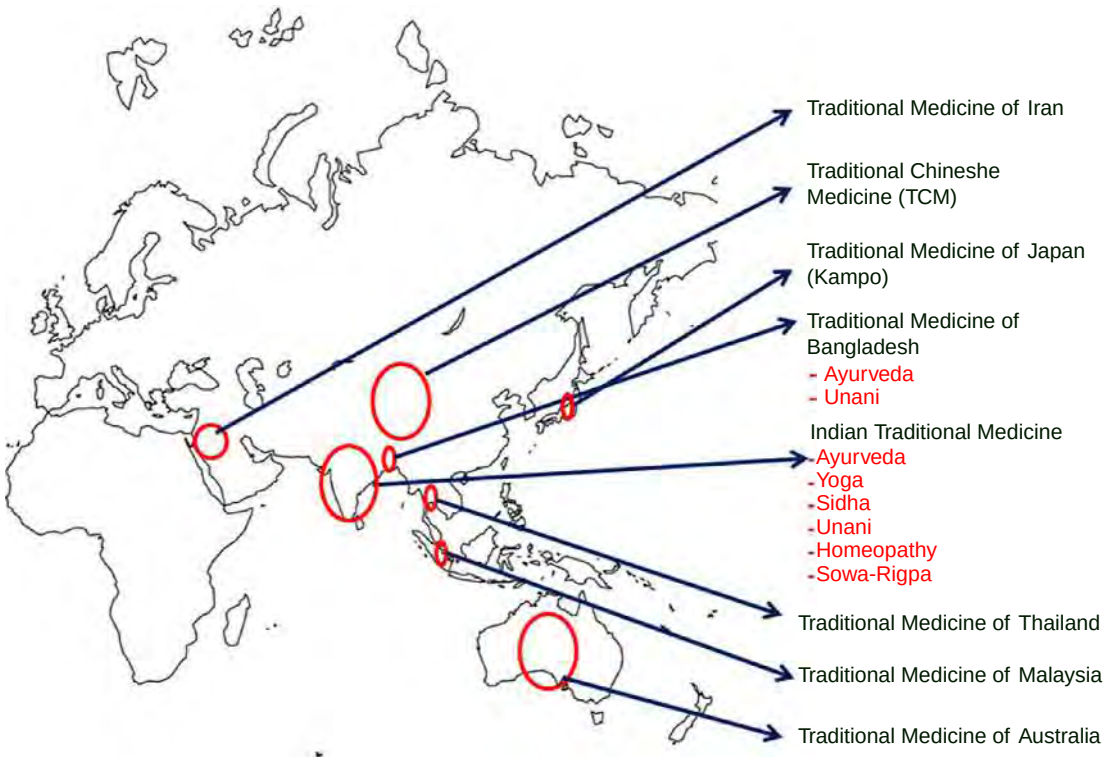


FIG. 1.1 Traditional systems of medicines in different countries.

1.2 AYUSH SYSTEMS OF MEDICINE

In India the major systems of TM, which have been practiced together for years, are Ayurveda, Yoga, Unani, Siddha, and Homeopathy (AYUSH), representing the Indian traditional healthcare system. The folklore practices and traditional aspects of therapeutically important natural products can be found in the book "*Materia medica*" of India. A conceptual singularity, which can be observed among the fundamental principles of these traditional systems of Indian medicine, is that they have a unique understanding of well-being. Because of its widespread influence throughout the world, it has been assigned major importance by the Government of India. The most common feature of TM is the use of medicinal plants, most of which are derived from various traditional systems and folklore practices. There are about 2000 drugs mentioned in the treatise "*Materia Medica*," many of them of vegetable origin, though some are derived from animal and mineral sources.

There are several molecules derived from plants, such as *Digitalis purpurea*, *Papaver somniferum*, the *Rauwolfia* species, and the *Cinchona* species, which are widely used in Western Medicine through the influence of traditional systems of medicine. The Chinese antimalarial preparation "Qinghaosu" helped in the discovery of Artemisinin, a potential antimalarial compound from *Artemisia annua*. Other examples include Artemether, for the treatment of drug-resistant malaria, and forskolin from *Coleus forskohlii*, a species used in Ayurvedic preparations for the treatment of cardiac disorders (Panda, 2010).

For the growth and development of Ayurveda and other systems of Indian Medicine, the Department of Indian Systems of Medicine and Homoeopathy (ISM&H) under the Ministry of Health and Family Welfare was established in March 1995 by the Government of India. It was renamed as the Department of Ayurveda, Yoga and Naturopathy, Unani, Siddha, and Homeopathy (AYUSH) in November 2003. Each letter of the acronym "AYUSH" represents officially recognized systems of medicine other than allopathic medicine. In 2014, a decision was taken to upgrade the Department of AYUSH to a full-fledged Ministry of AYUSH with a separate Minister. The Ministry is working toward the growth and development of Indian drugs. The Ministry is liable for policy formulation and implementation of schemes and programs for augmenting the domain of the AYUSH sector in comprehensive terms. Sowa-Rigpa, the medical system of the people of the Himalayan mountains, has also been recognized by AYUSH (Ministry of Health and Family Welfare, 2012–17) (Katoch et al., 2017). There are several organizations for promotion of AYUSH in India. Details about this are available in the Ministry of AYUSH website ayush.gov.in.

The Indian systems of medicine was first considered within the National Health Policy of 1983, later being emphasized within the National policies of 2000 and 2002. Ultimately the National AYUSH Policy (2002) envisaged that Ayurveda would play a substantial role on account of various qualities that can serve the poorest of the poor (National Health Policy, 2002). The policy also includes the consolidation of the Ayurveda knowledge base to shield it from industrial exploitation, piracy, and misappropriation by foreign entities. It promotes measures to confirm cheaper health services and medicines that are safe and efficacious. The combination of AYUSH within the provision of national programs, with optimum use of the large infrastructure of hospitals, dispensaries, and physicians, is considered in the policy (Samal, 2015). The proper use of resources within the National Rural Health Mission has been upgraded into a flagship program known as the National Health Mission (Shrivastava et al., 2015). As a result of these initiatives, the general public perception is evolving and opportunities have been enhanced in AYUSH. A statutory body within the Indian Drugs Central Council Act of 1970, called the Central Council of Indian drugs (CCIM), regulates Ayurveda to determine, guide, develop, and sustain the conservation of standards and the quality of educational study programs and seek to develop it further to international standards through resource allocation, smart governance, and management.

There are 12 assessment parameters in the pharmacopoeia standards of Ayurvedic medication, including identity, purity, strength, confirmed identification, chemical constituents and permissible limits of serious metals, chemical residue, aflatoxins, and microbic load. To ensure the quality of Ayurvedic medicines used in health facilities across the country, an essential drug list of Ayurveda, containing over 250 medicines compiled by the Ministry of AYUSH, has been published. The Government of India and the different states of the country are supported in obtaining such medicines for free public distribution to patients through dispensaries and different medical centers (Anonymous, 2013). The work on the development and revision of standards of Ayurvedic medication is supervised by the Pharmacopoeia Commission of Indian Drugs and the Ayurvedic Pharmacopoeia Committee. The Pharmacopoeial Laboratory for Indian Medicine (PLIM), the foremost laboratory under the provisions of the Drugs & Cosmetics Act, 1945, as well as numerous other scientific laboratories, are engaged in the work of customization and the development of standards in the operational procedures for the quality testing of Ayurvedic medication with subtle instrumentation and analytical tools. As a matter of fact, vital achievements have been made using the prevailing pharmacopoeia standards; a unified pharmacopoeia infrastructure is meant for higher coordination and quality outcomes. For the development of pharmacopoeial standards, studies by laboratories or establishments licensed by the government have been enhanced. This effort can substantiate the prevailing pharmacopoeial standards

of single and multiingredient medications of plant, mineral, metal, and animal origin (Mukherjee et al., 2010). Standard operating procedures for the manufacture of formulations and assays, as well as an atlas of chromatography and a pharmacognosy atlas, have been made to the quality standards of medicine to facilitate the testing procedures and the estimation of marker compounds and phytochemical standard materials. For precise authentication, DNA barcoding or fingerprinting of Ayurvedic herbs should be developed and incorporated into the existing system of quality standards. The Drug Control Cell of the Ministry of AYUSH takes care of regulative and internal control matters of Ayurvedic medication under the provisions of the Drugs and Cosmetics Act 1940 and the rules there below. The drug control cell coordinates with the state licensing authorities, drug controllers, and drug testing laboratories with the aim of directing the legal provisions of the internal control of Ayurvedic and different Indian medicines. The amendment of regulative provisions may be continuous in accordance with the requirements and rising trends in the quality control of natural products.

The global promotion and propagation of Ayurveda has been a crucial focus of the Ministry. It has created AYUSH educational chairs at overseas universities and institutes. The AYUSH Information Cell has been opened on the premises of the India Missions/ICCR Cultural Center and has been entrusted with publicizing authentic information on AYUSH. The achievements of international cooperation have been tremendous and have led to a rise of Ayurveda consciousness worldwide. Globally, ancient medication regained its status when Professor Youyou Tu was awarded the Nobel Prize in 2015 in medicine for the use of ancient Chinese medicine (TCM) in the discovery of an antimalarial drug and the UN selected June 21 as World Yoga Day. It was indeed a matter of triumph when "Traditional Medicine: Delhi Declaration" was adopted on a resolution of the World Health Organization Regional Committee for Southeast Asia SEA/RC67/R3 as proposed by AYUSH and the Indian government. It has now been adopted by the countries within the entire Southeast Asia region. The people of the Indian landmass possess a robust faith in AYUSH and are convinced of its effectiveness. Knowledge-based analysis, standardization, internal controls, and validation of Ayurvedic medicine is required to determine a knowledge base to satisfy international standards. The Ministry of AYUSH is making progressive efforts toward the promotion and development of Ayurveda both across the country and internationally.

1.2.1 Traditional Knowledge Digital Library

It is imperative to safeguard the sovereignty of ancient Ayurvedic data from being misused in patents. Although this information is within property rights, the Patent and Trademark Office Database does not have a mechanism to access this information to deny patent rights. The Traditional Knowledge Digital Library (TKDL) program assists with the documentation of the existing data of the Ayurvedic systems of drugs. It is both very costly and time consuming to fight patents that are granted to other people or organizations. Thus, conveyance of such data into a simply accessible format to forestall wrongful patents is essential and was actualized through the TKDL (http://www.tkdl.res.in). It inspired proprietary information, absolutely protected by national and international laws of property rights. The core stress of the project is on an innovative approach to Traditional Knowledge Resource Classification (TKRC) that permits the conversion of 140,000 pages of data containing 36,000 formulations delineated in 14 texts of Ayurveda into a patent-compatible format in varied languages, for example, the translation of Indo-Aryan shlokas in Hindi, as well as in English, French, German, Spanish, and Japanese. The knowledge includes the names of plants, the Ayurvedic description of diseases beneath their distinctive names, therapeutic formulations, and so on (Mukherjee et al., 2010). The Ministry of AYUSH launched the National AYUSH Mission (NAM) with implementation through states/Union Territories (UT). It envisages higher access to services, strengthening academic establishments, the social and internal control of medicine, and the continuous availability of raw materials within the States/UTs (Anonymous, 2015; Katoch et al., 2017). The general public health outreach activity has been to concentrate on increasing the awareness of the utilization of Ayurveda in managing community health issues.

1.3 AYURVEDA

Ayurveda can be called a primeval (before 2500 BC) Indian systems of healthcare involving a holistic view of man, his health, and wellness. An Ayurvedic treatment of an illness consists of the salubrious use of medication, diet, bound practices, and medicative preparations from invariably complex mixtures, primarily based on plant products. Around 1250 plants are presently employed in numerous Ayurvedic preparations. Several Indian medicative plants have come back under scientific scrutiny since the middle of the 19th century, though in a very infrequent fashion. The primary vital contribution from Ayurvedic pharmacological medicine came with the isolation of an antihypertensive compound from the *sarpagandha* (*Rauwolfia serpentina*), for the treatment of high blood pressure, insomnia, and psychopathy. This was the prime ancient-modern concordance in Ayurvedic plants. There has additionally been concordance for a number of alternative Ayurvedic crude medications similar to *Asparagus racemosus*, *Cedrus deodara*, and many others.

In the early development of contemporary drugs, biologically active compounds from higher plants played an important role in providing medicines to combat pain and diseases. To illustrate, within the British Pharmacopoeia of 1932, over 70% of the organic monographs were on plant-derived products. However, with the arrival of synthetic medicines and antibiotics, the role of plant-derived therapeutic agents declined considerably (mostly) within the economically developed nations. However, plant-derived medicinals still occupy a very important position within the treatment of diseases worldwide. Thus, most of the recently introduced plant-based medication are innovative in character and represent outstanding contributions to medicine (Dev, 1997). These medications embody many antitumor agents similar to podophyllotoxin, paclitaxel, and camptothecin and its derivatives, with each of these categories of compounds exhibiting their antitumor activity by distinct mechanisms (Beecher et al., 1989).

There has been a replacement trend in the preparation and selling of herbal medicine and medicinal plants (Bisset, 1994). These preparations, labeled as “herbal medication” or “phytomedicines” or single plant extracts or fractions therefrom, are distinct from the pure chemical entities of molecular medication. These new plant-derived products are fastidiously standardized and their pharmacological activity and safety for a particular application are evaluated. Thus, plant-based therapeutic agents still have scientific, social, and business significance and seem to be gathering momentum in the health and wellness arena.

The materia medica of written material, and other similar repositories of information from alternative cultures, represent valuable resources for the development of not only medicinal preparations but also nutraceuticals and cosmeceuticals. However, these claims should be critically evaluated in terms of contemporary scientific parameters. Once such a project is envisaged, some conditions should be critically evaluated in terms of contemporary scientific parameters. It is vital to precisely correlate the description of the illness in the ancient literature with the recent etiology and clinical pathology to confirm a correct correspondence. In the Ayurvedic literature, there is much emphasis on the gathering of plant materials at a selected season, from a selected section of the forest, and at an explicit time of day. It is currently well established that the concentration and profile of secondary metabolites in every plant depend upon environmental, biological-process, and photo-periodicity factors. Medicinal plant preparations have vital historical background; hence, it is ethical to clinically judge these first and then collect appropriate pharmacology knowledge. In any case, vital categories of compounds essential for bioactivity should be portrayed. All of this information is essential for the correct standardization of product (Mukherjee, 2001).

1.3.1 Origin of Ayurveda

Ayurveda is accepted to be one of the oldest treatises on medicine and came into existence in about 900 BC. The Sanskrit word "Ayurveda" comprises two words: “Ayus” meaning life and “Veda” meaning learning or science. Hence, "Ayurveda” signifies “Knowledge of life.” Ayurveda is claimed to be an Upaveda (part) of the Atharvaveda sacred text and also the Charaka Samhita (1900 BC), which is the primary recorded piece of Ayurveda absolutely dedicated to its ideals. It describes 341 plants and plant products that can be used in drugs. It was followed by another landmark of Ayurvedic literature, the Sushruta Samhita (600 BC), which emphasizes surgery in particular. It mentions the medicinal use of 395 plants, more than 50 medications of animal origin, and around 60 minerals and metals as therapeutic agents. Sushruta, claimed by the Indian ancient history of medicine as the father of surgery, lived and practiced surgery in Varanasi, India just about 2500 years ago. In India, the historical backdrop of healthcare reaches back to 5000 years BC when health service needs and sicknesses were noted in ancient literary works, such as *Rig-Veda* and *Atharva-Veda*. Later, in about 1000 BC, Ayurveda was described in the *Charak Samhita* and *Sushruta Samhita*, in which the use of plants and polyherbal formulations for human services was featured. Indian *Materia medica* (c.2000) incorporates medications from common sources that are derived from various traditional systems and folklore practices (Mukherjee et al., 2017a, b).

Atharva-Veda was recorded around 5000 years ago and contains references to Ayurvedic medication and healthcare. It incorporates each feature of one’s presence, including physical, mental, and social well-being. It clarifies what is useful and what is destructive to life, and how a happy life can be accomplished and a hopeless life be improved; all of these imperative inquiries and lifestyle issues are nicely presented in Ayurveda (Mukherjee et al., 2012). Indian Hindu culture has four compendia known as Vedas written by the Aryans—*Rig-Veda*, *Sham-Veda*, *Yajur-Veda*, and *Atharva-Veda*. Among these, the *Rig-Veda*, the oldest, was written between 3000 and 1500 BC. Ayurvedic mostly details polyherbal formulations, containing plant and animal parts, minerals, and metals. Old scriptures, such as *Rig-Veda* and *Atharva-Veda*, and official compendia, such as the *Ayurvedic Pharmacopeia* and *Ayurvedic Formulary*, show the strength of plant-derived medicines (Mukherjee et al., 2017a). The *Charak Samhita* (900 BC) is the principal documentation, giving ideas and therapeutic processes with regards to Ayurveda and with an emphasis on therapeutics; it lists 341 plants and plant items for use in medication (Mukherjee et al., 2015).

Another notable specialist of Ayurveda was Vagbhata. His work, *Ashtanga Hridaya* (c.700 AD), is viewed as being unrivaled for the standards and processes of pharmaceuticals. The Madhava Nidana (800–900 AD) was the following critical point of reference; it is the most well-known Ayurvedic text discussing illnesses. The praised scholar of Ayurveda, Bhava Mishra of Magadha wrote the treatise *Bhava Prakasha* around 1550 AD. It is highly regarded by current Ayurvedic experts for its portrayal of approximately 470 therapeutic plants. In addition to these amazing treatises, more than 70 "Nighantu Granthas" (drug compendia) were composed, for the most part between the 7th and 16th century AD. *Raj Nighantu* by Narhari Pandit and *Madanpala Nighantu* by Madanpala are considered gems on the topic of medicinal plants (Mukherjee, 2001). Along with Ayurveda, there are a few other integral and elective frameworks of medication in the Indian systems of medicine, such as the homeopathy, Siddha, and Unani frameworks, which have been created and honed over the course of time.

The most important of the Ayurvedic texts include *Charak Samhita*, *Susrut Samhita*, *Ashtanga Hridaya*, and the "brihat trayi" (higher triad) texts, while *Sharangdhar Samhita*, *Madhav Nidan*, *Bhavprakash*, and the "*laghu trayi*" are the concise triads. *Kashyap Samhita* and *Bhaisajya Ratnavali* are also important books of *Ayurveda*, which introduce broad information on the counteractive action of diseases, the advancement and safeguarding of well-being, and the treatment of diseases in all-encompassing terms (Mukherjee and Wahile, 2006). Ayurveda can effectively oversee lifestyle disorders, drug abuse, degenerative ailments, immune system illnesses, and metabolic and hypersensitive diseases with its special treatments, body–mind–constitution-based way of life, and medicaments. It has a multidimensional scope of treatment and is useful in treating various conditions, such as headache, Parkinsonism, neurological disorders, and musculoskeletal diseases (Mukherjee et al., 2010). The Ayurvedic system is appreciated for its use for the treatment of the vast majority of illnesses associated with different systems of the body with its novel treatments, known as *Panchakarma* (Debnath et al., 2015). The all-encompassing methodology of Ayurvedic healthcare has been combined in a way that individuals can utilize as an adjuvant for treatment and personalized healthcare. In India, Ayurveda is the most widely used and culture-bound tradition of healthcare and its learning is accessible in both systematized and unclassified structures.

1.3.2 Ayurvedic Drugs and Pharmaceuticals

The manufacturing of Ayurvedic medicines takes place under license. The medicines are derived from the formulae mentioned in the authoritative books, as prescribed within the individual schedules of the Drugs & Cosmetics Act 1940. Considering their methodology of preparation, palatability, bioavailability, and therapeutic values, formulations have been grouped in numerous dosage forms, for example, Avleh, Churna, Asava, Bhasma, Ghrita, Taila, Kupipakva, Gutika, Guggulu Modaka, Louha, and Pisti, as supported by the Ayurvedic Formulary of India. In four volumes of the National Ayurvedic Formulary, 265 standardized formulations from classical texts are revealed. In two parts (I and II) of the 13-volume Ayurvedic Pharmacopoeia of India, 645 monographs on the quality standards of single medications and 252 monographs of the quality standards of multiingredient formulations are revealed.

1.3.3 Medicinal Plants in Ayurveda

In India, there are about 15 agroclimatic zones and 17,000–18,000 species of flowering plants. Of these, around 6000–7000 are used in folk medicines and alternative systems similar to Ayurveda. Around 960 species of medicinal plants are calculated to be in use and 178 species have annual consumption levels of more than a hundred metric tons. To handle these problems, the National Medicinal Plants Board (NMPB) under the Ministry of AYUSH was set up in November 2000 by the Indian government. The board supports policies and programs for the expansion of trade, export, conservation, and cultivation of medicinal plants. The NMPB provides support for surveys, inventories, conservation, herbal gardens, linkage with joint forest management programs, research, and development. The Department of Biotechnology and the Department of Forests in India undertake diversity conservation, as well as cultivation, postharvest processing, and storage of medicinal herbs. Threatened, endangered, and vulnerable medicinal plant species are being protected in numerous ways. Restrictions are enforced for rampant deforestation for the collection of the raw materials of medicinal plants from wild sources.

1.3.4 Globalization of Ayurvedic Medication

The domestic and international acceptance of Ayurveda has resulted in enormous market potential. It is changing into a vital healthcare system in India and all over the planet. The Ministry seeks to identify and promote Ayurvedic Institutions engaged in drug development and research, education, and clinical analysis. Such institutions may be governmental, nongovernmental, or nonprofit enterprises and the goal is to support them and boost their functions and facilities to the

excellence of Ayurveda. These Centers of Excellence (COE) are intended as establishments and organizations specializing in one or several areas of clinical research in hospitals, nursing homes, on Ayurveda treatment, or in developing a knowledge base in medical science, pharmacies, or drug development to bridge Ayurveda and science, or in other specialized areas of AYUSH. Another aim is to feature new international functions and make significant qualitative enhancements within the existing services, as well as supporting human resources and adding infrastructure and equipment. The government is supporting the present facilities of the COE organization in upgrading to National Certification Board for Hospitals, National Certification Board for Testing and Activity Laboratories, Good Laboratory Practices (GLPs), or Good Manufacturing Practices (GMPs).

For globalizing the system and its product, the Ministry of AYUSH has targeted its attention on the standardization and quality control of Ayurvedic medications. GMPs are noted under "Schedule T" of the Drugs and Cosmetics Act and Rules thereunder. For export functions, the presence of heavy metals, such as mercury, arsenic, lead, and chromium, are strictly controlled in herbal and Ayurvedic medication within their daily safety limits (Anonymous, 2013; Katoch et al., 2017). These measures have been introduced to provide a bigger impetus to consumer awareness, patient and doctor information, acceptance within globalized markets, and to confirm safety—a topic of utmost concern for Ayurveda medicines. Clinical safety and the affectivity of documentation of Ayurvedic medication has been a persistent and increasing demand on a worldwide basis (Katoch et al., 2017). Clinical trials in Ayurveda are controlled by the principle of Good clinical practice (GCP). The safety and therapeutic effectiveness of Ayurveda, Siddha, and Unani (ASU) medication needs to be readdressed to assure their quality and scientific documentation. Hence, a program of clinical research along these lines will help to develop a quality knowledge base acceptable to authorities for product registration or approval for sale as modern or nongeneric formulations. The AYUSH GCP guidelines mention several ethical criteria for treating a patient with medical/surgical interventions. The need for proof for effectiveness in the licensing of patented or proprietary ASU medication for the enactment of the Drugs and Cosmetics Rule (Section 158 B) since August 2010 has necessitated the use of GCP.

1.3.5 Ayurvedic Treatment and Practice

Ayurveda advocates treating the entire body as a single unit. It does not think in terms of individual organs. It considers that the body is composed of multiple organs. The principles of treatment in Ayurveda consist of Shodhana (elimination of morbid factors) and Shaman (Palliative treatment) Karmas. Shodhana Karma is also widely known as Panchakarmas, for example, Vamama, Vicrchana, Vasti, Nasya, and Raktamok shama. These are important procedures to eliminate morbid factors, such as Vikriladoshas, Dustadhatu, or Malas, and to maintain homeostasis in the body. An approach of this nature is unparalleled in the annals of medical science. Stabilizing the chemical composition and physicochemical properties of the internal environment is an important feature of the organism in higher animals; this state is called homeostasis. Homeostasis is expressed by a number of biological constituents, that is, stable quantitative indices characteristic of the normal state of the organism. They include the values of body temperature, the osmotic pressure of blood and tissue fluids, the content of sodium, potassium, calcium, chlorine, and phosphorous ions, the levels of protein and sugar, the concentration of hydrogen ions, and a number of other indices.

The various organs and systems of the body, that is, the digestive system, circulatory system, and respiratory system, have different roles in maintaining homeostasis. Even a slight disturbance of homeostasis can give rise to a pathological condition. Shodhana Karmas play a vital role in eliminating toxic substances from the gastrointestinal tract, to eliminate the products of decomposition of organic substances (dusta dhatus and mala), and to maintain a constant level of doshas in the body. Thus, the principles of Shodhana Karma play a vital role in maintaining the internal environment, the buffer system (blood and tissue fluids), and in homeostasis of the body. Hence, the contribution of Shodhana therapy shows the excellence of the Ayurvedic ideology.

The principle, the treatments, and the philosophy of Ayurveda are among the best systems that fulfill the essential needs of humans. Ayurveda has many medicines that have minimal side effects when used judiciously. In view of advancing technology, there is much to rediscover and attention should focus on the application of technology in perusing the principles of the Dosha Dhattu Mala theory. Thus, Ayurveda formulates a holistic approach to treatment by encompassing the body as a whole, as a result of which Ayurveda treatment may be time consuming but with long-lasting results.

Almost all of the ancient text from Ayurveda divides medical knowledge into eight sections (Astanga). Plant-derived drugs have been classified by treatment and evaluated based on their therapeutic action since ancient times. It is in this way that Ayurveda can be considered as a scientifically organized discipline. Ayurvedic texts are greatly respected in neighboring countries and have been translated into other languages. *Charak Samhita* classified plant drugs into 50 groups based on their Sanskrit names, an example of which is shown in Table 1.1.

TABLE 1.1 Classification of Drugs According to *Charak Samhita* Based on Therapeutic Implications

Therapeutic Implications (Sanskrit)	Therapeutic Usage (English)
Balya	Promoting strength
Jivaniya	Promoting longevity
Dipaniya	Promoting digestion
Lekhaniya	Promoting antiobesity
Verne	Promoting complexion
Krmighna	Promoting anthelminsis
Stanyajanana	Galactogogue
Vamanopaga	Emetic
Kasahara	Antitussive
Svayathyhara	Antiinflammatory
Javarahara	Febrifuge
Vedanasthapana	Analgesic
Vayahsthapana	Antiaging

Ayurveda is classified into eight sections (limbs), which are called "Astanga Ayurveda" (see Table 1.2). Kayachikitsa tantra is the Ayurvedic branch that deals with treatments based on internal medicine, such as digestion and metabolism. Ayurveda also links the concepts of seasonal and climatic variations to overall health. The concepts of Dinacharya (daily regimen), Ritucharya (night regimen), Rasayana (rejuvenation), and Vajikarana (sexual health) are among the contributions of Ayurveda to medical science.

The second aspect of Ayurveda is *Aturasya Roganuth*, which deals with curing disease (medical). When the body is stable, the soul is clean and works in harmony with the mind, a condition known as *Prasanna Atmendriyamana*.

Diseases, according to Ayurveda can arise from the body and/or the mind because of internal factors or intrinsic causes. Ayurvedic treatment considers the patient as an organic whole, and treatment consists of the salubrious use of drugs, diets, and practices. Ayurvedic medicinal preparations are complex mixtures that include plant- and animal-derived products, minerals, and metals. The basic concept of diagnosis and drug development in Ayurveda is based on Tridosha theory, which includes the aspects of Vayu, Pitta, and Kapha (Sastri, 1996).

TABLE 1.2 Components of Astanga Ayurveda

Treatment Systems in Ayurveda (Sanskrit)	Modern Systems (English)
Kaya Chikitsa	Medicine
Salya Chikitsa	Surgery
Salakya Chikitsa	Ear, nose, and throat treatment
Bala Chikitsa	Pediatric treatment
Jara Chikitsa	Treatment related to genetics
Rasayana Chikitsa	Treatment with chemicals
Vajikarama Chikitsa	Treatment with rejuvenation and aphrodisiacs
Graham Chikitsa	Planetary effects
Visha Chikitsa	Toxicology

- *Vayu (Vata)*: Explains the entire biological phenomenon, which is controlled by the functions of the central and autonomous nervous systems. The malfunction of Vayu is a major factor in developing diseases, either by itself or coupled with other functional disorders due to Pitta and kapha.
- *Pitta*: The manifestation of energy (Tejas) in the living organism that helps digestion, assimilation, tissue building, heat production, blood pigmentation, activities of the endocrine glands, and so on. Many of these processes are thermogenic and metabolic.
- *Kapha (Sleshma)*: The function of thermotaxis or heat regulation and the formation of various preservative fluids, for example, mucus and sinovial fluid. The main function of kapha is to provide nutrition to body tissues to bring about the coordination of the body system and the regularization of all biological processes.

The primary position is relegated to the equilibrated state of doshas. As far as healthy living is concerned, this indicates their importance in the maintenance of the health and well-being of the body. It is also understood that a disturbance in the equilibrium of the doshas should be considered as leading to the development of disease. Health, according to Ayurveda, can be visualized as the physiological maintenance of all the functions of the living being, with disease being a disturbance in the physiology.

Bringing all these factors into balance is called *Aturasyaroganuth* and maintenance of the balance is called *Swasthya rakshana*. It is based on Trividha Pariksha (threefold diagnosis), which completely depends on the *Monah Budhi Indriyas* (knowledge) of the physician, and does not depend on the use of laboratory and other investigations. Hence, the diagnostic methods of Ayurveda are considered superior. Ayurveda considers that measuring disease and health in parameters is not always correct; instead it relies upon the Budhi of the Bhishak (the knowledge of the physician).

To formulate and assess the action of drugs, the theories of Rasa (taste), Guna (properties), Virya (active principles), and Vipak (biotransformation) were established. This means a physician may formulate medicine according to the patient's needs and not necessarily depend on pharmaceutical preparations. There are five basic methods of preparation of medicines (known as "panchavidha kashaya kalpana") to suit the palate of the patient and to elicit the required potency of the drug; the system also advocates drugs that have synergistic action and that act as vehicles for the drug to reach the target organ (known as "anupanas"). For example, in all vatarogas (mostly neurological disorders) "Sneha dravyas" (lipids) are formulations used to contain such disorders. Lipid-soluble drugs are rapidly absorbed and quickly reach the site of action. Additionally, the lipids readily cross the blood–brain barrier, so that the action of the drug will reach the cerebrum and spinal cord, and they even cross the placenta and breast epithelium. Therefore, there is a superiority in the formulation and administration of drugs exhibited through the Ayurvedic system. Hence, we are able to treat the disease of more complicated cases of nervous and hepatic disorders. The concept of Raktadushti (reddening of skin) in causing many skin disorders is most convincing and by correction of such dushti, we are able to control many uncontrollable and chronic skin disorders. There are innumerable theories and principles of Ayurveda regarding Samprapti (pathology) and Chikitsa (treatment), including formulations for relief from many chronic and incurable disorders.

1.4 UNANI

The Unani system of medicine originated in Greece, starting with the Greek philosopher–physician Hippocrates (460–377 BC). Hippocrates (460–377 BC) freed drugs from the realm of superstitious notions and magic and gave them the standing of science. Greek and Arab scholars, such as Galen (AD 131–212), Raazes (AD 850–925), and Avicenna (AD 980–1037), developed the system considerably. The theoretical framework of Unani drugs supports the teachings of Hippocrates and the principles put in place by him. Here, a disease is thought of as an activity and its symptoms are the reaction of the body to the disease. These basics of Unani are similar to a part of Ayurveda. This method incorporates the belief that each person has a distinctive humoral constitution that represents a healthy state (Siddiqui, 1996). Unani considers the material body to be created from seven components: Arkan (elements), Mizaj (temperaments), Aklath (humors), Anza (organs), Arawh (spirits), Quo (faculties), and Afal (functions). Each of them have an in-depth relationship with the state of health of a person. A physician takes these factors into consideration before diagnosis or prescribing treatment.

Hippocrates was the first physician to introduce the strategy of taking medical histories and this gave rise to the "humoral theory." The body–substance theory incorporates the presence of many humors: Dam (blood), Bhalgham (phlegm), Safra (yellow bile), and Souda (black bile) within the body. The temperament of a person is expressed by the words sanguine, phlegmatic, choleric, and melancholic according to the preponderance of the several humors. The humors themselves are allotted temperaments: blood is hot and moist; phlegm is cold and moist; body fluid is hot and dry; and humor is cold and dry. The Unani system incorporates the belief that each person has a distinctive humoral constitution, which represents his/her healthy state. Any imbalance in this state affects his/her health. There is an influence

of preservation or adjustment referred to as "medicatrix naturae," which strives to revive disturbances at the intervals the treatments are prescribed according to the constitution of the person. If this power weakens, an imbalance in body substance composition is certain to occur and this causes sickness. In Unani drugs, a good deal of reliance is placed on this power. The medicines utilized in this method, in fact, facilitate the body in regaining this power to an optimum level and thereby restore the humor balance. Thus, Unani drugs are the result of the fusion of the various thoughts and experiences of nations with an ancient cultural heritage, such as Egypt, Arabia, India, Iraq, Iran, and different countries of the Near East and Far East.

In India, the Arabs introduced the Unani system of drugs and, within a short period of time, it took firm hold. Once the Mongols had ravaged Persian and central Asian cities, such as Shiraz, Tabrez, and Geelan, students and physicians of Unani drugs fled to the Republic of India. The rulers of Delhi before the Mughal Emperors, the Khiljis and Tughlaqs, provided state patronage to the students and even registered some as state staff and court physicians. Throughout the 13th and 17th century, Unani drugs had a presence in the Republic of India. Abu Bakr Bin Ali Usman Kasahani, Sadruddin Damashqui, Bahwabin Khwas Khan, Ali Geelani, Akabl Arzani, and Mahound Hoshim Alvi Khan are among those who made valuable contributions to the present system. The scholars and physicians of Unani drugs who settled in the Republic of India were not content with the known medication. They subjected Indian medication to clinical trials. In Unani drugs, a single medication or mixtures in raw form are more popular compared with compound formulations. The system offers remedies for disorders that have been clinically tried. Such drugs, which are poisonous in crude form, are processed and sublimated in many ways before use. The system offers wonderful remedies for various disorders that have been tested over many centuries (Mukherjee, 2002). Though the origins and development periods of these systems of drugs are completely different, the elemental principles and practices of using plants and plant-based formulations within healthcare are similar (Ramakrishnappa, 2002). During British rule (1757–47), Unani drugs suffered a black eye and development was hampered due to the withdrawal of governmental patronage. It was primarily for the efforts of the Sharifi Family in Delhi, the Azizi family in Lucknow, and the Nizam of Hyderabad that Unani drugs survived throughout the British period. An outstanding physician and scholar of Unani drugs, Hakin Ajmal Khan (1868–1927) championed the system in India. Even before independence, many committees were appointed that underscored the long running role of traditional systems of drugs. In 1969, the Indian Government established a Central Council for Indian Medicine and Homeopathy (CCRIMH) to develop research in numerous branches of Indian systems of drugs, such as Unani drugs, Ayurveda, Siddha, Yoga, treatment, and homeopathy. The research activities in these systems continued under the aegis of the CCRIMH until 1978 when it was divided into four separate analysis councils, one each for Unani, Ayurveda, Siddha, and Yoga. Since its inception, the Central Council for Research in Unani Medicine (CCRUM) has been seeking to produce a scientific basis for the Unani system and to develop viable solutions to the health issues of the population.

1.4.1 Therapy and Formulation of Unani Medicine

Unani considers the human body to be created from seven components: Arkan (elements), Mizaj (temperaments), Aklath (humors), Anza (organs), Arawh (spirits), Quo (faculties), and Afal (functions). A physician takes these factors into account before diagnosis or prescribing treatment. There are many kinds of medical aid (treatments) that are prescribed within the Unani system of drugs, including regimental therapy, dietotherapy, and pharmacotherapy.

- *Regimental therapy*: Includes body process, diuresis, Turkish bath, massage, purging, and emesis.
- *Dietotherapy*: Aims at treating certain ailments by the administration of specific diets or by controlling the amount and quality of food.
- *Pharmacotherapy*: Deals with the employment of herbal medicine, principally with an herb of individual components. The naturally occurring medicines utilized in this method are symbolic of life and are usually free from adverse effects. In this method, the temperament of the individual is considered to be important; hence, the medicines administered match the temperament of the patient and accelerate the process of recovery. The basic principles of the Unani system of drugs correspond to written texts. This method incorporates the belief that each person contains a distinctive humor constitution that represents a healthy state (Siddiqui, 1996). The Unani system of drugs, with its own recognized practitioners, hospitals, and academic institutions, forms an integral part of the national health care system. Presently, the Government of India is providing increasing support and funds for a multipronged development of Unani medication that focuses on the benefits of this method in providing healthcare to the Indian population.

1.5 SIDDHA

The Siddha system constitutes a major part of the traditional medical practices that are used in the southern part of India (Mukherjee et al., 2007). Siddha is basically a system whereby attention is given to minerals and metals instead of plant constituents. Herbs are prepared as triturates and calcinates of metals into their *basmam* and *chendooram*. *Agasthiar* is regarded as the originator of Siddha bioscience. In the Siddha system, man is not just a composite of bones, muscles, and tissues, but rather there is an in-depth nexus between man and nature. The Siddha system centers on a five-component theory that refers to the earth, water, heat, air, and ether of the external world and of internal man as elementary principles for creation, preservation, and destruction. This system describes 96 principal constituents of people that incorporate physical, physiological, ethical, and intellectual components. Any imbalance or slight deviation with these units causes disease. The diagnostic methodology in the Siddha system is eightfold and includes an examination of the pulse, tongue, complexion, speech, and scrutiny findings. The Siddha drugs are principally made of minerals and metals instead of plant constituents.

The word Siddha comes from the term "Siddhi" meaning "attainment of perfection." This medical specialty resembles Ayurveda. It is an ancient system of drugs developed by 18 Siddhars (Pillai, 1998). The "Siddhars" were angelic figures who achieved mastery of drugs through their practices. Similar to Ayurveda, it uses natural resources for healthcare. The Siddha system is one of the oldest systems of healthcare in India. The formulations of Siddha drugs embody herbal products, inorganic substances, and animal products to prepare completely different formulations, such as *Chendooram* (a blood-red fine-grained medicine), *Choornam* (powdered drugs), and *Chunam* (medications ready by calcination). For example, if the liver is not functioning properly, it is believed that the concentration of a particular mineral/metal element in the liver is low and that this results in dysfunction. If the particular metallic element is rich in Siddha medicine and targeted against the liver, it seems to correct the dysfunction of the liver. All the metals and minerals used in the Siddha system are in an entirely detoxified state as per the method known as "Shodhana." During this process, the metal molecules are rendered nontoxic. Further, on boiling with several herbs and animal products, the inorganic metal is converted into an organic compound.

Within the Siddha system, detoxification may be a common drug regime to extend therapeutic efficiency, thereby minimizing the toxicity, popularly called "Suddhi Seithal." These compounds, on purification, are called "Suddhi Seithavai." As per the Siddha language, substances of plant origin are called *Thavra vargam*, those of animal origin are called *Jeeva vargam*, and those of mineral origin are called *Dhathu vargam* (Formulary of Siddha Medicines, 1993). In Ayurvedic language, medicines with mineral mixtures are called "*Bhasmas*" and in the Siddha system they are referred as "*Parpams*" (predominantly of a Ca and phosphorus composition). However, the detoxification methods stay common for each of the systems (Mukherjee et al., 2015).

1.6 HOMEOPATHY

The principle of Homeopathy has been known since the time of Hippocrates of Greece, the founder of medicine, around 450 BC. Homeopathy, as it is practiced today, was developed by the German physician, Dr. Samuel Hahnemann (1755–1843). Homeopathy is based on the idea that "like cures like"; that is, substances that cause certain symptoms in a healthy person can also cure those same symptoms in someone who is sick. This so-called law of similar gives homeopathy its name: "homeo" for similar, "pathy" designating disease. In his experiments, Hahnemann developed a method of "potentizing" homeopathic remedies by diluting them in a water–alcohol solution and then vigorously shaking (or succussing) the mixtures. The results convinced him that a high degree of dilution not only minimizes the side effects of the remedies but also simultaneously enhances their medical efficacy (Mukherjee et al., 2007).

Homeopathy merely means the treatment of diseases with remedies that are capable of manufacturing symptoms identical to the disease when taken by healthy individuals. Perhaps, if Peruvian bark containing an antimalarial is taken by a healthy person, it would produce symptoms that precisely mimic intermittent fever (now referred to as malaria). Homeopathy has been practiced for more than 150 years in India. It has therefore blended so well into the roots and traditions of the country that it has been recognized in concert with the Indian Systems of Medicine (ISM) and it plays a vital role in providing healthcare to the public (Mukherjee et al., 2007).

Modern homeopaths usually prescribe doses at dilution ratios ranging from 1× (1 part substance to 9 parts dilution medium) to 200c (200 repetitions of diluting 1 part substance with 99 parts of dilution medium); higher ratios are indicated with an *m*, for a 1–999 ratio. Critics of homeopathy argue that, in extreme dilution ratios (which are considered high potencies), the remedies may not contain even a single molecule of the original healing substance. Nonetheless, studies have shown that homeopathic remedies may be effective for certain disorders, such as childhood diarrhea, hay fever, asthma, and the flu. Further research is now being conducted under the auspices of the National Institutes of Homeopathy.

Most homeopathic remedies have undergone "provings," or medical observation, in which healthy individuals are given doses of undiluted homeopathic substances. Mental, emotional, psychic, and other details of the patients are most important. This leads the physician to a better understanding of which remedy will best suit a particular set of symptoms.

The majority of homeopaths practice "constitutional" homeopathy, based on the idea that each person's constitution or mental, physical, and emotional makeup may need to be treated along with any specific ailments. Classically, only one homeopathic medicine is used at a time. An extensive patient history is taken and the physical and psychological symptoms of the patient are observed, then an initial prescription is made. If the medication does not have the desired effect or if the symptoms persist, a second analysis is done and a second prescription is given. This process continues until the correct medication for the underlying ailment is found. Constitutional treatment is generally used for chronic problems; acute or short-term ailments are usually treated with remedies specific to the illness. Recently, over-the-counter combination homeopathic remedies have become available for a variety of common ailments. These products contain several of the most common remedies for a particular problem and can be useful for self-treatment of minor conditions. For prolonged or serious illness, a professional homeopath can prescribe specific single remedies.

1.7 SOWA-RIGPA

"Sowa-Rigpa" originated in Tibet and is commonly known as the Tibetan system of medicine. It is one of the oldest, living medical traditions of the world and is well documented. It has been popularly practiced in India, Nepal, Bhutan, Mongolia, and Russia. The Tibetans made use of their abundant natural resources of flora and fauna in the fight against disease. The history of the Tibetan medical system dates back some 3800 years and it has continued to evolve since then to the time of the strong emergence of Buddhist culture in India. The 7th and 8th century AD saw real development in the field of Tibetan medicine. Ayurveda has contributed a great deal in enriching Tibetan medicine. The *Gyud-shi* (the *Four Great Tantras*), which is the most authoritative classic of Tibetan medicine, bears ample proof of its loyal allegiance to Ayurvedic classics, such as *Charaka*, *Susruta*, and *Astanga hydra* of *Vaghbhata*. One of the unique features of the Tibetan medical system is its ideological structure of medical theory and practice in the image of a tree known as the Allegorical Tree (Mukherjee et al., 2007).

The majority of the theory and practice of Sowa-Rigpa is similar to Ayurveda. The first Ayurvedic influence came to Tibet during the 3rd century AD, but it became popular only after the 7th century with the introduction of Buddhism to Tibet. Thereafter, this trend of exportation of Indian medical literature, along with Buddhism and other Indian arts and sciences, were continued until the early 19th century. India, being the birth place of Buddha and Buddhism, has always been a favorite place for learning Buddhist art and culture for Tibetan students; many Indian scholars were also invited to Tibet for the prorogation of Buddhism and other Indian arts and sciences. This long association with India has resulted in the translation and preservation of thousands of texts of Indian literature in the Tibetan language on various subjects, such as religion, sciences, arts, culture, and language. Out of these, around 25 texts related to medicine are also preserved in both canonical and noncanonical forms in Tibetan literature. Much of this knowledge was further enriched in Tibet with the knowledge and skills of neighboring countries and their own ethnic knowledge.

Pulse diagnosis and urine analyses are distinctive features of Tibetan medical system. Although references to these diagnoses can be found in Ayurveda, Siddha, and the Chinese systems, the method of pulse reading that the Tibetan physicians evolved is elaborate and highly sophisticated. From the beginning, Tibetan physicians have considered pulse diagnosis to be the most reliable diagnostic method for ailments. The fundamental concepts of Tibetan medical systems, similar to Ayurveda and Siddha, revolve around the five cosmo-physical energies and three humoral energies. The working concepts of Ayurveda, such as panchabhautica, tri-doshas, sapta dhutas, and malatraya, also constitute the main features of Tibetan medicine. The most distinctive features of Tibetan medicine are its integrated Buddhist approach to the relationship between mind and body, the application of "Marigpa" or ignorance, and the three inborn mental poisons of attachment, anger, and delusion being the main cause of all suffering (Mukherjee et al., 2007). In India, this system is widely practiced in Sikkim, Arunachal Pradesh, Darjeeling (West Bengal), Dharamsala, Lahaul, and Spiti (Himachal Pradesh), and the Ladakh region of Jammu and Kashmir.

1.8 TRADITIONAL CHINESE MEDICINE

Traditional Chinese medicine (TCM) started in ancient China and developed over more than a 1000 years. TCM specialists utilize herbal medicines and different mind and body actions, for example, acupuncture therapy and judo, to treat or avert medical issues. Acupuncture is thought to be safe when performed by accomplished professionals using sterile needles (Mukherjee et al., 2007). Improperly performed acupuncture can cause possibly serious reactions. Tai Chi and Qi Gong,

two mind and body practices that are a part of TCM, are for the most part safe. There have been reports of Chinese herbal products being contaminated with drugs, medications, poisons, or heavy metals, or of not containing the ingredients that are listed. A portion of the herbs used as part of Chinese medicine can contradict other medications, have serious reactions, or be dangerous for individuals with certain therapeutic conditions. For instance, the Chinese herb ephedra (ma huang) has been connected to serious health difficulties, including heart attack and stroke. For most conditions, there is not sufficient thorough logical confirmation to know whether TCM strategies work for the conditions for which they are used. TCM envelops a wide range of practices, including needle therapy, moxibustion (burning an herb over the skin to apply heat to acupuncture points), Chinese herbal medicine, Tui na (Chinese remedial back rub), dietary treatment, and Tai chi and Qi gong (practices that combine specific movements or postures, coordinated breathing, and mental focus). TCM was established on the old theory of Taoism and goes back over 2500 years. Traditional frameworks of medicine likewise exist in other East and South Asian nations, including Japan (where the traditional herbal medicine is called Kampo) and Korea. Some of these frameworks have been impacted by TCM and are similar to it in some ways, yet each has created particular highlights of its own.

1.8.1 Concepts of Traditional Chinese Medicine

Traditional Chinese medicine is a contemporary medicine in practice in China and an integral part of Chinese civilization. The numerous entries within the Chinese medical specialty are composed on the premise of philosophical background. This philosophy has sustained the continuity of Chinese drugs throughout the past four millennia and TCM has maintained its own identity up to the present day. With thousands of years of expertise in treating diseases with natural materials, TCM still plays a crucial role within the healthcare system of contemporary China and is formally recognized, not only in China, but also in Japan and in other eastern and southeastern Asian countries that have identical cultural traditions. The theory underlying the use of Chinese medical materials forms an integral part of TCM, which describes medication in terms of taste (flavor), property, channel reaction, compatibility, reason, toxicity, and process. There is a theoretical link between the flavoring tastes and characteristics on the one hand and the therapeutic effects on the other hand. A very comprehensive discussion of this subject will be found in a book from this era entitled Yao Xing Lun (Materia Medica of medicative Properties, c. AD 600). This book covers the topics of combination, reaction, taste, temperature, toxicity, function, primary clinical application, process, and preparation. The characteristic application of Chinese medical materials are the combined use of medicine with the thought of accelerating or promoting therapeutic effectiveness, of minimizing toxicity or side effects, of accommodating advanced clinical conditions, and of changing the actions of the medication. A principal drug is directed against, and has the foremost impact on, the causes or the majority of symptoms of the illness and is indispensable to the formula of the prescription. Several formulae encompass solely a principal and one or two associate ingredients. If the principal and also the associate ingredients do not seem harmful, there are corrective adjuvants. The principal ingredient focuses on the amount and site of the disorder, precluding the necessity for a carrier. An associate drug either aids the principal drug in treating the cause or the majority of symptoms or is the major ingredient directed against synchronic symptoms. An adjuvant drug has a role in all the subsequent three completely different functions. First, it reinforces the impact of the principal drug or the associate drug, or directly treats a less severe side of the illness. In this case, it is referred to as a useful adjuvant. Second, it moderates or eliminates the toxicity or harsh properties of the principal or associate drug. In this case, it is referred to as a corrective adjuvant. Third, it may have an opposite impact to the principal drug; however, it produces a supplementary impact within the treatment and is employed in terribly serious and sophisticated disorders. In this capacity, it is referred to as an opposing adjuvant. A carrier drug will either focus the actions of the formula on an exact space of the body or harmonize and integrate the actions of the opposite ingredients of the formula. Not all formulas contain the total hierarchy of ingredients. In fact, it is quite uncommon for a formula to incorporate all the assorted styles of associate, adjuvant, and carrier ingredients. However, a formula might typically contain one principal drug targeted at most of the symptoms of the illness (Zhang, 1990; Bensky and Barolet, 1990).

When considering ancient therapeutic frameworks, for example, TCM, it is critical to isolate questions regarding traditional hypotheses and ideas of well-being and health from inquiries concerning whether particular mediations may be useful with regards to present day science-based medicine and health-promotion practices. The old beliefs on which TCM is based incorporate the following points:

- The human body is a smaller than expected variant of the bigger, encompassing universe.
- Harmony between two contradicting yet reciprocal powers, called yin and yang, bolsters well-being, and sickness comes about because of an irregularity between these powers.

- Five components—fire, earth, wood, metal, and water—emblematically speak to all phenomena, including the phases of human life, and clarify the working of the body and how it changes during illness.
- Qi, a crucial vitality that flows through the body, plays various rolls in looking after well-being.

TCM professionals utilize an assortment of strategies with the end-goal of advancing well-being and treating ailments. These strategies include:

1. *Chinese herbal medicine*: The Chinese *Materia Medica* lists a large number of therapeutic substances, fundamentally plants, yet in addition a few minerals and animal items. Distinctive parts of plants, for example, the leaves, roots, stems, blooms, and seeds, are utilized. In TCM, herbs are regularly combined in recipes and given as teas, capsules, fluid concentrates, granules, or powders.
2. *Acupuncture*: Acupuncture is a group of methodologies, including the incitement of particular foci on the body, utilizing an assortment of strategies. The acupuncture system that has been regularly examined scientifically includes penetrating the skin with thin, solid, metal needles that are controlled by the hands or by electrical means.
3. *Tai chi*: A centuries-old mind and body practice. It includes delicate, dance-like body movements with mental concentration, breathing, and relaxation.

1.8.2 Quality Evaluation of Chinese Herbal Medicine

Chinese TM research is concentrated on the isolation and structural determination of pharmacologically active substances from TCM, followed by determination of their therapeutic profile. Chinese herbal medicine research is focused on the discovery of medicinally important TCM drugs, on the pharmacological basis of their standard uses, and on the development of techniques for quality control of the various herbs and preparations used in TCM medicine. Serious efforts have been made in the analysis of Chinese medical materials since the 1920s, with resulting progress. The Chinese government inspired this analysis of Chinese medication through the National Projects of Science and Technology to establish a high-quality system. The wide utilization of TCM in China and within the West drives the need for the development of comprehensive scientific monographs. These monograph documents are developed by establishments recognized by the New Drug Analysis Committee of the Ministry of Public Health, China. On the premise of the results of contemporary research projects, new rules are introduced in China as a part of a high-quality assurance program for TCM. Thin-layer chromatography is used for comparing the fingerprint of the sample and authentic material is needed for those plants for which other assays of active constituents have not been developed. The manufacturers are obligated to produce documents of each herbal products regarding their pharmacodynamics, toxicology, quality control methodology, effectiveness, and safety (Zhu and Qiao, 1989a, b).

1.9 JAPANESE TRADITIONAL MEDICINE

Japanese traditional herbal medicine (Kampo medicine) was inspired by a period of long historical development in Japan. In Japan, the administration of herbal medication goes back by over 1500 years. Later decades have seen a rediscovery of Kampo medicine in medical practice, together with a scientific reconsideration and examination of its importance in current healthcare (Watanabe et al., 2011). The expression "Kampo," which actually means "technique from the Han time period" (206 BC to AD 220) of ancient China, refers to its birthplace in old China. The fundamental helpful handbook for the utilization of herbal solutions was the *Shang hanlun*. In the Edo-period from AD 1600 onward, the particular Japanese attributes of Kampo evolved. Japan's isolation from the outside world prompted regularly increasing alterations from the predominantly Chinese ideas. The gigantic assortment of Chinese traditional medications was decreased, with the most solid medications consequently consolidated into ~300 remedies. From a realistic perspective, Japanese doctors criticized the very hypothetical and theoretical nature of Chinese drugs as being unable to meet the issues of regular practice. The most critical appraisal originated from Yoshimasu Todo in the 18th century who stated, "In clinical solution, we should just depend on what we really have seen by examination of the patient." For Yoshimasu Todo, one approach to collect information on the state of the body was to inspect the abdomen area, for which he built up a refined palpation strategy (fukushin). The results of this palpation would give vital clinical data with a specific endpoint to choose the most suitable herbal medicine for the patient. Yoshimasu Todo's practical outlook and the abdominal palpation procedure (fukushin) as a diagnostic method have impacted Kampo treatment up to the present day.

Kampo TMs have been incorporated into the Japanese National Health Insurance drug list since 1971. About 148 Kampo herbal medicines have been recorded to date. The use of Kampo has steadily increased and, as indicated by a study by the *Nikkei Medical* journal, over 70% of doctors recommend Kampo medications today. The Japan Society for Oriental

Medicine is the largest society for Kampo medicine and has 8600 individuals and 2600 certified board members. In 2001, Kampo medical education was incorporated into "the model core curriculum" by the Japanese Ministry of Education, Culture, Sports, Science, and Technology (Maegawa et al., 2014). The development of present-day ready-to-use forms was directly connected with the large increase in Kampo use, for the most part as spray-dried granular concentrates of the traditional formulae. They have progressively replaced the traditional decoction of crude medications, despite the fact that they are covered by the national insurance framework. Other than being simple to administer, industrial production has empowered its advantages. The quality control of the purity and toxicity is standardized in Japan, following the Japanese pharmacopoeia and globally established regulations for GMP and GLP. The standardization of the main components has turned out to be possible and this is a precondition of clinical research. Today, extract preparations make up to 95% of the Japanese Kampo market.

1.10 THAI HERBAL MEDICINE

Thai TM is a national identity, handed down from the wise Thai ancestors. These people had accumulated their healthcare experiences in fighting against the problems of illness and disease since the period of Sukhothai (more than 700 years ago). Ranikhanihaeng, a great king at that time, had ordered the establishment of a large medicinal plant garden to serve as a source of drugs for the treatment of the ailments of his people. Thai TM can be categorized into four major systems (Luangpirom, 2015):

- traditional herbal medicine;
- psychological treatment;
- traditional massage and physical therapy; and
- traditional herbal medicine based on the element or humor theory.

Traditional herbal medicine was widely practiced in ancient Thai society. It is based on the personal experiences of the people in a village or a community in a particular culture. This form of knowledge has existed and been inherent from one person to another, from one family to another, or even from one culture to another. They tend to use medicinal herbs frequently found at or close to the home, by application, sniffing, or oral routes. The fruits of the ebony tree (*Diospyros mollis* Grift), for example, may be used for the treatment of intestinal worms.

Thai traditional massage has become increasingly popular in Thailand, even though its history can be traced back to the time when Buddha was still alive. It is believed that the founder of the ancient art of massage was Jivaka Kuniar Bhaccha, who was a contemporary of Buddha and also his personal physician over 2500 years ago. In Thailand, the knowledge of Thai traditional massage was revived by King Rania III, who ordered the medical texts on this subject to be engraved at Phra Chetuphon Temple (Wat Pho) in 1832. Even today, one of the most outstanding massage schools in Thailand is located at Wat Pho in Bangkok (Disayavanish and Disayavanish, 1998).

Thai traditional massage can be used to promote health and to treat some diseases in combination with vapor or steam baths and herbal medicine. In some rural areas, the folk or traditional healers have demonstrated their expertise in treating bone fractures. They tend to use bamboo sticks to make splints and most also use magical powers to promote the healing process. The only system accepted by the Thai government or the Ministry of Public Health is herbal medicine based on element or humor theory. In the body, there are the following elements:

- Earth (solidity)
- Water (fluidity)
- Fire (heat)
- Wind (motion)

Most of the theories of Thai traditional medicine have originated from the four elements. In every part of Thailand, schools for Thai traditional medicine exist and produce a small number of qualified traditional doctors annually. These doctors need to be given a traditional medical license approved by the Ministry of Public Health before they can go into practice (Chuthaputti, 2007).

1.11 TRADITIONAL MEDICINE IN BANGLADESH

The systems of TM indigenous to Bangladesh are Unani-Tibb and Ayurveda. TM has a long history in Bangladesh, practiced in and acknowledged by both provincial and urban populations. Medications fundamental to the practice are discovered in the soil, mainly without serious long-term side effects, and viable in specific situations in which present-day

medicine has failed. Officially recognized by the government of Bangladesh after its independence, Unani and Ayurvedic drugs were brought under a drug control framework in 1982 to provide oversight of manufacturing and marketing. Training institutions have grown in number from 2 in 1974, to 12, all recognized and assisted by the government, which runs a 4-year degree program. Given the achievements and extensive presence of TM in Bangladesh, the administration is thinking about linking it to the primary healthcare (PHC) system. Such activity is viewed as a cost-effective, relatively convenient way of providing healthcare to an extensive area of the country.

When Bangladesh constituted the eastern part of Pakistan, the Pakistani Board of Unani and Ayurvedic Systems of Medicine was in operation. Following independence, the Bangladesh Unani and Ayurvedic Practitioners Ordinance of 1972 restructured this body to be the Board of Unani and Ayurvedic Systems of Medicine, Bangladesh. The Board is in charge of maintaining educational guidelines at teaching organizations, organizing the enlistment of appropriately qualified persons (delegating a register), and also the institutionalization of Unani and Ayurvedic Systems of medicine. A research establishment has been working under the Board since 1976. The Bangladesh Unani and Ayurvedic Practitioners Ordinance of 1983 forbids the practice of the Unani and Ayurvedic systems of medicine by unregistered practitioners. A noteworthy element of the Ordinance is the exclusion of an arrangement contained in the enactment that made it an offense for an Ayurvedic or Unani expert to sign birth certificates and physical-fitness certificates.

Control over the education of Unani and Ayurvedic medicine rests with the Board of Unani and Ayurvedic Systems of Medicine. There are nine teaching institutions under the Board, five for Unani medicine and four for Ayurvedic medicine. They offer diplomas after a 4-year program. The Registrar of the Board also serves as the Controller of Examinations. TM is popular among family households. Generally speaking, 48% of families looked for treatment from traditional practitioners for themselves or for any of their relatives who were poor, characterized as those whose monthly family wage was under 10,000 Taka. Families sought treatment from traditional practitioners mostly for women and children experiencing fever, pain, the common cold, and for general afflictions, for example, anemia, helminthiasis and nutrition, eye infection, regular dental ailments, and ear issues. Others looked for treatment for nontransferable illnesses, for example, diabetes, cardiovascular infection, hypertension, heart diseases, and hypertrophy of the heart. The significant reasons for seeking traditional suppliers were low cost, no side effects, timely services, and especially local availability, which makes the administration effectively open. It has been found that unskilled and minimally educated people are the primary customers of TM. An impressive number of existing customers are observed to be acquainted with TM and have been utilizing it for a long time. Hence, for the purpose of adequacy, the customers were happy with TM. The majority of the customers noted that they never had any unwelcome side effects after utilizing TM. Suppliers likewise disseminated handouts in well-known areas, undertook advertising on local TV, and made wall paintings to draw in less-educated individuals. It was apparent, despite existing law and strategy in regards to the production and practice of TM in Bangladesh that inadequate checks prompt the improper preparation of medicine with low quality or even the manufacturing of medicines without legitimate authorization because of the inaccessibility of appropriate medicine testing research facilities for TMs (WHO, 2001).

1.12 IRANIAN TRADITIONAL MEDICINE

Traditional Iranian Medicine (TIM) goes as far back as 8000 BC. It is a division of Arabic-Unani medication as referred to by Cyril Elgood, the English medicinal history specialist. TIM includes a wide range of medical experiences used in the prevention, diagnosis, and treatment of diseases based on the humor theory of temperament in which the liver is one of the most important organs in the body (Akbarzadeh et al., 2015).

Among Persians, traditional science had a high status. Persian conventional sciences enjoyed royal support and had immense importance because Persian kings were magnificent scholars. After the murder of Darius and the control of Kelikiyeh by Alexander, the incalculable Persian books and sciences were passed to the Greeks; thus, the historical backdrop of this medicine is associated with old Greece (Zeinalian et al., 2015). Some of the earliest practices of ancient Iranian drugs are documented within the sacred Avesta and the various Zoroastrian (the ancient pre-Islamic religion) texts. Throughout the Achaemenid era (559–330 BC), 21 books of sacred texts encompassing 815 chapters were a reference for science, consisting of drugs, astronomy, law, scientific discipline, philosophy, cognition, logic, and biology. It is inferred from these books that Zoroastrians placed much importance on personal hygiene, public health, and also the prevention of contagious diseases. The foremost scholars of drugs and pseudoscience were Iranian Magi and Mobeds (Zoroastrian priests) who passed their data on to their pupils from one generation to the next. Herbs, such as wild rue and gum, were continuously burned within homes to kill insects and bacteria, a custom that continues to the present day. The sacred Avesta has mentioned many medicinal herbs, including basil, chicory, English violet, and peppermint, whereas Bundahishn cites the names of 30 sacred medicative plants. Avestan texts list not only the varied parts of plants used for treatment, such as roots, stems, scales, leaves, fruit, and seeds, but also indicate that the plant is the remedy for every illness (Akbarzadeh et al., 2015).

According to the Zâdspram, a Pahlavi text of the 9th century AD, there are thousands of species of medicinal plants created by the Persian deity (the highest spirit idolized in Zoroastrianism) for the prevention of thousands of sicknesses created by Ahriman and the simplest of those plants is haoma (Vedic soma). Haoma (*Ephedra vulgaris*) is native to the Iranian upland and contains an oversized amount of alkaloid that is effective in the treatment of respiratory and cardiac diseases. Garlic was used to scale down high pressure, combat heart conditions, and treat infections. Rue was once a preferred remedy for aches, easing shaking fits, and for joint pain; it was also used to disinfect the house. Bangha, extracted from Indian hemp (*Cannabis indica*) seeds, has psychoactive effects and was used as an anesthetic.

Frankincense was used for medicative inhalation. Aloes wood was used for cardiac health and in the treatment of irregular heartbeat. Several modern Iranian herbalists use traditional books in which borage, sweet marjoram (*Majorana hortensis*), fenugreek, and chicory are used in treatment. Ancient Persian physicians believed that health is that the result of the "right" proportions of humor, whereas illness is the product of their excess or deficiency. Therefore, the medication of the body consists of keeping the body in health and reestablishing balance. The Vendidad tells of three types of drugs used: drugs by the knife (surgery), drugs by herbs, and drugs by divine words. A Mazdean physician-in-training is required to treat and cure three non-Mazdean patients before receiving permission to treat Mazdeans. During this time, physicians are tutored to treat any and every patient, whether friend or foe. Avestan scriptures did not limit giving treatment to Mazdeans alone. The Ordibehesht Yasht classified physicians into five categories:

- *Health doctors (Ashoo Pezeshk)*: They were accountable for the well-being of a town, preventing the spread of contagious diseases by quarantining, keeping the four sacred components of water, wind, earth, and hearth free from contamination, and ensuring that the sanitation of homes was maintained.
- *Medical examiner (Dâd Pezeshk)*: Just like modern pathologists, their duties included examining the dead, autopsies and the provision of burial licenses, and ascertaining the reason for death with a watch toward finding cures for future cases.
- *Surgeon (Kard Pezeshk)*: Archaeological excavations within the burnt town in Sistan have yielded skulls that show signs of surgery. Surgical procedures, troublesome and dangerous even now, were far more so in the past when it was difficult to properly anesthetize patients and when medical instruments were rudimentary.
- *Herbalist (Gyâh Pezeshk)*: The origin of herbal drugs predates the event of agriculture and cultivation in Persia; nonetheless, some believe that the traditional Persians were the first to document the properties of herbs and to cure diseases.
- *Psychiatrists (Mantreh Pezeshk)*: This doctor used holy words and prayers to cure patients with an illness of body and soul that could not be cured with herbs. Treatment consisted of verbal communication, the reading of poetry, paying attention to music, and also the recitation of prayers, including ones from the holy books of different nations, which were designed to console and heal the patient. Avestan texts tell of consultation among the surgeons, herbalists, and psychiatrists that indicates a sort of medical association at the time.

The first doctor as documented by Avestan texts was Vivangahan, followed by Abtin, Atrat, and Purshaspa. Mani, Roozbeh, and Bozorgmehr are among the opposite notable Persian physicians named within the sacred Avesta. Shahpour I developed the international university in 271 AD, which was a center of learning and study within the fields of science and drugs. The antique faculty continues to be a medium of information in Khuzestan Province in southwestern Persia. It was a crucial cultural and scientific center of the Sassanid era (AD 226–652).

Iranian drugs, which combined medical traditions from the Greece, Egypt, India, and China for 4000 years, became the inspiration for the medical practices of European countries throughout the 13th century. Among the torchbearers of ancient Persia's scientific heritage are Mahomet Zakaria Razi, Abu Nasr Farabi, Omar khayam, and Avicenna, who used these data to make additional discoveries of profit to all mankind.

- Razi, legendary within the West as Razes (AD 865–925), thought to be the father of pediatric medicine and a pioneer of neurosurgical processes and eye specialties, discovered and refined the use of ethanol alcohol in drugs.
- Farabi, also legendary in the West as Alfarabius (AD 872–951), is noted for his contributions to psychological science. He wrote the primary treatises on psychology.
- Avicenna (AD 980–1037), a prolific genius, introduced systematic experimentation into the study of physiology, experimental drugs, evidence-based medicine, clinical trials, risk correlation analysis, and the idea of a syndrome. He is known for his contributions in clinical pharmacology and neuropsychiatry.
- Khayyam (AD 1048–1131) was a notable astronomer who contributed to arithmetic and calendar reform.

Considering the definitions given by the WHO, TIM can be separated into two areas that are composed of conventional pharmaceutical and verbal customary medicine. Traditional Iranian Medicine has two fundamental branches, in particular a hypothetical branch and a practical branch, which can each be separated into subbranches:

- *The theoretical branch of TIM*: A science that discusses the status and changes of the human body and the causes and signs of health and disease.
- *The practical branch of TIM*: Although it may remind us of practical procedures, it is really the science of how to keep health and how to bring it back after the occurrence of disease.

The Seven Natural Affairs in TIM, physiological functions of the human body, are considered to be based on seven factors, known as "Umoor-e-Tabee-e-ya." These are Arkan (elements), Mizaj (temperament), Akhlat (humors), Azaa (organs), Arwah (spirits), Quwa (faculties or forces), and Af'al (functions). TIM gives more consideration and significance to the counteractive action of illness instead of its cure. Maintaining well-being was important to the point that the primary obligation of doctors was to keep individuals healthy and to treat them on the off-chance that they became sick. There are six variables fundamental for the upkeep of good health that are called "Setteh-e-Zarurieah." These basic components are air, food and drink, rest and attentiveness, evacuation and maintenance, body movement and rest, and mental development and rest.

An irregularity in body disposition and humors causes the beginning of a disease condition. In this manner, treatment depends on the redress of temperament and humors to accomplish a balanced state. Each illness has a particular temperament. In this way, the medication used for the treatment should have the contrary disposition to that of the infected humor, bringing about normalization of the mind. For example, a disease that is cold in nature can be cured by a medication that has a hot disposition. It should be noted that not all of the ailments in TIM are dealt with in light of the demeanors, because there are three types of illness as indicated by TIM:

1. Distemperaments (distortion of the temperament of a single organ up to the whole body).
2. Disfigurements (distortion of the anatomy or the structure of an organ).
3. Disconnections (distortion of the correlation and connection between organs).

Distemperaments are mostly treated by the correction of temperament and the latter two are mostly treated by special drugs or manipulation, including surgery. TIM physicians have four modes of treatment: Tadbirba Sitteh-e-Zarurieah (correction of the six essentials), Tadbirba Ghaza (diet-o-therapy), Tadbirba Dawa (pharmacotherapy), and Aamaleya daavi (manipulation or physical therapy). These modes might also be considered as levels of treatment; if a level fails or is inadequate, the next level is considered (Emtiazy et al., 2012). The Faculty of Traditional Medicine in Persian medicine is a part of the culture and history of Iran and today the Islamic Republic of Iran is moving toward discovering its useful medical treasures to share with mankind all over the world and to take its role in the worldwide move toward integrative medicine.

1.13 MALAYSIAN TRADITIONAL MEDICINE

In Malaysia, traditional and complementary medicine (TCM) is classified into six major groups, namely, traditional Malay medicine, traditional Chinese medicine, traditional Indian medicine, homeopathy, complementary medicine, and Islamic medical practice. Malaysian studies have reported that 69.4% of the Malaysian population have used TCM in their lifetime, and about 55.6% of people have used TCM within a 12-month period (Kew et al., 2015). TM practices brought by Indian and Chinese trades and migrants supplemented, but did not supplant, the indigenous medicinal framework in Malaysia.

The variety of therapeutic systems in Malaysia mirrors the differing populations of Malay, Chinese, Indian, and indigenous legacy. Notwithstanding allopathic medicine, the significant systems of medicine honed in Malaysia incorporate Ayurveda, Siddha, Unani, traditional Chinese medicine, and traditional systems of medicine that are given by TM specialists, mystics, bonesetters, traditional birth orderlies, and other people who utilize home cures. Therapeutic choices likewise incorporate homeopathy, naturopathy, reflexology, aromatherapy, and chiropractic. Traditional Malay medical practices can be followed fundamentally to Indonesia. These medicinal practices are particularly well known among Malay in country regions; they depend on practical experience and perception and are passed on orally. Therapeutic treatment may incorporate saying spells over water and offering it to the patient to drink, or administering herbs orally or remotely. More than one of these alternatives might be utilized and more than one TM professional might be called upon (WHO, 2001).

Chinese traditional medicine is accepted to have been brought into Malaysia by Chinese migrants working in the tin mines. These migrants brought natural medicines and different types of treatment, including acupuncture. Chinese medical professionals hold high status and are known as "Sinseh." Today, traditional Chinese medicine is likewise used in urban areas. All traditional Indian therapeutic systems are practiced in Malaysia. The larger part of medicines utilized as part of these systems comes from vegetable, mineral, and animal sources. Herbal arrangements and herbal items are imported from India as therapeutic tablets, oils, ointments, metals, mineral inventions, and natural powders. The 1996 National Health and Morbidity Survey II found that 2.3% of the general population surveyed consulted a traditional or correlative/elective medicinal specialist and 3.8% utilized both allopathic medicine and traditional Chinese medicine. Although no measurements

are accessible, TM is primarily practiced by suppliers of TM, although allopathic medicinal suppliers practice corresponding/elective medicine and additionally allopathic medicine (Sultana et al., 2014).

In Malaysia, sales of traditional and traditional/complementary medicines are estimated to be 1000 million Ringgit (Malaysian currency) every year compared with a market worth 900 million Ringgit for allopathic pharmaceuticals. The official healthcare system adopted and actualized by the Malaysian Government is an allopathic one. Subsection 1 of Section 34 of the Medical Act of 1971 contains the accompanying broad exception:

> *Subject to the arrangements of subsection 2 and directions made under this Act, nothing in this Act might be considered to influence the privilege of any individual, not being a man taking or utilizing any name, title, expansion or depiction figured to prompt any individual to trust that he is qualified to practice medicine or surgery as per current logical strategies, to rehearse frameworks of therapeutics or surgery as indicated by absolutely Malay, Chinese, Indian or other local techniques, and to request and recoup sensible charges in regard of such practice. Subsection 2 restrains the treatment of eye sicknesses to professionals of allopathic medicine. In like manner, the Poisons Ordinance of 1952 confines the utilization of specific substances to professionals of allopathic medicine. (WHO, 2001)*

There are no other laws influencing traditional health practice in Malaysia; however, there are various laws that control the production and sale of TMs. These are the Poison Act of 1952, Sale of Drug Act of 1952, Advertisement and Sale Act of 1956, and the Control of Drugs and Cosmetics Regulations of 1984. Since 1992, TM items have been registered. The Malaysian Government has published the *Malaysian Herbal Monograph and Compendium of Medicinal Plants Used in Malaysia* and also established an information hub on integrated medicine for the world through Malaysia utilizing strategic partnerships with other agencies. Several guidelines for the levels and types of evidence to support claims for therapeutic products, for the clinical evaluation of TCM interventions, intellectual property management, and for standardization of herbal medicinal products have been set out by the Malaysian Ministry of Health. The Drug Control Authority is in charge of product registration, including quality and safety. Each producer of TM is required to consent to GMPs, and importers are required to conform to good storage practices. The Ministry of Health has set up the Steering Committee on Complementary Medicine with a multisectoral participation to advise and help the Minister in planning approaches and techniques for checking the practice of traditional Chinese medicine in the nation (Xiang-dong et al., 2010).

A national policy is being drafted on traditional Chinese medicine to urge established practitioners to shape their own self-regulatory bodies. These bodies will empower a system of authority to register the member-practitioners. To guarantee that the capabilities of experts are recognized and can be certified for formal enrollment, the bodies are required to set formal benchmarks, including training, for their own particular practices. They are likewise urged to update the aptitudes and knowledge of their members. The Unit of Traditional Chinese Medicine has been set up at the Primary Health Care Section, Family Health Development Division, and Ministry of Health. It will be in charge of observing and encouraging the use of the Ministry's arrangements and also strengthening national and universal coordination. The umbrella body for traditional Chinese medicine has issued a Practice Approval Certificate for specialists who have taken its courses or courses from a recognized college. This endorsement is required for a Business License Certificate. Homeopathy will be presented as a training option at the recently established Faculty of Biomedicine. Neither national healthcare protection nor private protection covers traditional Chinese medicine in Malaysia. Malaysia is committed to seeing TCM integrated into the Health System.

1.14 TRADITIONAL MEDICINE IN AUSTRALIA

In Australia, complementary medicines (also known as "traditional" or "alternative" medicines) include vitamin, mineral, herbal, aromatherapy, and homeopathic products. Complementary medicines may be either listed or registered, depending on their ingredients and the claims made. The national policy on TM/CAM was issued in 1999. Regulations on TM/CAM in the form of the Therapeutic Goods Act were issued in 1989. The national program and national office for TM/CAM, the Office of Complementary Medicines, was established in 1999; the office is administered by the Ministry of Health. In 1997, the Complementary Medicines Evaluation Committee was established as the expert committee. In 1989, Australia began regulating herbal medicines by means of the Therapeutic Goods Act, which contains partly the same regulations as those issued for conventional medicines. Herbal medicines are regulated as over-the-counter medicines for self-medication; the specific categories are "registered goods" and "listed goods" and form part of the Australian Register of Therapeutic Goods. Medical, health, nutrient content, and structure/function claims may be made for herbal medicines by law (Oliver, 2013).

There are 1500 natural medications listed in Australia; none is incorporated on the national basic medication list. The review system has included unfavorable impact evaluation since 1970. In Australia, natural medications are sold in pharmacies as finished medications, in special outlets, by authorized specialists, and without confinement. Regulatory necessities

for herbal medications incorporate adherence to data in pharmacopeias and monographs and the same GMP controls as those used for conventional pharmaceuticals. Execution of these prerequisites is guaranteed through the GMP required for finished goods manufacturers. Safety requirements for herbal medicines incorporate indistinguishable prerequisites from those for conventional pharmaceuticals, and also uncommon necessities of conventional use without proven harmful effects and reference to reported logical research on comparable items. Consistence with these necessities is guaranteed through "compositional guidelines" for endorsed integral prescription substances that portray the character tests and points of confinement for contaminants and buildups, despite the fact that these are not lawfully official on manufacturers. Other control components incorporate postadvertising surveys, assessment of toxicological information on new proposed herbal substances, and history of utilization information.

The issue of control of the Western herbal solution and naturopathy callings has been around for a long time. In Australia, there is a national registration system for fully and partially enrolled health experts called The Australian Health Practitioner Regulation Authority (AHPRA). AHPRA's operations are administered by the Health Practitioner Regulation National Law, and are in each state (the National Law). This Law became effective on 1 July 2010 and has empowered that without precedent for Australia, 14 health callings have possessed the capacity to be directed by broadly predictable enactment under the National Registration and Accreditation Scheme. AHPRA bolsters the 14 National Boards that are in charge of controlling the health callings. The essential part of the National Boards is to ensure the general population and set principles and approaches that all enrolled health professionals must meet. In 2011, this was trialed by mostly enrolled callings like TCM. In the third and last round, the working party was requested to think about the need to direct any unregulated health callings, including cultivators and naturopaths.

The TGA orders generally safe medications as recorded drugs. TGA does not evaluate each recorded prescription to decide if it will work in the way the provider claims it will. The listed medicine is permitted to be marketed in Australia if:

- It contains nothing but preapproved low-risk ingredients. These preapproved ingredients have been evaluated by the TGA for quality and safety but not for evidence that they will work.
- The manufacturing site (if in Australia) is inspected and licensed by the TGA or if manufactured in a facility overseas the site has been assessed by the TGA and determined to meet appropriate standards. This means there are systems in place to control the quality of the final medicine.
- It does not make claims or imply that it will be useful in the treatment or prevention of serious illnesses that would require the involvement of a health professional.

Sponsors of listed medicines must hold evidence that:

- It supports the health benefits or claims they are making about their product.
- The medicine has been manufactured by a facility that has been authorized by the TGA.
- The medicine only contains preapproved low-risk ingredients.

Many listed medicines, for example, multivitamins, have multiple ingredients that go into the final medicine. Before an ingredient can be used in a listed medicine, TGA scientists review the ingredient's safety. The TGA does not assess whether there is any evidence to show that the ingredient in the listed medicine is effective in the way claimed by the sponsor of the medicine. In undertaking a review to establish the quality of an ingredient for use in listed medicines, the TGA will look at, among other things:

- composition of the ingredient (what the ingredient is and its purity);
- stability of the ingredient (how long the ingredient remains stable at a particular temperature without deteriorating significantly—this is used to determine shelf life and recommended storage temperature); and
- to ensure an ingredient is safe for use in listed medicines the review includes looking at the history of use; biological activity (how the ingredient interacts with systems in the body) and the likelihood of the ingredient being harmful, based on available published evidence and any reported adverse reactions.

Therapeutic goods must be entered on the Australian Register of Therapeutic Goods (ARTG) before they can be lawfully supplied in Australia. Prior to listing on the ARTG the TGA does not evaluate the final product (in contrast to the ingredients); the label and the therapeutic claims made for the medicine or the evidence the sponsor may have to show the product will do what they say it will do.

Traditional medicine practice (TMP) inside Aboriginal Australia incorporates a holistic perspective that mirrors the WHO definition of health, which is one of "physical, mental and social prosperity and not simply the nonappearance of disease or illness" (WHO, 2002). This perspective recognizes good health as a complex framework including interconnect-

edness with the land, acknowledgment of soul and lineage, and social, mental, physical, and emotional well-being both of the individual and the community. Indigenous Australians see illness as the consequence of one of three causes: a characteristic physical reason, a spirit causing damage, or infection due to divination. The effect of colonization and the resulting uprooting and disengagement of individuals both from their traditional lands and later from their traditional families has been huge in its subsequent impact on the utilization of traditional practices, including TM.

The Alma-Ata declaration on PHC by the WHO in 1978 saw a reaction from a few nations to enhance their TM use and regulation of use within the essential primary health care model. PHC for Aboriginal and Torres Strait Islander Australians is at present tended to by either government-controlled health services or community-controlled health services (ACCHS) that offer biomedical healthcare and work to trained Aboriginal Health Workers. ACCHS are started and administered by the a nearby Aboriginal Community to empower conveyance of comprehensive and socially proper healthcare to the separate group(s). This holistic methodology in the evolution from essential therapeutic care to primary health care as adopted by the Alma-Ata assertion in 1978 has been praised; however, there has been no mention of the incorporation of TM use within the outline of these health administrations as other countries have. It is recognized that in remote areas in other countries it is likely for TM to coincide with biomedical healthcare as a major aspect of a pluralistic medical system. It is unclear if this additionally applies to Aboriginal Australia and provided that this is true, to what degree TM is practiced and how it sits with the use of biomedical healthcare.

The Office of Aboriginal and Torres Strait Islander Health (OATSIH) has provided details regarding the arrangement of health services in 2010–11 incorporating traditional healing and bush medicine service arrangements. It is one thing for TM to be practiced in conventional ways at a neighborhood level and another for it to be recognized as a major part of a national social insurance procedure. In Australia, there are no national government associations for Aboriginal TMP. There is one national nongovernmental association currently in operation. The Indigenous NGO Aboriginal and Torres Strait Islander Healing Foundation Ltd. have supported new undertakings for Indigenous healing, for example, the Angangkere Healing Project in Alice Springs, and the Rumbulara Traditional Healing Centre in Victoria, running as a part of the Rumbulara Aboriginal Co-agent Ltd., which will be built up separately to the present therapeutic administration PHC facility. Conversely, New Zealand has a National Board of Maori Traditional Healers and in 1999 the NZ Ministry of Health distributed a set of guidelines for conventional Maori healing, while in the United States in 2002 the Association of American Indian Physicians affirmed a resolution recognizing and supporting Native American traditional healing and solution as a component of the range of social insurance appropriate for Native Americans. The most recent reaction to the improvement of a National Aboriginal and Torres Strait Islander health plan was for an increased recognition and incorporation of Aboriginal conventional medication inside the health plan. It has been discovered that managed administration structures can possibly enhance the nature of TMP, including the reduction or elimination of deception by specialists. Enhancing the quality and amount of research inside Aboriginal Australia could possibly add to essential human services for Aboriginal and Torres Strait Islander Australians (Oliver, 2013).

1.15 NATURE'S NATURAL PRODUCT LIBRARY FOR NEW DRUG DISCOVERY

In spite of the considerable chemical variety from the improvement of combinatorial sciences and high-throughput screening strategies in recent years, natural products and related structures continue to be critical components of pharmacopeias. Going forward, natural products and related structures may become considerably more essential for the advancement of enhanced and new pharmaceuticals because of the assortment of practically applicable secondary metabolites of microbial and plant species, whose concoction and genetic variety are being uncovered by DNA sequencing and related genomics and bioinformatics technology (Mukherjee et al., 2016).

Up until now, strategies for recognizing and describing the exercises of secondary metabolites have been wasteful and frequently bleak; however, the latest advances in genomics, informatics, and related 21st century omics innovations are drastically quickening the pace of discovery and investigation. Refined fractionation techniques hyphenated to current spectrometry and spectroscopy techniques can characterize the metabolomes of cells, tissues, and even life forms (Mukherjee et al., 2017a, b). Multivariate investigations and network modeling empower complete distinguishing proof and assessment of natural products of assorted varieties and usefulness; and when incorporated with network approaches, it is possible to profile molecular changes caused by transformation and by pathogens and other ecological stressors, and accordingly to anticipate the objectives and mode(s) of activity and toxicities of natural products and subordinates. This commitment of natural products appears to be moving, particularly against a background in which interest in natural products by significant pharmaceutical organizations has been minimal. There are a few reasons for this lack of excitement among businesses. The United Nations Convention on Biological Diversity has concerns in regards to the regulation of natural products and their access worldwide. This can be managed by following regular best practice.

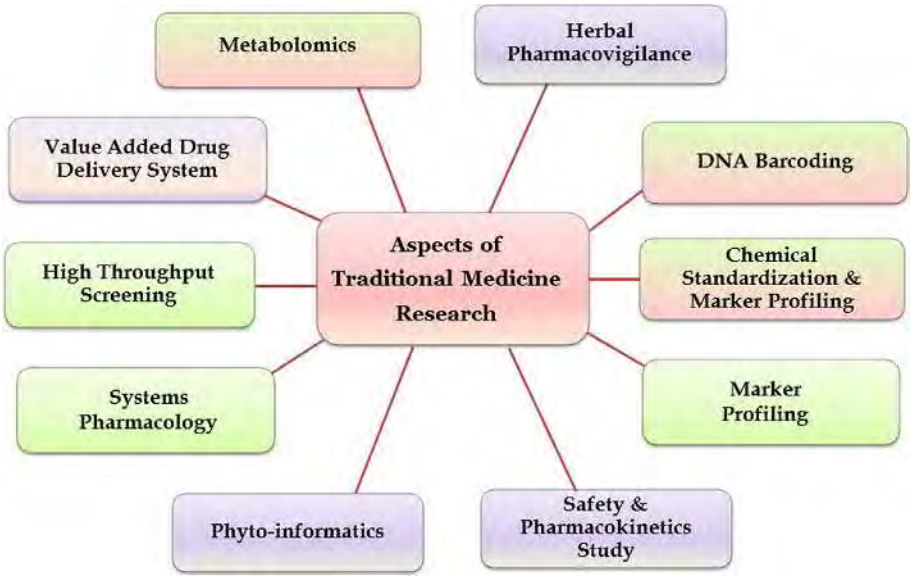


FIG. 1.2 Different aspects of research and development of traditional medicine.

The natural product compilation uncovers a broad scope of pharmacophores with a high level of stereochemistry. These benefits can add to the capacity to give hits, even those contrary to the new difficult screening targets, for example, protein–protein interaction. However, natural products may demonstrate some extra advantage over manufactured synthetic versions of natural metabolites: aggravates that are prospering as drugs have been found to demonstrate the property of metabolite resemblance. This implies natural products can be organically dynamic and furthermore liable to be substrates for at least one of the numerous transporter networks that deliver the drug to the intracellular site of activity. Several aspects of research for TMs have been highlighted in Fig. 1.2. These focal points have roused a redeveloping enthusiasm for natural product research in drug discovery (Mukherjee et al., 2017a, b).

1.16 DRUGS DERIVED FROM TM

In most countries today, the practices of allopathic and traditional systems of medicine exist side-by-side in a complementary way. Consequently, efforts should be made to harmonize the process of their evaluation and quality control for the optimum utilization of botanicals. An urgent need exists for TM to direct natural product research. Approximately 70% and 42% of cancer and rheumatic patients used complementary and alternative medicine. Garlic, Ispaghula, Ginseng, ginger, gingko, St. John's wort, and saw palmetto are a few examples of scientifically validated botanicals that are regularly prescribed by modern physicians. Ensuring the accurate identification and authentication of the herbs is the first crucial step to avoid confusion, admixtures, or adulterations in the botanicals. In order to ensure quality assurance, plant identification is crucial to guarantee that the correct plant raw material has been used. The identification of medicinal plants may be achieved by combining methods, including macroscopic and microscopic examination, chemical fingerprinting, and DNA-based characterization. The useful active compounds in plants for medicinal preparations are among the huge diversity of secondary plant products that are often specific for certain plants or plant groups (Mukherjee et al., 2009). In order to ensure the correct identification and authentication of TM herbs, some specific measures are considered (Mukherjee, 2002). There are several important integrated strategies that need to be considered for validation in traditional systems of medicine, such as Ayurveda (Mukherjee et al., 2015). The important aspects of evidence-based validation of TM are shown in Fig. 1.3. Malaria is a perpetual killer of humankind. Quinine (Fig. 1.4.) has been used in the treatment of malaria for over 400 years (Achan et al., 2011). It was first isolated from the bark of Cinchona species (*Cinchona officinalis*) by two drug specialists from France (Buss et al., 2003). This was later substituted by manufactured versions of chloroquine and mefloquine. However, the final lead came from traditional Chinese medicine in the form of *Artemisia annua* (Quinhaosu) as malarials.

In 1971, Chinese researchers focused on discovering antimalarial agents by utilizing ethnopharmacological information from TCM, leading to artemisinin (Cragg and Newman, 2013). They started with over 2000 Chinese herb formulations and distinguished 640 hits with possible antimalarial potential. After a long trial, they recognized qinghao (the Chinese name of *Artemisia annua* L.), which showed good effect against plasmodium parasites. However, their results were not

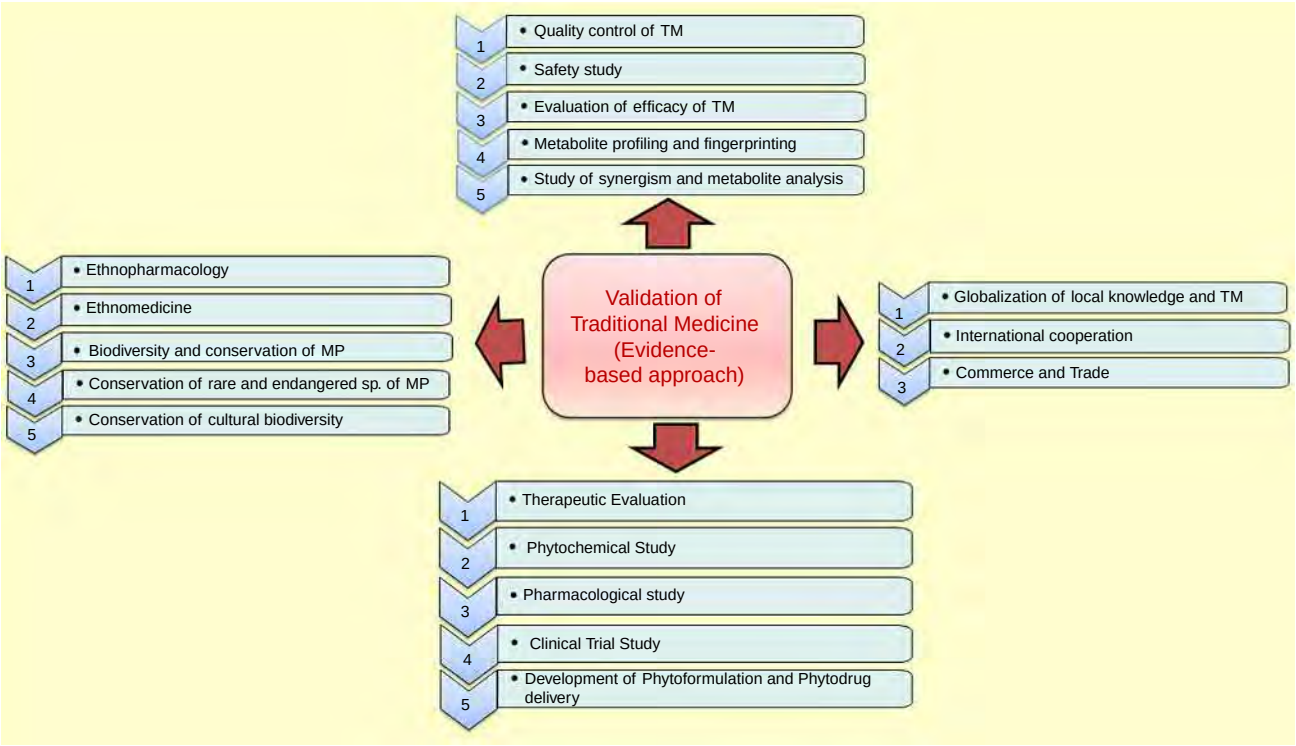
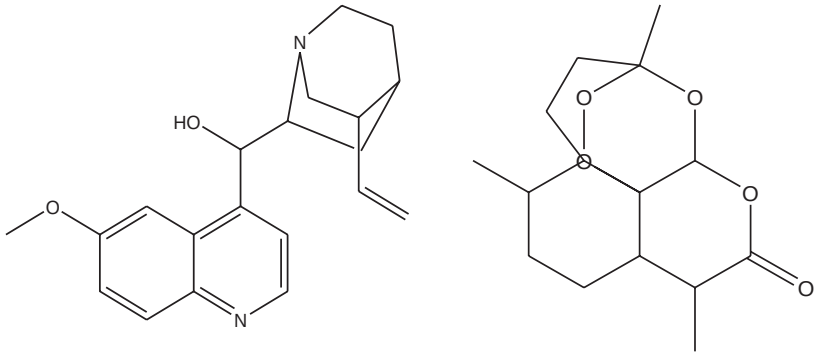


FIG. 1.3 Evidence-based approaches for validation of traditional medicine.



Quinine (i) Artemisinin (ii)

FIG. 1.4 Natural products as drugs.

reproducible. At some point, they found traditional Chinese literature references and discovered an exclusive reference in Ge Hong's *A Handbook of Medicine for Emergencies*, which stated, "A modest bunch of qinghao inundated with 2 liters of water, wring out the juice and drink everything." This sentence made them rethink the standard process of including heat in the extractions. However, prior to getting great preclinical outcomes without clinical trial offices, Youyou Tu and her associates fearlessly volunteered to be the primary individuals to take the *Artemisia* concentrate. They led clinical trials in the Hainan region to validate the clinical viability with patients inoculated with both *Plasmodium vivax* and *P. falciparum*. This study was concluded in 1971 and the treatment was implemented in 1982. In 2015, this critical discovery, which was inspired from TM, received acknowledgment worldwide and Youyou Tu was awarded the Nobel Prize in Medicine. India has an ancient system of Ayurvedic medicine that provides a wealth of information on the folklore practices and traditional aspects of therapeutically important natural products. Among the major challenges of Ayurvedic medicine are quality, safety, and efficacy issues and these aspects need to be emphasized more (Mukherjee et al., 2016). Globalization and reinforcement of Ayurvedic medicine is necessary for the establishment of evidence-based healthcare claims.

1.17 HARMONIZATION OF TRADITIONAL SYSTEMS AND HEALTH PRACTICES

Traditionally, knowledge has been transmitted orally, by observation, and/or direct duplication. Oral and written knowledge transmission is a steady trade. However, written information may bring about changes to conventions, which are then passed on orally—or the other way around—hence, starting a dichotomization. Written materials provide notations and references over a long period of time, over expansive geographical areas and various social foundations. In general, written content permits a more preservationist-based information transmission and may cause a homogenization of learning. An example of this is the products and related learning from Indian and Chinese *Materia medica* that were traded along the Silk Road, which can be seen in the Mediterranean healing customs from the 5th to the 4th century BC onward. An accumulation of 51 birch-bark leaves known as the Bower Manuscript is viewed as the oldest content describing Hindu medicine yet in existence. It was found in Kucha (Chinese Turkestan) on the Silk Road in 1889, translated by the German-British orientalist Rudolf Hoernle (1841–1918), and dated to the 5th century AD. Even opium, which around this time was among the most important medications in the Mediterranean and the Near East, is specified in the Bower Manuscript. Despite the fact that much of this knowledge of antiquated Hindu medication has now been lost over time, such medicinal information inside India has principally been passed on by Ayurvedic practitioners and traditional healers (Mukherjee et al., 2012). Traditional healing practices on the Indian subcontinent started to mix with the Arab system of drug usage (Unani-Tibb) from the 12th century onward, and toward the end of the 18th century the British population started to introduce the Western system of treatment. The principal European therapeutic school in India, the Calcutta Medical College, was opened in 1835. On account of worldwide commercialization and intercultural learning and knowledge exchange, patients progressively can decide between various therapeutic systems for their social health needs with access to doctors concentrated in various medicinal systems. In view of the historical and economic development in a nation or area, a country's traditional system(s) of medicine is frequently supplemented with biomedicine (Ngo et al., 2013).

With regard to India, Western culture and drugs, together with indigenous TM and pharmaceuticals, are practiced. The role of the government combined this advancement where interest in indigenous choices was enhanced, despite their success, which received limited attention from the national experts. India has a pluralistic system of medicine and patients may choose between various medicinal systems. However, there are a limited number of institutions in which authorities from various therapeutic systems work together. One good example of this cooperation is a cutting edge facility for doctors in Thailand, at which Thai TM is coordinated with biomedicine into an advanced healthcare benefit. The key elements for effective coordination that have been recognized are information transmission of Royal Thai traditional medicine and privately undertaken herbal pharmaceuticals to the clinic experts, and a healthcare group comprising individuals with various specializations, together with an efficient supply of natural drugs.

Coordinating therapeutic systems is not just a test for a hospital facility. Well-being informatics (health information systems) is now firmly connected to the measurements of Western biomedicine. As the pattern toward concurrence of various medicinal systems will undoubtedly expand, it is suitable to outline health informatics applications consistent with the qualities of various therapeutic systems. The pros and cons for picking a specific therapeutic system in a specific health condition, for instance, the mixing of medicinal systems into health informatics, may assist a patient to make an educated choice. In this regard, there is an absence of clinical studies concentrating on comparative effectiveness of conventional herbal drug and food products. Because coprescription of therapeutic plants and biomedicine is often assessed, experts and clinicians are urged to keep an eye out for contra-indications and to produce useful communications. Clinical studies, rather than concentrating only on viability, could assess effectiveness by including a more heterogeneous grouping of members and embracing research settings that reflect regular heath circumstances. The changing worldwide economic scene and changing worldview in medication improvement may leverage in the endeavor to officially register traditional medications with legislative offices (Leonti and Casu, 2013). Harmonization needs knowledge exchange and empowerment. The salient aspects are highlighted in Fig. 1.5.

There has been a push for mainstream utilization of traditional and complementary medicine around the world. In some nations, local healers remain the sole or principle health providers for many individuals living in rural areas. For example, the proportion of traditional health professionals to residents in Africa is 1:500, while the proportion of modern therapeutic specialists to natives is 1:40,000. In the Lao People's Democratic Republic, 80% of the population lives in provincial territories, with every town having perhaps one or two traditional health professionals. More than 100 million Europeans are currently clients of T&CM, with one-fifth being customary clients; many tend to pick health services that incorporate T&CM. In a national study in China, professionals of traditional Chinese medicine received 907 million visits from patients in 2009, which represents 18% of all visits to practitioners. Additionally, the number of traditional Chinese medicine inpatients was 13.6 million, or 16% of the aggregate in all clinics examined. In a few nations, certain kinds of TM have been totally incorporated into the social insurance system, including China, the Democratic People's Republic of Korea

FIG. 1.5 Harmonization of traditional medicine.

(North Korea), the Republic of Korea (South Korea), India, and Vietnam. In China, for example, traditional Chinese medicine and regular medicine are held closely to each other at each level of the human services administration, and open and private protection cover both types of treatment. In numerous different nations, TCM is in part coordinated into the national health system, while in a few nations there is no coordination at all (Lao Ministry of Health and World Health Organization, 2012).

T&CM changes, difficulties, and needs have moved on since the latest WHO worldwide strategy document published in 2002. An ever-increasing number of nations are coming to acknowledge the commitment that T&CM can make to the well-being and prosperity of people and the completeness of their healthcare systems. In the period 1999–2012, the quantity of WHO and national agreements covering TM has increased significantly. This gives nations better control of natural medicines or makes national research organizations consider T&CM. Governments and consumers have greater access to extensive parts of T&CM practices and can consider them as an incorporated part of health providers. In Africa, the quantity of national administrative systems expanded from 1 in 1999/2000, to 28 in 2010. The Ministry of Health in Brazil has built up a national policy on integrative and complimentary practices. In the Mediterranean area, five states have reported that they have regulations particularly for T&CM experts. Some states in the Southeast Asia region are currently seeking a better way to deal with the training, practice, research, documentation, and control of TM; in Japan, 84% of Japanese doctors utilize Kampo every day. In Switzerland, certain TM treatments have been reestablished into the essential medical coverage making them accessible to every Swiss citizen.

Regardless of noteworthy advances, the control of TM items, practices, and specialists is not happening at an equivalent pace. Several countries have reported that speedier advances are being made for the development of herbal medicines, but advances in T&CM practices and professionals is lacking. A matter to consider is that the well-being, quality, and viability of T&CM administrations cannot be guaranteed if there is not proper control of practices and specialists. This circumstance presents a genuine test for some, in which an absence of information and experience exists with respect to the plan for a national strategy, prompting weak or missing regulation, and an absence of proper coordination of T&CM administrations into the healthcare administration and delivery system. It additionally reflects the need for all countries to harmonize strategies on TM. The WHO Traditional Medicine Strategy: 2014–23 is responding to the necessities and difficulties recognized by member states and expanding on the work done under the WHO Traditional Medicine Strategy 2002–05. The refreshed procedure for the period 2014–23 dedicates more consideration than before for better administrations and systems, items, practices, and specialists (Burton et al., 2015).

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Chapter 2

Ethnopharmacology and Ethnomedicine-Inspired Drug Development

Chapter Outline

2.1 ETHNOPHARMACOLOGY AND INTEGRATIVE MEDICINE

Ethnopharmacology is an interdisciplinary field of research and it is characterized by ideas from various disciplines and scientific methods. Ethnopharmacology is the process of drug development from ethnomedicine that commences when a botanist, ethnobotanist, ethnopharmacologist, or plant ecologist, accumulates traditional knowledge and identifies plant(s) of pharmacological importance. The study of ethnopharmacology depends on plant science, chemistry, and pharmacology (perception, distinguishing proof, characterization, and experimental examination), along with the crucial involvement of different disciplines. In view of these observations, ethnopharmacology may be characterized as "the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man" (Mukherjee et al., 2010a).

Through ethnopharmacology, countless concepts are utilized to depict research, which regularly utilizes generally comparative techniques and ideas. However, each of these is recognized by being set in a certain convention of research. Phytotherapy research, for instance, concentrates on plant-based treatment through a science-based therapeutic practice from other, more traditional, uses of medicinal plants (Heinrich, 2013). Ethnopharmacology also joins sociocultural ideas and techniques. In the broadest sense, ethnopharmacology depends on ideas nearer to the sociocultural sciences than the natural sciences/medicine. Accordingly, any authentic idea should be founded on the advancement of this logical approach. In any case, written records of useful natural medicines and of the more extensive medicinal practices are obviously accessible from numerous cultures. Essentially, this definition encompasses the everyday therapeutic practice and the related observations of practitioners. Such portrayals of medications, and reflections about their value, are especially a part of traditions, such as Siddha, Ayurveda, Kampo, Unani, Aztec medicine, Arabic medicine, European herbalism, TCM, or some other locally or socially characterized therapeutic modality. Plainly, a significant number of these unique practices do not survive. Contrasted with medical practice in ethnopharmacology, there is an additional core interest in an experimental assessment of such remedial therapies. Various type of pharmacological use and "medicinal testing" of herbal remedies for therapeutic use may be viewed as a major thrust area of ethnopharmacology. Physician William Withering (1741–99) efficiently investigated the medicinal properties of foxglove (*Digitalis purpurea* L.), which allegedly was utilized to treat dropsy by English people. He utilized British folk herbalism to develop a pharmaceutical used for medical treatment.

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Before these investigations, herbalism was more of a medical tradition keen on the wellbeing of patients than a methodical investigation of the efficacy and chemical properties of therapeutic plants.

Ethnopharmacology utilizes an approach in which the "anecdotal adequacy" of therapeutic plants is put to the test in a research facility (Gertsch, 2009). While specialists are starting to prescribe alternative (herbal) remedies for their patients when modern medical drugs do not work, the fact that herbal medicines lead to fewer adverse reactions and are less dangerous and habit forming compared with allopathic prescriptions, for which harmful symptoms may even lead to death, also holds true. Interestingly, there is a developing body of evidence to demonstrate that elements of therapeutic plants act synergistically. There are advantages and disadvantages to both herbal medicine cures and modern drugs, and such factors ought to be considered on an individual basis (Mukherjee et al., 2010a, c).

Surveys have shown that the percentage of natural products in the modern drug armamentarium is considerable, with estimates varying from 35% to 50%. Almost every class of drug includes a model structure derived from nature, exhibiting the classical effects of that specific pharmacological category. A great number of these natural products have come to us from the scientific study of remedies traditionally employed by various cultures. Most of them are derived from plants, pilocarpine, vincristine, emetine, physostigmine, digitoxin, quinine, atropine, and reserpine are a few well-known examples (Farnsworth and Bingel, 1977).

Evidently, the ethnopharmacological impulse to modern medicine can lead to many novel useful drugs, but modern and traditional uses may be entirely different. For example, the plant material studied at the National Cancer Institute (Bethesda, MD) has been collected at random, but the analysis performed by Spjut and Perdue (1976) showed that if antitumor screening had been guided by the knowledge of medicinal folklore and poisonous plants, the yield of active species would have been greatly increased. In this study, plants were classified as "active" regardless of the tumor system or whether the results were obtained from in vivo and in vitro studies. It is generally accepted that ergot has constituted a gold mine for finding therapeutically active components (naturally occurring or modified chemically) against many different diseases. It is by no means inconceivable that the cannabinoids may play a similar role in the future. Derivatives are already being tested for various purposes in clinical medicine.

Traditional medicine is a general, powerful source of biological activity. Ethnopharmacology is not just a science of the past using an outmoded approach. It still constitutes a scientific backbone in the development of active therapeutics based upon the traditional medicines of various ethnic groups. The ultimate aim of ethnopharmacology is the validation of these traditional preparations, either through the isolation of active substances or through pharmacological findings. The information gathered about indigenous drugs will permit feedback to traditional medicine. Harmful practices can be discouraged, such as the use of plants containing tumor-producing pyrrolizidine alkaloids. Knowledge of active constituents in indigenous drugs may lead to substantial improvements in traditional therapy. WHO has emphasized the importance of scientific investigations into indigenous herbal medicines (WHO, 1978).

2.2 ETHNOBOTANY IN DRUG DEVELOPMENT

Ethnobotany is the study of plant-human interrelationships embedded in dynamic ecosystems of natural and social components. Put another way, ethnobotany is the study of contextualized plant use. Plant use and plant-human interrelationships are shaped by history, by physical and social environments, and by the inherent qualities of the plants themselves. The object of ethnobotanical inquiry is actually a sort of "text" (Ricoeur, 1971), the meaning of which is derived partially from the natural, social, and cultural contexts in which the text is played out. An ethnobotanical text revolves around the use and management of vegetation in a human community.

The roles played by plants reflect the biological and physical properties of the plants, the biological and perceived needs of humans, the natural and anthropogenic communities of which the plants are a part, and the genetically limited responses of plants to human disturbance. Species that are elements of the vegetation disturbed by humans are each manipulated and used in different ways. At the same time, different members of the human community participating in this interactive text vary slightly in their behavior. Each person uses his or her personal knowledge of plants, agriculture, house building, and medicine in a slightly different manner, and each responds somewhat differently to changes in the environment. Nonetheless, variation plays around clear, central patterns that define the ethnobotanical text. In an industrial society, plants are primarily esthetic parts of the urban environment and secondarily known to provide food, raw materials, habitats for wildlife conservation, and genetic resources for bioengineering (Anderson, 1952; Posey, 1984).

In its early days, ethnobotany was implicitly shaped by imperialist motives (Brockway, 1979); collectors were sent to gather useful plants from areas occupied by traditional cultural groups and the collected plants were used for commercial exploitation by the modern world. In today's developing world, however, ethnobotany is shaped by an explicit concern for collecting data within a framework in which those data will contribute to the development of all classes in all nations

and, especially, to planned development in the region from which the data are collected. Policy makers need information about economically valuable natural resources and the ways in which those resources are used so that they can predict the outcome of development programs and facilitate the development and introduction of new, locally adapted crops and agricultural techniques (Alcorn, 1992). Today's ethnobotany responds to all these needs and modern ethnobotany is concerned with the "totality of the place of plants in a culture" (Ford, 1978).

Plants have been a rich source of medicines because they produce a host of bioactive molecules, most of which probably evolved as chemical defenses against predation or infection. Microorganisms and fungi that inhabit the soil and are easy to collect and culture have provided a dazzling array of antibiotics. Advances in synthetic chemistry and molecular biology promised to supply new means for designing drugs in the laboratory (Balick et al., 1994). The ethnobotanical approach is actually one of several methods that can be applied in choosing plants for pharmacological studies. It is estimated that 265,000 flowering species grace the Earth. Of these, very small number of plants have been studied exhaustively for their chemical composition and medicinal value. In a world with limited financial resources, it is impossible to screen each of the remaining species for biological activity. Some kind of collection strategy is needed. Investigators, for example, can gather vegetation randomly in an area supporting rich biological diversity. Unfortunately, random searches yield relatively few new drug possibilities. One notable exception is Taxol. In 1992, the Food and Drug Administration approved Taxol, derived from *Taxus brevifolia* (the Pacific yew tree), as treatment for ovarian cancer, and in 1994 approved it for treating metastatic breast cancer unresponsive to other therapies. Taxol was found in the course of a random-screening program conducted by the National Cancer Institute (NCI), which has maintained a plant-screening program with varying degrees of energy since 1960 (Cox and Balick, 1994).

2.2.1 Ethnobotany in Traditional Medicine and Modern Therapy

In recognition of the political, economic, and social barriers slowing the delivery of modern biomedical health care to most of the world's population, the World Health Organization (WHO) has embarked upon an ambitious program to evaluate herbal medicines (WHO, 1978; Penso, 1980; Akerale, 1985). This project ultimately hopes to circumvent the problems of developing and distributing appropriate pharmaceuticals by encouraging the cultivation and use of locally adapted medicinal plants with proven empirical value. Ethnobotany can contribute to this strategy in two ways. First, ethnoecological studies may provide models for profitable and environmentally sound multiple use land management programs. Second, ethnobotanists can invoke the considerable economic potential of as yet undiscovered or undeveloped natural products (Myers, 1983; Balick, 1985). Of an estimated 75,000 edible plants, for example, only 2500 have ever been eaten with regularity, a mere 150 have entered world commerce, and a scant 20, mostly domesticated grasses, stand between human society and starvation (R.E. Schultes, pers. com.). To diversify this resource base is one goal of ethnobotany, and numerous promising crops that can be exploited in ecologically sound ways have already been identified (Balick, 1985).

Possibly, the greatest economic potential of ethnobotany lies in the area of folk medicine. Annually, worldwide sales of plant-derived pharmaceuticals currently total over $20 billion, and a great many of these drugs were first discovered by traditional healers in folk contexts (Farnsworth, 1982). The gifts of the shaman and the sorcerer, the herbalist and the witch, include such critical drugs as pilocarpine, digitoxin, vincristine, emetine, physostigmine, atropine, morphine, and reserpine (Farnsworth, 1988). The forests of tropical America have yielded scopolamine, cocaine, quinine, and D-tubocurarine. An impressive 70% of all plants having antitumor properties have been found in tropical forests (Myers, 1983).

Yet, if ethnobotanists are to seize upon traditional knowledge as a means of rationalizing the preservation of threatened rain forests, they must do far more than search for new wealth. Millenia ago, men and women had entered that forest, and through adaptation, hundreds of cultures emerged, the complexities of which rivaled even those of the dense vegetation out of which they were born. To stay alive, these men and women invented a way of life and, lacking the technology to transform the forest, they chose instead to understand it. The experimental process that originally led to the manipulation and combination of these morphologically dissimilar plants, and the discovery of their unique chemical properties, is far more profound than the phrase "trial and error" suggests. The patterns that any researcher—and the shaman most certainly has earned that title—observes in nature depend on cognitive constructs and an intellectual synthesis, and reflect, in turn, culturally patterned thoughts and values. Sensitivity to nature is not an innate attribute of a South American Indian. It is a consequence of adaptive choices that have resulted in the development of highly specialized perceptual skills. Those choices, in turn, spring from a comprehensive view of nature and the universe in which humans are perceived as but an element inextricably linked to the whole.

Despite the enormous availability of medicines and, above all, of pharmaceutical specialties, plants have a place in current therapy. There is renewed interest in using plants in therapy. Such is the case of *Artemisia* (Klayman, 1985), a source of quinine. Behind the therapeutic success of chloroquine and its synthetic derivatives in the treatment of malaria, the use of

quinine passed into a chapter in the history of medicine. Through a biological phenomenon that is now well studied, even at the molecular level, bacteria and parasites can develop resistance to chemotherapeutics; that is, they undergo selection that results in resistance to a particular chemical compound. This process has occurred, in part, with a species of Plasmodium, the causative agent of malaria, to a point that synthetic antimalarial drugs have lost such a significant part of their efficiency in the last quarter of the 20th century that it has often been necessary to return to the use of quinine. Currently, there is such a great demand for the plant alkaloid that extraction laboratories cannot satisfy the growing demand, maximized now that malaria has again become a great health risk in tropical areas. The demand has been accentuated further by the resistance of the insect vector, the Anopheles mosquito, to insecticides that were used in the 20th century in sanitation campaigns.

Traditional medicines depend on a number of plants that are currently used in scientific medicine, although they have not yet been improved upon. Such is the case of *Digitalis purpurea* L. and *D. lanata* Ehrh. Many other drugs exist to which therapeutic effects have been attributed. As is well known, synthetic chemistry has until now had little success in obtaining drugs effective in the treatment of various viral diseases; even though immunotherapy has achieved great success, there are no vaccines for all viral diseases. It is possible that plants may be useful to treat these diseases. An example from Ecuador is Lanigua (*Margyricarpus setosus* Ruiz and Pavon), the roots of which, in infusion, are used in the symptomatic treatment of measles. Other examples are nachag (*Bidens humilis* Sesse and Moc), the flowers of the branches of which, in infusion, are used in the treatment of infectious hepatitis, or the latex of several species of Euphorbiaceae, especially of the genera *Croton* and *Euphorbia*, which in topical form are used in the treatment of common warts. Furthermore, numerous plants are known for certain antineoplastic effects (Cassady and Douros, 1980), modifiers of fertility (Moreno and Schwartzman, 1975), and other effects (Perdue and Hartwell, 1969).

Controlled studies are needed, for example, by the double-blind system, to confirm the therapeutic effect of these plants of traditional medicine in therapy. Nonetheless, in folk medicine, these plants are employed with apparently favorable results, and above all without causing detectable unfavorable side effects.

An inventory of medicinal plants compiled by WHO (1978) and encompassing only 90 member countries gave the large figure of 20,000 species, of which only 250 were of widespread use or had been analyzed to identify their main active chemical compound(s). That sample, even though a partial one, reveals the enormous empirical traditional knowledge about medicinal plants. Most of this knowledge is verbal and only incompletely incorporated in historical and folklore works. Aboriginal knowledge is the fruit of centuries and, in some cases, millennia of plant use. While the capacity of chemists to modify a molecular structure is almost unlimited, the capacity to invent or create new structures with therapeutic properties has been limited. In the meantime, the plant kingdom offers us thousands of new molecules (Evans et al., 1982; Gottlieb, 1982). The study of those molecules identified as "active compounds" is indispensable. Phytochemical investigations carried out during the 1970s and 1980s have discovered a number of alkaloids and other pharmacologically active substances that are currently being studied and that can possibly serve as models for new synthetic compounds (Barz and Ellis, 1980).

2.2.2 Drug Discovery From Ethnobotany

All the wonderful progress of synthetic chemistry and of science in general, unfortunately, has not served to alleviate and cure all the sickness in the world. According to surveys and other research carried out in different countries, scientific (or standard) medicine in developing countries (Naranjo, 1981) serves only a minority (estimated at 30%–50% of the total population), while the rest of the population attends to its health needs through the process called traditional medicine, aboriginal medicine, or folk medicine, processes based essentially on the use of low-cost medicinal plants that are easily accessible to the entire population.

One positive aspect of the use of medicinal plants is their low cost compared with the high price of new synthetic drugs, which have become totally inaccessible to the vast majority of people. Another consideration in favor of the use of medicinal plants, when they are the only recourse available, is that they have comparatively few side effects. Synthetic drugs, in general, have very potent pharmacodynamic effects; but as they are active, many also have strong and possibly dangerous and harmful side effects. Between 3% and 5% of patient hospital admissions are attributed to the side effects of synthetic drugs. Direct administration in folk medicine offers little risk. Thus, there exists a wide field for research in the phytochemistry of those hundreds of plants that are used in folk medicine in each country, research confirming the presence of pharmacodynamic chemicals, such as alkaloids, glucosides to a lesser degree, and essential oils and other substances, indispensable knowledge that justifies the practices of naturalist and folk medicine. Natural products (botanicals) have played a major role in drug discovery.

The relationship between man and plants has been very close throughout the development of human cultures. Throughout history, botany and medicine were, for all practical purposes, synonymous fields of knowledge, and the shaman, or witch doctor—usually an accomplished botanist—represents probably the oldest professional man in the evolution of human

culture. At no time in the development of mankind, however, has there been more rapid and more deeply meaningful progress in our understanding of plants and their chemical constituents than during the past quarter century. And this is curious, especially in view of the somewhat earlier deprecation in pharmaceutical chemistry of any emphasis on plants. The gradual sophistication of phytochemistry in the last half of the 19th century and the exaggeration of hope for specific remedies from vegetal sources for any and all ills set up a counter-current, a tendency to disparage any data concerning the potential value of physiologically active natural products.

This situation results, in part at least, from the rather contemptuous attitude that certain chemists and pharmacologists in the West have developed toward both folk remedies and drugs of plant origin. Needless to say, the more enlightened members of these professions have avoided so crude an error, realizing that the humblest bacterium can synthesize, in the course of its brief existence, more organic compounds than can all the world's chemists combined. Then, the discovery, almost within a decade, of a series of so-called "Wonder Drugs," nearly all from vegetal sources, sparked a revolution. It crystallized the realization that the plant kingdom represents a virtually untapped reservoir of new chemical compounds, many extraordinarily biodynamic, some providing novel bases on which the synthetic chemist may build even more interesting structures. The startlingly effective drugs that have come from this decade or two of discovery are scattered throughout the plant kingdom. They range from muscle relaxants from South American arrow poisons, antibiotics from moulds, actinomycetes, bacteria, lichens, and other plants; rutin from a number of species; cortisone precursors from sapogenins of several plants, especially from *Strophanthus* and *Dioscorea*; hypertensive agents from *Veratrum*; cytotoxic principles from *Podophyllum*, *Vinca* and other sources; khellin from *Ammi visnaga*; reserpine from *Rauwolfia*; hesperidin from the citrus group; bishydroxycoumarin from *Melilotus* and others—not to mention the numerous psychoactive structures of potential value in experimental psychiatry, some new, some old, from many cryptogamic and phanerogamic sources. Not only have new drugs from vegetal sources been discovered, but new methods of testing and refined techniques have led to the finding of novel uses for older drugs.

As a result of these advances, nearly one-half of the 300,000,000 new prescriptions written currently in the United States contain at least one ingredient of natural plant origin. Even if the antibiotics and steroids are excluded, well over 17% of all American prescriptions filled in 1960 used one or more kind of plant product—either produced directly from plants or discovered from plant sources and later synthesized. A more up-to-date analysis of American prescriptions covering over 1 billion written in 1967 gives the following breakdown; 25% contained principles from the higher plants; 12% were microbiology-derived products; 6% were animal-derived substances; 7% had minerals as the active ingredient; and 50% of the active principles were synthetic.

Linnaeus wrote that the number of plants in the whole world is much less than is commonly believed, "calculating that their number" hardly reaches 10,000. Another early estimate was made by Lindley who, in 1847, credited the plant kingdom with a total of nearly 100,000 species in 8935 genera. He assigned 1194 species to the algae; 4000 to the fungi; 8394 to the lichens; 1822 to the bryophytes; 2040 to the ferns and fern allies; 210 to the gymnosperms; and 80,230 to the angiosperms. The intensification of exploration during the last century obliged taxonomists gradually to increase their horizons. However, the estimates have not kept pace with botanical collecting and taxonomic research. Most of the currently accepted calculations have not been substantially altered since the early years of this century. They allow the plant kingdom between 250,000 and 350,000 species. This aggregation is usually thought to have the following distribution: Algae—18,000; Fungi (including Bacteria)—90,000; Lichens—15,000; Bryophytes— from 14,000 to 20,000; Pteridophytes—6000–9000; Gymnosperms—about 675 species in 63 genera; Angiosperms—about 200,000 species in some 300 families, of which 30,000–40,000 are Monocotyledons.

In view of the continued description of 5000 new species and varieties each year, this estimate of the total is unrealistic. Perhaps it is significant that botanists with long field experience in the tropics are unhappy with contemporary calculations. May not even the highest currently accepted census for the angiosperms, 200,000 species in 10,000 genera and some 300 families, be deficient. Richard Spruce, the British explorer of the Andes and Amazon for over 15 years during the last century, estimated that the vascular plants of the Amazon Valley numbered some 60,000 species, and, considering the scarcity and superficiality of plant collecting up to his days, he wrote that there might "still remain some 50,000 or even 80,000 species undiscovered." In the early years of the present century, Jacques Huber, the Swiss specialist on the Brazilian Amazon, set the obsolescent flora of the eastern part of this area at some 2500 species.

2.2.3 Ethnobotany and Drug Development

Man, and perhaps some of his closer relatives, have always made use of plants to treat illness, and many of these remedies have real beneficial effects. The need to document plant usage and to attempt to confirm efficacy remains urgent and must be undertaken with the same rigor, as would any other scientific study.

Various approaches can be taken in using information about traditional medicinal plants. It is necessary to verify that the traditional knowledge has been accurately passed from generation to generation, and it is probably sensible to concentrate on regions with a diverse flora. Perhaps not surprisingly, there has been a concentration on Chinese traditional medical products, but there is increasing interest in other ancient systems of medicine (e.g., the Ayurveda in India) and those found in more primitive societies (Mukherjee et al., 2010b).

It is hard to establish the success rate of a screening approach based on ethnopharmacological information. Few wide-ranging studies have been performed, and it is not known whether negative results are published as enthusiastically as positive ones. Additionally, it must be remembered that ethnopharmacological investigations may lead to the discovery of unusual biological activities or unique chemistries, which may, in turn, lead to the development of analogs suitable for pharmaceutical development. As an example, forskolin has unique actions to active adenylate cyclase, and analogs may be useful in the treatment of glaucoma, and as cardiotonics. Forskolin is the major active component of the Ayurvedic plant makandi (*Coleus forskohlii*). In another example, a plant (*Homalanthus nutans*) used in Samoa to treat the viral disease, yellow fever, has provided a phorbol ester, prostratin, which was discovered by the National Cancer Institute to have anti-HIV activity.

The ethnobotanical lead developed from the leaves of *Premna schimperi* (Verbenaceae) from Ethiopia was the recognition of the antimicrobial activity of and the subsequent identification of 12-xox-10β, 17α-cleroda-3, 13(16)-dien-15-oic acid 1 as the active principal. This diterpene proved to have appreciable activity against some important Gram-positive pathogenic bacteria. Although not particularly exciting as an antibacterial, the value of this extract for local use was confirmed. As a consequence of this finding, a study was initiated on other Ethiopian Premna species. From *P. oligotricha*, lactone was obtained, which proves to be a more active antibacterial than the acid and the structurally unusual peroxide. The latter has no antimicrobial activity but does have structural novelty.

There is an obvious need to be aware of possible simple answers to observations of biological activity and to rule out the possibility of this before embarking on a major phytochemical separation exercise. However, even when the active compounds do prove to be organic molecules, disappointments can still occur. The purgative action of the seeds of *Croton penduliflorus* was eventually determined to be due to free long-chain fatty acids, but this took extensive bioassay-guided separation in order to isolate these very common natural products.

2.3 ANTHROPOLOGICAL ASPECTS OF ETHNOPHARMACOLOGY

The anthropological study of people and the plants that they use, grow, forage, think about, and imagine is a rich way to more fully understand the ways that people enact their humanness. This is not to say that the study of plants and people is more important or valuable than the study of people and birds (Hage and Miller, 1976), or any other aspect of ethnobiology (Anderson et al., 2012). Anthropology has shown us that human beings "operate in an environment as they perceive it, not as it is" (Brookfield 1969, cited in Cotton, 1996). Research has come a long way from Harshberger's definition of ethnobotany more than 100 years ago, which emphasized the utilitarian aspects of plants, toward a discipline that has embraced peoples' perception and management of the natural world, in addition to their use of those natural resources. In order to understand why and how people use and manage plants, there is a need to understand first how they perceive their environment. There are many ways to learn about how plants are culturally perceived. The most obvious way is first-hand experience in the field, but there are information sources less conventionally accessed by ethnopharmacologists. Literature, such as epics, poetry, and novels, which appreciates the social use of some well-known plants, can be an wonderful source of ethnopharmacology knowledge. Sources such as these shows that detailed cultural and contextual information about plants as observed by the keen human eye can be found everywhere. Hopefully, ethnobotanists and ethnopharmacologists alike may continue to find joy and scientific wisdom in exploring the diverse array of ethno-related knowledge, beliefs, and practices, both from conventional and nonconventional sources, with an open mind.

2.4 ETHNOMEDICINE IN HEALTHCARE

Ethnomedicine has an enormous effect in the developed world and in developing nations on account of wide biological activities, as well as higher safety margins, and lower economic cost than manufactured medications (Nema et al., 2011). Ethnopharmacology research is not centered on the portrayal of medicinal impacts in the substance of a treatment (or medicinal case histories), yet here again fuses bioscientific research. The definition utilized here is in this way fairly more engaged and features the reconciliation of exploratory research on the impacts of a traditionally inspired drug with sociocultural strategy.

The "discovery" of curare presents a notable case of ethnopharmacological research that has led to new pharmaceuticals. The investigation of the organic origin of the arrow poison, curare, its physiological (and poisonous) impacts, and the compound responsible for these, gives a captivating case of an early ethnopharmacological approach. Curare was used by "certain wild clans in South America for harming their bolts" (von Humboldt, 1997). Many different travelers documented this use and the toxic substance captivated the two scientists and the wider public. Especially notable are the point-by-point depictions of the procedure used by Alexander von Humboldt (1769–1859) in 1800 to prepare poisoned arrows in Venezuela. There, von Humboldt met a gathering of indigenous individuals who were celebrating their arrival from a mission to get the crude material for making the toxin. In a later advance, *Chondrodendron tomentosum* Ruiz et Pavon was recognized as being the organic source of tube curare (named on account of the Graminaeous tubes utilized as storage compartments). Different types of the Menispermaceae (Chondrodendron spp., Curarea spp., and, Abuta spp.) and types of the Loganiaceae (Strychnos spp.) have also been used as a part of the creation of curares.

The use of thorough ethnographic field approaches that have been refined over the course of time will continue to improve our appreciation of the social development and social exchange of healing in different societies. A coordinated, hypothesis-, and issue-driven ethnopharmacology will move from multidisciplinary (parallel streams lacking coordination), to interdisciplinary (some methodological and theoretical exchange across fields), to transdisciplinary strategies that incorporate the views, goals, and equipment of different fields. The different aspects of ethnopharmacology and ethnomedicine with a central theme based on learning from Local Health Traditions (LHT) and their validation is shown in Fig. 2.1**.** There is a need to coordinate traditional and correlative medicine. Although traditional systems of medicine are recognized, their joining and mainstreaming in medicinal service delivery systems and their incorporation into national projects, remains a test. The accessibility and moderateness of traditional cures for individuals need to be guaranteed.

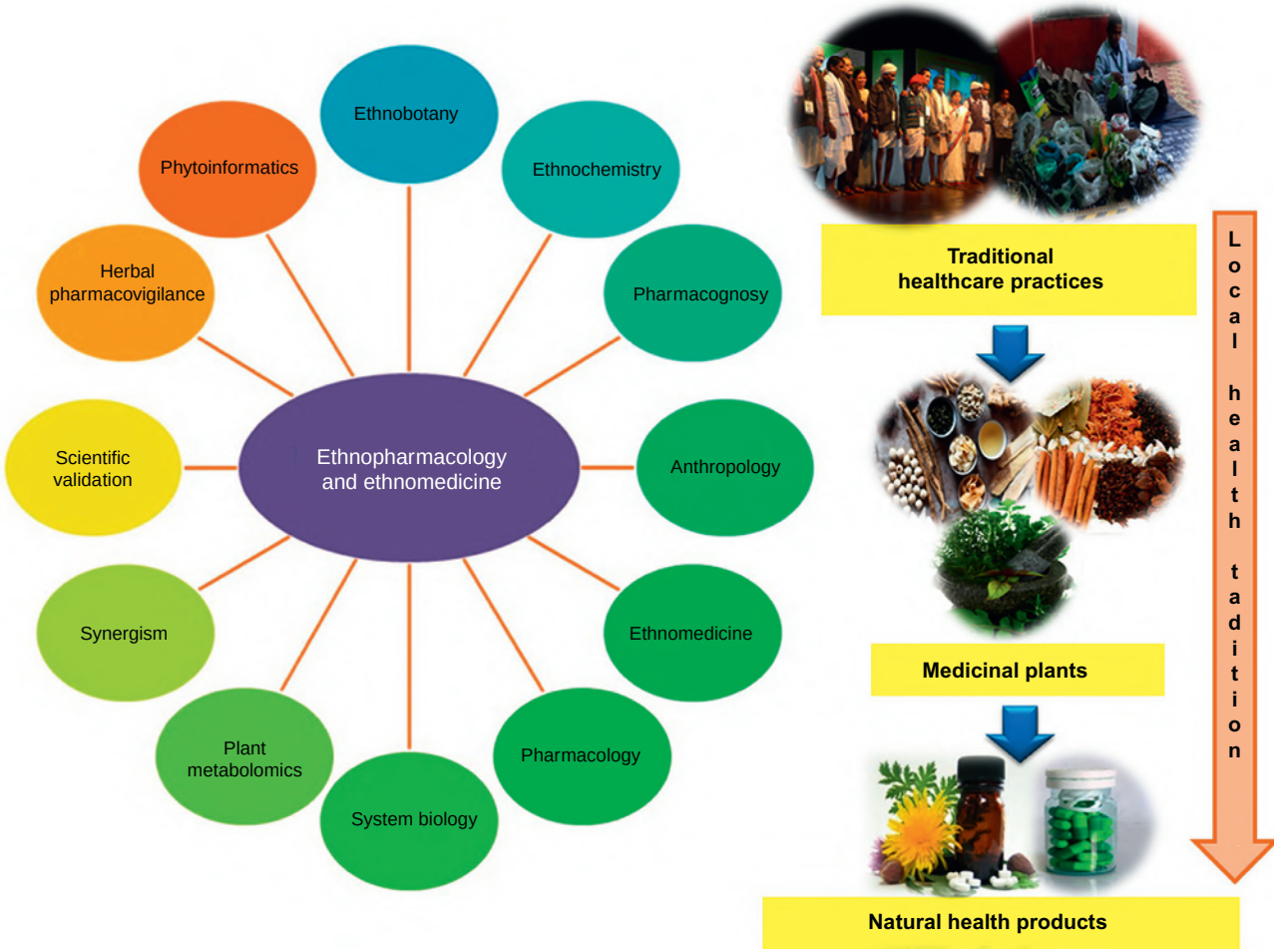

FIG. 2.1 Ethnopharmacology and ethnomedicine integrated with local health traditions.

There is an incredible opportunity for business development of selected medicinal plants in India because there is dependable demand in the market. It is essential to characterize the therapeutic plants appropriate to our soil and climatic conditions and advance them among farmers. The farmers and villagers of ancestral territories can assume a noteworthy part in social business enterprise activities for developing different therapeutic plants required by pharmaceutical ventures. Agriculturists ought to be left to develop, while independent associations should concentrate on postcollection preparation and capacity, together with a system for promotion. Other than development in their common environment, every single other action should, if possible, be coordinated to guarantee full value expansion. Business openings in this area are gigantic because of expanded use of plant-derived molecules and formulations in pharmaceuticals, food, and agro-chemical ventures. There is a need to understand the possible pharmacological uses of medicinal plants and urge pharmaceutical companies to use natural molecules from therapeutic plants in their products.

Advances in research facilities and clinical sciences will keep enabling ethnopharmacologists to provide a greater level of specificity: the constituents and mechanisms of medicinal plants (and different substances); how varieties in the collection and quality of plants, and their formulation, influence pharmacological profiles; synergism among constituents of single—and multiple therapeutic preparations, and between traditional herbal medications and modern pharmaceuticals.

The use of plants, plant concentrates, or plant-derived pure chemicals in treating diseases in different conditions has stood the test of time. Indeed, today, numerous pharmacological classes of medications incorporate a characteristic model. Plant-derived medicine (atropine, ephedrine, digoxin, morphine, quinine, reserpine, and tubocurarine) are cases of medicines, which were initially found through the investigation of traditional cures and information on indigenous medication from individuals in a society. There is a revival of enthusiasm for herbal medicine worldwide and conventional medicine is now starting to acknowledge the use of botanicals once they are experimentally demonstrated. Ethnopharmacology has officially assumed an essential part in the advancement of regular drugs and will be playing a major role in the years to come. A collaboration among ethnobotanists, ethnopharmacologists, doctors, and phytochemists is essential for productive results in research on plants with therapeutic value. While the ethnopharmacologists have a more prominent part to play in defending a mix of activities, the role of the phytochemists will move somewhat toward the institutionalization of botanicals. Research on traditional medicaments has found the circumstances and the end-results relationship of numerous plant-based treatments in decades. Reserpine, an alkaloid from *Rauvolfia serpentina* Benth. (Apocynaceae) received worldwide attention for the twin impact of bringing down hypertension and as a sedative (Woodson et al., 1957). Shatavarin-I, a glycoside isolated from an Ayurvedic drug from the roots of Asparagus racemosus Willd. (Liliaceae), is prescribed for preventing miscarriage (Gaitunde and Jetmalani, 1969). "Triphala" is a traditional case of a polyherbal plant mixture in Ayurveda having synergistic and counterbalancing properties, which contains dried products of *Emblica officinalis* Gaertn. (Euphorbiaceae), Terminalia belerica Roxb. (Combretaceae), and *T. chebula* Retz. (Combretaceae) in the proportion (1:1:1), recommended as a diuretic in vascular blockage, colon detoxifier, and as a rejuvenator. These three natural products were blended and the polyphenols of those plants together created synergistic impact (Ponnusankar et al., 2011; Mukherjee et al., 2011). In Ayurveda, an impactful blend of three herbs known as trikatu, comprising *Piper longum* L. (Piperaceae); *P. nigrum* L. (Piperaceae); and *Zingiber officinale* Rosc. (Zingiberaceae); in the proportion of 1:1:1, is used to advance absorption and digestion (Gertsch, 2009) because of the alkaloidal constituent piperine. This bioactive compound has a number of pharmacological properties, including mitigating movement. These cases of Ayurvedic medicine make it more attractive to specialists and academic groups for additional research (Mukherjee et al., 2015).

2.5 ETHNOMEDICINE IN COMPLEMENTARY THERAPEUTICS

The ethnomedical approach requires an ethnobotanist, a modern physician, an ethnopharmacologist, a natural product chemist, an anthropologist, and a traditional healer. It requires the collection of detailed information. The therapeutic value of a medicinal plant is gaged by the evaluation of specific ethnomedical and botanical information. It becomes obvious that the ethnomedical approach is based on field research, that is, it must be conducted on the site on which the healer operates. It depends on healer interviews, disease prevalence interviews, ethnomedical interviews, observed patient responses, and case discussions with local practitioners. This information allows us to evaluate the natural product and its effect on a selected disease. The botanical data include: (1) the names of the plant; (2) voucher numbers and specimen locations; (3) the collection site coordinates, latitude, longitude, and altitude; (4) the description of the habitat that is associated with the collected species; (5) the type of terrain and soil; and (6) physical descriptions of the life cycle of the plant. The ethnomedical data include the actual sources of the ethnobotanical data, that is, the interviewer and the interviewee, as well as: (1) the number of plants in the medicine; (2) how and when it is collected; (3) the plant part/s used in the preparation of the

medicine; (4) a description of the disease (signs and symptoms); (5) patient demographics; (6) the therapeutic activity of the medicine; (7) the preparation of the medicine; (8) any additives that might be used; (9) the dosing (amount, route of administration, duration); and (10) the side effects. The ethnomedical approach results in a deeper and more comprehensive understanding of local diseases and the phytomedicines that are used to treat them. The ethnomedical database has all the information that is required to prioritize and select potential leads from natural products for screening and also makes the recollections, if necessary, easy to accomplish. Historically, ethnopharmacology was the origin of all medicines. Therefore, natural products were the most important source of drugs. In the 20th century, synthetic chemistry and then biotechnology offered alternatives to natural products. Due to technological convenience, the efforts of drug-discovery scientists have tended to be directed toward synthetic sources along with natural products.

Ethnopharmacology has provided some very notable past successes, including morphine (isolated in 1804), quinine (isolated in 1820), digitoxin (isolated in 1841), ephedrine (isolated in 1897), and tubocurarine (isolated in 1935). These compounds, or their analogs and derivatives, are still in widespread use. Developments undergoing trials and with an ethnopharmacological association include artemisinin for malaria, components from marigolds for psoriasis, flavones as antianxiety compounds, prostratin as an antiviral, and the South African appetite suppressant being developed by Phytopharm. It is regularly guaranteed that using ethnopharmacological data will enormously increase the odds of finding new medications. However, it is not clear from distributed data whether this attestation is legitimate. Two large-scale studies furnish a few pointers to progress rates with normal item screening. The National Cancer Institute (NCI) anticancer screening program uncovered that screening of random samples gave a hit rate of 10.4%, while plants gathered based on some ethnopharmacological data gave a hit rate of 19.9%. Nonetheless, plants that were known to be harmful had a considerably higher introductory hit rate, 50.0%. The second illustration originates from the Central Drug Research Institute in Lucknow, India. On an extensive variety of measures, randomly gathered plants had a hit rate of 18.9%, though plants with related ethnopharmacological uses had a hit rate of 18.3%. Ethnopharmacology also helps to explore the advantages and dangers of local and traditional medicinal plants with the objective of adding to their number and promoting them with sustainable use (Heinrich, 2006, 2010).

Presently, research following an ethnopharmacological approach is ordinarily led by the rising economies of Asia, America, and Africa. Around 10% of respiratory, gastrointestinal, and dermatological ailments are treated by ethnopharmacological means. All others are of lesser significance and, about 5% of all investigations incorporate central nervous system (CNS) research and even fewer examinations incorporate psychedelic impacts (<2%). As one would expect, questions relating to the toxicity of local and traditional medications are focused upon in many investigations. It has been observed that, awareness of basic reasonable and methodological guidelines in the field has expanded in a progression of basic surveys endeavoring to characterize good practice as it identifies with particular methodological and theoretical aspects of the field (Heinrich, 2015).

Compounds and substances from plants have been utilized to treat human ailments since the beginning of medicine. Right now, the human services situated in ethnopharmacology is still the pillar of around 75%–80% of the total population, and a significant piece of traditional treatment includes the use of plant extracts. Unmistakably, ethnopharmacology remains an important hotspot for the discovery of new medications and it is assessed that 13,000 plant species worldwide are known to have been used as a part of drug development. Around 60% of anticancer and 75% of antiinfective medications approved from 1981 to 2002 could be followed to natural sources (Patwardhan and Mashelkar, 2009). Studies on the sources of new medications from 1981 to 2007 reveal that half of the medications approved since 1994 depend on natural products (Harvey et al., 2015). Currently, it is assessed that 80% of molecules used as a part of medications sold worldwide are obtained from the ethnopharmacological approach and that more than a hundred new natural-product-based leads are in clinical development. In addition, regardless of the gigantic improvement of chemical synthesis today, 25% of prescribed drugs on the planet are of vegetable origin. Aspirin, atropine, artemisinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, Taxol, tubocurarine, vincristine, and vinblastine are examples of what therapeutic plants have given us before. The greater part of these plant-based drugs was initially found through the investigation of traditional cures and folklore of indigenous individuals and some of these could not be replaced regardless of the tremendous headway in engineered science.

Because of growing medicine discovery from ethnopharmacology, specialists and pharmaceutical enterprises have shown growing enthusiasm for traditional health practices utilized far and wide. This appreciation has been growing for a considerable length of time because of methodical demonstrations that plants are the richest asset of medications of traditional systems of medicine, current solutions, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates, and chemical elements for engineered drugs. Because the reported information so far accessible on plants is relatively small compared with the whole population of plants, ethnopharmacologists, botanists, microbiologists, and natural product chemists the world over are always searching for plants and phytochemicals that have therapeutic viability.

Moreover, the wide range of medicinal action makes the natural products an alluring possibility for additional research. In this specific situation, another acknowledgment has been given to ethnopharmacology and traditional, complementary, and alternative medicines, which while re-emerging as new vital alternatives in considerations of health, have given significant insights into plants with bioactive compounds possibly usable in the generation of new medications (Harvey, 2000). The World Health Organization's commission on protected innovation and advancement in general health has properly recognized the promise and role of traditional solutions in creating moderate medications for the treatment of medical issues. The ethnopharmacological approach is as of now utilized to research various medicinal plants and vegetable from traditional ethnic groups. In spite of the fact that the clinical viability of these arrangements is accounted for by traditional practices, they have not been experimentally approved. In this way, ethnopharmacologists normally create working theories from field observations, having as one of the principle objectives to improve the learning of local groups in joining scientific discoveries to traditional records. In this unique situation, the main question that directs ethnopharmacological research is whether a particular plant extract utilized as a part of the social setting to cure some illness shows a pharmacological basis that clarifies the impacts generally demonstrated. In this procedure, ethnopharmacological discoveries begin with field observations and finish in new pharmacological knowledge (Gertsch, 2009). In this way, ethnopharmacology research is transdisciplinary, addressing areas, such as anthropology, ethnobiology, and, as the name suggests, pharmacology. A few focal points can be accomplished with the use of ethno-directed techniques for screening bioactive compounds. Due to ethnopharmacological utilization of vegetable items in particular groups for the prevention or treatment of different health conditions, it is possible to meet preparatory criteria for safety for human use and any unfavorable impacts of such use. The better social adequacy of natural products and lower cost is empowering for both the consuming public and national human services organizations in considering plant medications as an integral practice with engineered drugs. Ethnopharmacology recommends itself to those medicinal and social insurance systems practiced in a traditional way and is not directly thought to be a part of traditional Western pharmaceuticals (Mukherjee et al., 2009b). This knowledge has advanced over the years by drawing on the religious convictions and social structures of various indigenous peoples, by exploiting natural products in their surroundings, and by creating and approving remedial and preventive methodologies utilizing a scientific strategy. In spite of the fact that TMs have not been generally confirmed with scientific documents as in customary prescription, it is very well known among individuals because of the practical advantages, traditional convictions, temperate preferred standpoint, and easy access and various other reasons having a provincial, religious, and social premise. Despite the fact that ethnopharmacology needs to go a long way in demonstrating its medicinal adequacy deductively, it is, because of its massiveness and wide relevance, applicable to an assortment of illnesses and targets (Mukherjee et al., 2009b).

2.6 VALIDATION OF ETHNOMEDICINES

With the worldwide increase in interest for medicinal plant or plant-derived medications, there is a call for guaranteeing the quality and safety of natural medications utilizing several scientific strategies. Substance constituents in natural prescriptions may shift, contingent upon harvest seasons, plant origins, drying forms, and other related factors. Accordingly, it is by all accounts important to decide the vast majority of the phytochemical constituents of herbal medicine products, keeping in mind the end-goal to guarantee the unwavering quality and repeatability of pharmacological and clinical research and to understand the bioactivities and possible reactions in order to improve the nature of herbal medicine products. The quality control of natural drugs aims to guarantee their quality, safety, and adequacy. The absence of chemical markers remains a noteworthy issue for the quality control of herbal medicine. By and large, there are no adequate chemical and pharmacological information of chemical markers. Further, there are numerous specialized difficulties in the generation of markers. For instance, temperature, light, and solvents frequently cause degradation, as well as a change in purified components; isomers and adaptations may likewise cause changes in the markers. In any case, an idea of understanding the complex standards of natural medication must be produced through marker profiling and related methodologies in order to create evidence-based practice with regard to herbal medicines.

The significant difficulties for the improvement and advancement of TM include chemoprofiling, safety assessments, quality control, and compelling administrative rules for natural prescriptions. An upgraded worldwide coordinated effort and administration are expected to modify the modern standards and develop new procedures for the improvement of TMs and dietary supplements. Research through joint efforts and participation across the country may help to a substantial degree in the advancement and improvement of TM for the comprehensive improvement of health services. The advancement and assessment of therapeutic plant-derived items are being controlled and implemented through different offices in various nations. This provides novel points of interest to researchers and pharmaceutical ventures to upgrade drug discovery and improvement.

2.7 ETHNOPHARMACOLOGY AND TRANSLATIONAL RESEARCH

Translational research spans over several disciplines connecting preclinical and clinical work. However, ethnopharmacology is focused on the patient, diagnosis, treatment, and outcomes, and then taking these annotations to the laboratory to explore the (plant-based) medicines concerned. This might lead both to new drugs and to identifying new targets, but most importantly it develops an evidence base for such preparations.

In this area of ethnopharmacology, there is significant evidence to do back-translation from patient to the laboratory, then forward translation through diagnosing different paramters, as well as in vitro experiments for elucidating the mode of action, in vivo studies, ADME, pharmacology, and clinical studies, to finally get the medication (or information on the medicine). In order to start within the forward translation, execution of the method is most crucial. It is necessary that the species investigated are exactly identified taxonomically with binominal name and authority. It needs botanical experience to confirm that a specific plant is set to the proper associated species "converted" from a common name (synonym) to the scientific name. The simplest way is to use www.theplantlist.org for checking botanic names and families. There ought not to be a difficulty with documentation; it ought to be a standard part of good laboratory practice (GLP) whenever diagnostic work on an artificial compound might have problems with contaminants from the manufacturing processes, just in case of plant extracts. The plant material used for traditional medication can, in most cases, vary in concentration of active constituents from batch to batch due to biological variation, geographical factors, climatic variations, and genetic factors. Ideally, it would be sensible to form a broad sampling of plants to match the variation before any research work commences. NMR-based techniques, which measure all metabolites, can be employed to examine patterns of similarity in cases for which the active constituent(s) are unknown. The results then have a higher probability of being representative of the species under investigation. Whereas clinical trials and in vivo experiments are controlled by strict regulation by authorities, the in vitro space is not.

However biological science has protocols that everybody uses. Several kit-based measures have been developed. Such results will then be compiled in worldwide databases, for instance GeneBank. Of course, in contrast to genes, which are created from a similar few parts in all living organisms, the variation within the plant matrix is so great that it would be difficult to use common means of extraction. The *Journal of Ethnopharmacology* has taken the lead with publication of a series of papers. The series includes the topics of ethnopharmacological field studies (Heinrich et al., 2009), antiinfective agents (Cos et al., 2006), diagnostic procedures in experimental polygenic disorder analysis (Matteucci and Giampietro, 2008), and animal models in polygenic disorders (Froede and Medeiros, 2008). Toxicology is a weakness of ethnopharmacology, while stressing on economical and safe traditional medicines; however, the toxicologic aspects of native and traditional medicinal plants are sometime insufficiently investigated. The task is comparatively clear, the testing of traditional medicine and many regulatory authorities have precise descriptions of what is needed for registering an herbal product. Most regulatory authorities need tests for acute and chronic toxicity, mutagenicity, and teratogenicity. Of equal importance is the analysis of the herbal substances that are used medicinally. The assessment of potential interactions of herbal medicines with different other medicines is also a very important area of investigation.

When a decent case has been synthesized on diagnosing information and a secure pharmacology profile, the obstacle is moving to a clinical test. Most of the research work on medicinal plants is based on in vitro screening, which should be supported by in vivo tests. The laboratory-based researchers concerned have very little contact with clinical scientists; the link in the translational chain is sometimes missing. It is imperative that scientists planning and running clinical trials have the correct knowhow. Clinical trials on traditional medication should meet the scientific standards set for clinical trials so as to determine that a clinical test has a bearing and one should make sure that the analysis is of a decent quality. This is important because usually metanalyses of clinical trials on herbal products return to the conclusion that the clinical trials are not of high enough quality and do not offer new insights. This can be boosted by the standard of the research work. It is encouraging to see that many programs have been initiated around the world to train clinical scientists to specifically run trials on traditional medication (Willcox et al., 2012).

2.8 BIOLOGICAL WEALTH OF ANCIENT WISDOM

The great biological wealth of different countries has been exploited based on ancient knowledge, though most of them have not yet been categorized using modern tools. In spite of the rich herbal wealth in many countries, there is no success in realizing the full potential of medicinal plants. The knowledge about cultivation, management, processing, and utilization of the products of medicinal plants is inadequate, primarily due to lack of research and awareness. There is a need to promote the rational use of plant-based drugs. Apart from this, the supply of selected seeds and manufacturing processes to produce world-class products with very high standards of quality control should be ensured (Mukherjee et al., 2012).

From an historical point of view, it is obvious that our ancestors did not have theories and hypotheses; they were very excellent observers with all their senses and based on that, via trial and error, they developed all kinds of things, from food and medicinal plants to dyes, fibers, construction materials, and poisons for, among other things, hunting and fishing. They did not use modern analytical instruments but were able to develop the cures for various diseases using different species of medicinal plants.

The demand for medicinal plants is increasing every day. The WHO has projected the global market for herbal products to increase to US $200 billion in 2008 and US $5 trillion in 2050. Of the global herbal product market of US $80 billion, China has a share of around US $6 to $7 billion, whereas India's share is not even US $1 billion. There are tremendous opportunities for growth in this area. India has similar potential for promoting floriculture and aquaculture in a big way. Knowledge-based value addition for these natural resources would mean exporting value-added products rather than merely the raw materials. Ancient knowledge is a unique resource of India for it has the treasure of a minimum of 5000 years of civilization. It is essential to leverage this wealth for national wellbeing as well as to seek a global presence for the nation.

Patients are looking for safe and effective medicines from herbal resources as an alternative for primary treatment against several infections and diseases. Traditional knowledge and experiences present so many new remedies, commercialized products, and information based on its application, which can help to develop and promote a diversity of cultural practices. Because we have a better understanding of how the body functions today, we are thus in a better position to recognize the healing powers of plants and their potential use for multifunctional chemical entities for treating various complicated health conditions. Today, in most of the society, use of traditional medicine continues side-by-side with allopathic medicines. Traditional knowledge has two potential values, one as an easy, accessible, and low-cost source of medicines for primary health care, and the other as a source for finding novel leads and/or targets for drug development. Based on the paradigm of "single compound single target and multiple compound multiple targets," the pharmaceutical industry has developed drugs for most targets. The efforts to incorporate knowledge from TM into modern healthcare to ensure that it meets safety and efficacy standards are far from completion.

India is one of the 12 mega biodiversity zones, covering 2.4% of world's territory but with 8% of worldwide biodiversity. It incorporates 15 agro-climatic zones containing around 47,000 plant species, including almost 15,000 medicinal plants (WHO, 2013). An expected 25,000 plant-based formulations are used as a part of folk remedies and known to rural communities in India There are more than 1.5 million professionals of traditional systems of medicines utilizing therapeutic plants for preventive, palliative, and therapeutic applications. There are more than 7000 medicinal drug-producing units in India (AYUSH, 2011), which expend around 2000 tons of herbs yearly. A few traditional healthcare systems have existed in India for hundreds of years and, out of all the traditional practices, Ayurveda, Yoga and Naturopathy, Unani, Siddha, and Homeopathy are the official traditional systems of medicine. These systems are known as Indian Systems of Medicine (ISM), now known by the acronym for Ayurveda, Yoga and Naturopathy, Unani, Siddha, and Homeopathy (AYUSH, 2011), which as a whole provide health services to most of the people of India and neighboring nations (Mukherjee et al., 2010a). This major diversity of traditional medicine resources has been depicted in Fig. 2.2. Commitments for development are made by people of history of science, clinicians, ethnographers, agronomists, organic chemists, specialists in veterinary solution, and different fields. For the future, one would trust that the multiple voices of the different disciplines that add to ethnopharmacology will make a dynamic pressure that energizes discourse and coordinated effort.

2.9 APPROACHES TO DRUG DEVELOPMENT INSPIRED BY TRADITIONAL MEDICINE

Traditional medicine (TM) is helpful in different aspects of drug development from natural resources. Several molecules with potential therapeutic applications have been isolated from plants, including vincristine, vinblastine, artemisinin, paclitaxel, etoposide, teniposide, camptothecin, and podophyllotoxin (anticancer). The pharmaceutical companies have engaged in large-scale pharmacologic screening of herbs from Ayurvedic plants. A confluence of spectacular advances in molecular biology, chemistry, genomics, and chemical technology and the related fields of spectroscopy, chromatography, and crystallography may influence several therapeutically potent lead compounds from TM.

Most of the populations of the Indian subcontinent practice complementary and alternative medicine, which are popular while verification of their safety and effectiveness is modest. Various strategic areas in medicinal plant research should be considered for global importance. This will revitalize herbal medicine in procession with modern medicine. Incorporation of herbal medicine and contemporary tools will help to fight against many complex diseases through the development of new entities and benefit their own development. Such committed efforts toward discovery would be advantageous with support from superior strategies.

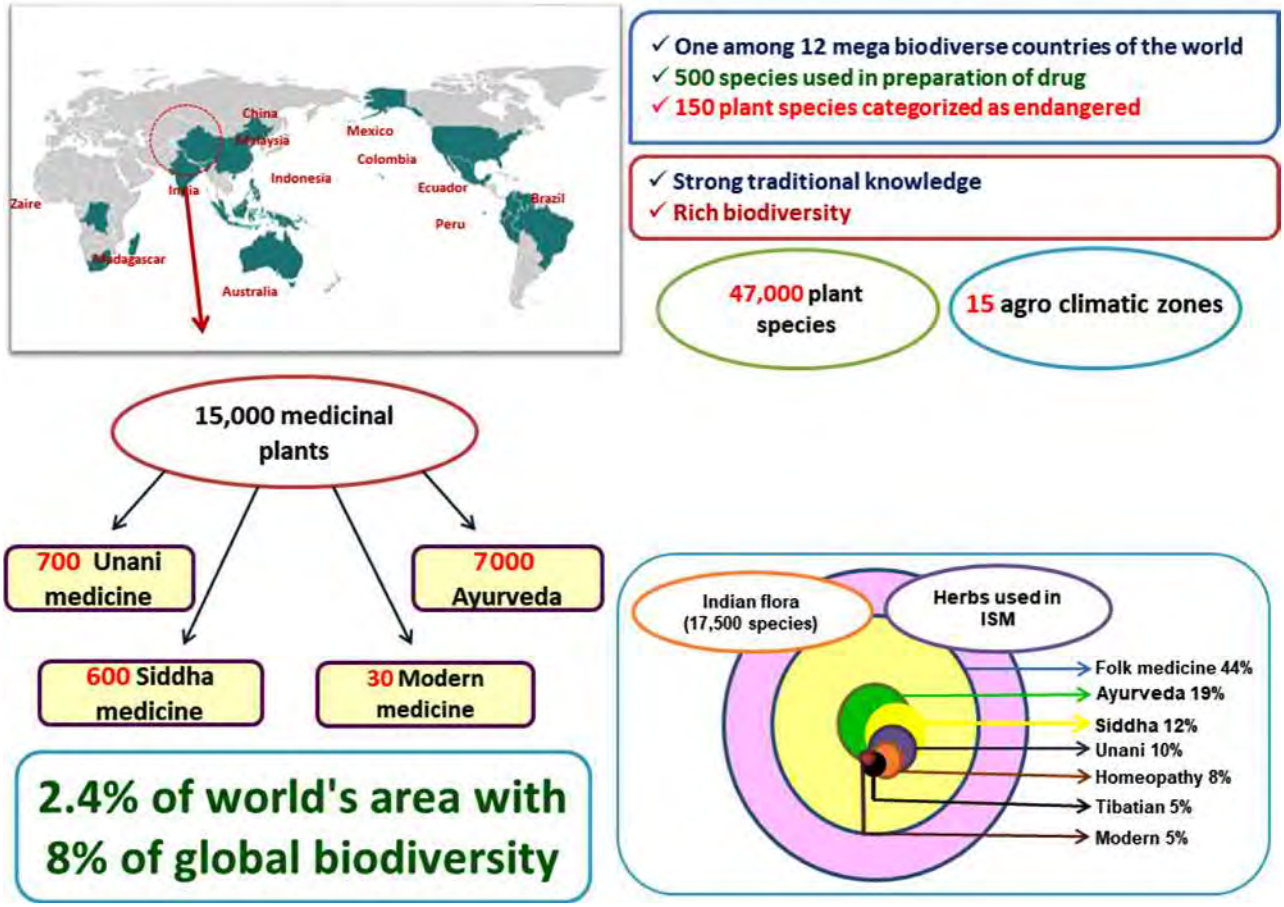

FIG. 2.2 Biodiversity and traditional medicine resources of India.

Evidence-based research is receiving greater acceptance in India and its subcontinents. The concept and methods of Ethnopharmacology research developed elements from diverse disciplines, such as Ayurveda, Siddha, ethnobotany/ethnomedicine, chemistry, pharmacognosy, pharmacology, biochemistry, molecular biology, and pharmacy. The main objective of Ethnopharmacology was to develop appropriate techniques to evaluate the traditional remedies in line with the orthodox concepts of Ayurvedic pharmacy and pharmacology. The scientific evaluation and standardization of traditional remedies using exclusively the parameters of modern medicine is both conceptually wrong and unethical. The evaluation of traditional remedies, particularly those of the classical traditions, has to be based on the theoretical and conceptual foundation of these classical systems of medicine, but may utilize the advancements made in modern scientific knowledge, tools, and technology (Mukherjee et al., 2009c). In fact, herbs/plants are the oldest friends of mankind. They not only provided food and shelter but also served to cure different ailments. By the middle of the 19th century, at least 80% of all medicines were based on ethnopharmacology. Even today, if we walk into any pharmacy, we will find that at least 25% of drugs are derived from plants. Indeed, today, many pharmacological classes of drug include a natural product prototype. Ethnopharmacology and TM are still important components of human healthcare worldwide. People rely on ethnomedicine for its cost-effectiveness, acceptability, biomedical benefits, and accessibility. There has been a continuous growth in the demand for herbal medicines globally. The demand has been increasing as a result of the growth of human population, habitat loss and alteration, overexploitation, overgrazing, deforestation, and the frequently inadequate provision of modern medicine. Presently, the use of ethnobotanical information in medicinal plant research has gained considerable attention in segments of the scientific community. The treatment of diseases with medicinal plants is more beneficial than synthetic and modern medicines because of ease of use, treatment efficacy, affordable cost, and minimal side effects. Plants are an integral part of life in many indigenous communities. Besides being a source of food, fodder, and fuel, the use of plants as herbal medicines in curing several ailments runs in parallel with human civilization. India is endowed with a rich wealth of medicinal plants, being perhaps the largest producer, and rightly acclaimed as the botanical

garden of the world. It has a strong base of many systems of medicines, including Ayurveda, Unani, Siddha, and other local health practices. The earliest mention of medicinal plants is found in Rigveda (67 medicinal plants), Yajurveda (81 medicinal plants), and Atharvaveda (290 medicinal plant species), with Charak Samhita (700 BC) and Sushruta Samhita (200 BC) as the traditional databases. Out of the estimated 422,000 flowering plants reported in the world, more than 50,000 are used for medicinal purposes (Govaerts, 2001). In India, more than 43% of the total flowering plants are reported to be of medicinal importance. Traditional medicine based on herbal remedies has always played a key role in the healthcare systems of many countries. Modern treatment facilities do not reach aborigines or the people who live far away from towns (WHO, 2003). In the developed countries, 25% of the medical drugs are based on plants and their derivatives. The Indian subcontinent is occupied by over 54 million tribal people residing in about 5000 forest-dominated villages spread across the country, comprising 15% of the total geographical area. Due to constant association with forests, ethnic people have immense plant related folklore, which they inherit and pass on from generation to generation just through oral conversation.

Among several approaches for the search of novel biologically active leads from botanicals, pharmaceutical scientist can evaluate the substance pharmacologically after new chemical constituents are found. However, this is often not thought of to be a really valid approach. A second approach is to just gather each accessible plant, prepare extracts, and check every extract for one or more sorts of medicinal activity. This testing can proceed within the standardization of extracts and, therefore, the bioassay-guided isolation of the active constituents. The phytoconstituents obtained will then be taken for more structure–activity relationship studies. While all these parameters are determined, the constituent/extract obtained is further investigated for its toxicity and safety, followed by clinical trials. This random assortment and in-depth screening methodology could be cheap and, therefore, the best approach that for eventually manufacturing helpful medications capable of being manufactured and developed by industry. The classic methodology of pharmacological screening involves successive testing of herbal extracts or phytoconstituents from biological materials in isolated organs followed by testing in whole animals, largely in rats and mice. A majority of the medications in use as therapeutic agents are found and evaluated in these ways. However, for the analysis of traditional medicine, we should not always follow the conventional approach, but rather return to the holistic in vivo approach. This may also be performed through clinical trials or animal experiments. Besides the classic physiological observations that may be created by in vivo experiments with physiological parameters, genomic, metabolomic, and proteomic investigations are gaining momentum. The systems biology approach combined with metabolomic knowledge for the various extracts of medicinal plants or fractions should be possible to find the combination mechanism between the compounds present within the extract and, therefore, the activity.

A very new world of potentialities with many new technologies have been opened for the evaluation of TM. They provide insight into the attainable changes within the organism in an exceedingly holistic approach in order to perceive the mode of action by scrutinizing the changes within the transcriptome, proteome, and metabolomic patterns compared with those ascertained with established medication. Such an approach is currently referred to as the systems biology approach. The metabolomic approach needs the applied statistical analysis of a huge amount of data by various techniques, such as variable and principle component analysis, to extract knowledge from these data.

Evidence-based drug analysis ought to be conducted with the involvement of patients and funding bodies to determine the task of medical practitioners. A widespread revolution in phytochemistry has been achieved through strengthening its importance with the latest technologies to reinforce the initial link between phytochemistry and TM. Evidence-based analysis includes developing policies, restrictive criteria, and technical markers that might guarantee and supply the continuing accessibility of high-quality, safe, and effective traditional medicinal products. Therefore, through harmonious international coordination, a number of rationalists, scientists, and scholars have dedicated themselves to the promotion and development of alternative systems for drug development from natural resources.

2.10 INTELLECTUAL PROPERTY (IP) RIGHTS IN ETHNOPHARMACOLOGY

Documented proofs regulatory authorization connecting pharmacopeias and monographs would make it simpler for herbal drug manufacturers to gain bigger access to regulated markets across the globe. Ethnopharmacological studies offer scientific rationality for the business use of natural products so that genetic resources can have some market value (Heywood, 2011); however, scientific data alone are not enough to confirm complete development. Patents, logos and copyright are the areas mainly thought to be covered by Intellectual Property (IP) rights. Intellectual property also embodies the knowledge base of ancient traditions as passed on by indigenous communities and rights of possession of genetic resources provided by the Convention on Biological Diversity (CBD) (CBD, 1992). The CBD was the first international accord to ascertain proprietary rights. Thus, the CBD envisages the business use of natural products and creates possession rights in genetic resources to facilitate their use. Those rights will then be supplemented by alternative types of IP, such as patents. Not

everybody agrees with the benefits of IP (Goodman, 1993). There are mixed views regarding whether or not business profit ought to be derived from natural products and whether IP is beneficial to the global economy or a mechanism that encourages biopiracy. The opposing views in this discussion appear to be immovable, but several of the perceived problems may well be resolved by improved dialog and a culture of partnership and transparency.

Sometimes IP rights and patents are mistrusted by native people on the basis that they may lead to legalized plunder of indigenous natural resources. However, patent protection is needed (Lagrost et al., 2010). The vital point to understand is that whoever owns the IP, the exclusivity adds value to genetic resources, so all parties can profit, together with the country of origin and also the indigenous communities. A partnership term must be looked at from both sides. A collaboration can only work if native stakeholders, particularly indigenous communities acknowledge the value that Western business interests will add to their possession rights of genetic resources. Nonetheless, licensing to international trade can be a competitive business. Although authorities' contacts are helpful, it is nonetheless advantageous for country stakeholders to also work with foreign partners, with the aim of commercializing native genetic resources. Additionally, the importance of intermediary organizations who safeguard both interests as they desire continuing transactions with drug companies and on the other hand with supply countries (Sampath, 2005). If any collaboration is to succeed, there must be a mutual respect for every partner's priorities. A distant partner should seek to respect the cultural identity of indigenous peoples and to bring value to the communities while not conflicting with the native culture, or with the continuance of ancient practices.

One of the key factors in a good partnership is to try for open and clear dialogue between the parties. A number of difficulties arise due to expectations and lack of expertise with trade; however, it is not difficult to speak openly, to know the structure of the communities, and to create a partnership of trust and dialog. It is useful to produce clear explanations of equitable profit and an explanation of how profit from the genetic resources can flow back to the countries of origin.

2.11 CONSERVATION OF BIODIVERSITY IN ETHNOPHARMACOLOGY

The huge diversity of plants, animals and microorganisms (both terrestrial and marine) that grow and live in natural or semi-natural ecosystems serve as a resource for ethnopharmacology, might doubtlessly be exploited for medication and also the traditional information regarding their use. Today, that resource base is progressively threatened, primarily from environmental loss or degradation along with development, growth within the human population, and also the impacts of invasive alien species; however, ethnopharmacologists have devoted insufficient attention to conservation and sustainable use.

Although the central goal of ethnopharmacology is to research on the social science explanation and also the pharmacologic basis of the medicative use of plants, animals, fungi, microorganisms, and minerals by human cultures (Leonti and Casu, 2013), it is progressively changing into a multidisciplinary field of inquiry. The linkages between ethnopharmacology, food, and nutrition are especially being recognized. As has been argued elsewhere (Arnason, 2005; Heywood, 2011, 2013), ethnopharmacology, diversity, agriculture, food, and nutrition are inextricably connected, but suffer from division and have poor collaboration that should be overcome if progress is to be made. Consequently, ethnopharmacology must extend its remit not only to medicative uses, but also take into consideration the food and nutritional aspects of the various species utilized in ancient societies. So, the distinction between food and medication is difficult to maintain at the interface between diet, medication, and natural products. Nearly all societies use plants and animals in more than one way, as food, medicine, and cosmetics' (Etkin, 2007).

The collection of plants and animals for medicines or food goes back to the beginning of human habitation of the world. The utilization of plants as medicines has been recorded from ancient civilizations, such as Egypt, China and Greece (Dias et al., 2012). Until 30 years ago, there were few restrictions on the liberty of people, organizations, or firms to gather, measure, and exploit biodiversity, primarily plants and animals, from around the world as sources of potential medication or medicines or as new food, fiber, oil, or energy crops. In addition to native use, the exploitation of plants by individuals not indigenous to the region where they grow may be a long-standing tradition that dates back to the expedition to Punt (probably today's Somalia) sent by Queen Hatshepsut of Egypt in 1495 BC to bring back trees with musky resin. Later, naturalists and biologists engaged in sampling for plants and animals that might be exploited, either within the country involved or by transporting them back to their own country for introduction to cultivation or domestication.

In 20th century some pharmaceutical firms invested heavily in the exploration of plants to find new sources of natural products that may have potential for the development of new medication and a huge number of species were screened for this purpose. The situation changed drastically after the Convention on Biological Diversity (CBD) came into effect in 1993. Before the CBD, the world's diversity of plants, animals, and microorganisms was considered the "common heritage of mankind" and exploited to a large extent. Throughout the negotiations leading up to the CBD, it had been forcibly explained that such an angle was utterly unacceptable and on the contrary the position that countries have sovereignty over the diversity and genetic resources within their own frontiers was enshrined within the CBD. It specifically mentions species

of medicinal value in the indicative list of classes of the elements of biological diversity to be known and monitored (CBD, 1994). It also includes measures to respect, preserve, and maintain the data, innovations, and practices of traditional and native communities for preserving and sustainably using diversity. In 2002, the Bonn guidelines on access to genetic resources were enacted, in which honest and equitable sharing of the profits from the use of genetic resources was envisaged. The CBD adopted the Nagoya Protocol in 2010, which aims at sharing the advantages arising from the use of genetic resources in a very honest and equitable manner, taking into consideration all rights over those resources and to technologies, to the conservation of biological diversity, and also the property use of its components (CBD, 2010).

In an extrapolation, from the proportion of national floras enclosed in 15 national pharmacopeias, Schippmann et al. (2006) calculated that 50,000–70,000 species of upper plants are used as medicines worldwide. So far, plants have provided more active compounds utilized in medicines than have animals, though marine organisms are progressively proving to be a vital supply. Newman and Cragg (2012), in their review, highlighted natural products as sources of medicine from 1981 to 2010 and it is clear that the role of microbes as sources of novel bioactive entities depends on the interaction with organisms from similar or sometimes different taxa. They cite the case of activations of natural product biogenesis in actinomycetes by mycolic acid-containing microorganisms (Onaka et al., 2011).

2.11.1 Ethnobioresources and Conservation Approaches

Until the 1980s, medicinal plant conservation was neglected and under-appreciated. A key milestone was the international consultation on the conservation of medicinal plants WHO-IUCN-WWF in Chiang Mai, Thailand in 1988 and, therefore, the Chiang Mai declaration issued by the consultation affirmed the importance of medicinal plants and referred to the UN, its agencies, and alternative international organizations to require action and to support conservation. A draft set of guidelines for the conservation and sustainable use of medicinal plants was approved and later revised and published (Heywood and Synge, 1993).

Different varieties of community conservation are usually adopted for medicinally vital species in ancient societies. Though usually lacking legal recognition or government support, community-based approaches are progressively receiving support from nongovernmental organizations (NGOs) and are helpful in combining both native and formal systems of medicine (Shukla and Gardner, 2006). Culturally protected forest patches or sacred groves are instances of community conservation. Structured scientific approaches to the conservation of medicinal and aromatic plants, such as genetic conservation, have developed considerably in the past 20–30 years. The conservation of target species within ecosystems is a complicated and tough method (Heywood, 2014) and has been tried to this point for a very small number of medicinal plants. Like agricultural plants, as well as wild food plants and crop wild relatives, the main target is on the genetic variation (hence the term "genetic conservation") that is responsible for providing the required characteristics. In the case of medicinal plants, the secondary metabolites are exploited. Genetic conservation involves technical procedures, such as ecogeographical measurement, population sampling, and analytical techniques for crucial genetic variation, and socioeconomic problems, such as wild harvest homes and property use of those resources, bioprospecting agreements, and the role of native communities and property rights. Specific attention has to be paid in the sampling of populations for genetic conservation to the variations within the constituents, such as essential oils, which can be full of several factors (Hunter and Heywood, 2011).

Intraspecific variation will occur as a result of differing soil conditions, altitude, atmospheric conditions, seasonal factors, and alternative environmental options, leading in some cases to the evolution of various chemical variants or chemotypes. This important chemical variation occurring in plants, particularly those with distinguished chemical parts, such as terpenoids, and alkaloids, has attracted the attention of the researchers and an intensive literature has been developed.

The ex situ conservation of germplasm of medicinal plants in seedbanks, living collections in botanical gardens, field gene banks, tissue, and cell culture is a crucial complement to in-place approaches. Several seed banks do contain samples of some medicinal plants; however, they are not typically the result of deliberate sampling campaigns. Botanical gardens have occupied a crucial role within the study of medicinal plants for the past 500 years (Heywood, 1987) and proposals for medicinal plant conservation have been created by Heywood (1991) and Hawkins (2008). A review of the techniques to preserve germplasm for ethnobiology is given by Dierig et al. (2014). The quantity of medicinal plant species in trade that are cultivated may be only a thousand (Mulliken and Inskipp, 2006). Bringing widely used medicinal plant species into cultivation might take the pressure off their wild populations by reducing the amount required to be harvested. It also permits higher species identification, improved quality control, and greater prospects for genetic enhancements. On the other hand, it is argued that the cultivation of medicinal plants might also, in some cases, result in a loss of wild habitat and scale back incentives to conserve and manage wild populations (Dulloo et al., 2014).

2.12 NETWORK PHARMACOLOGY IN ETHNOPHARMACOLOGICAL RESEARCH

Drug discovery from ethnopharmacological resources is moving toward a systems level polypharmacology method to deal with circumstances, for example, absence of viability and protection of single-targeted compounds (Hutchinson and Kirk, 2011). System pharmacology is put forward as a new change in outlook in drug discovery (Hopkins, 2008). It presents methodologies to seek novel remedial prospects to re-purpose approved drugs, which are being created and connected continually. In actuality, the idea of system pharmacology is consistently associated with pharmacognosy and ethnopharmacology or traditional medicine. In traditional medicine, such as Ayurveda and Traditional Chinese Medicine (TCM), the idea of utilizing blends of plants (herbal medications) to cure is quite famous (Gertsch, 2011). Formulations, such as "Triphala" used in Ayurveda, comprise a mix of herbs in which various dynamic phytometabolites focus on numerous targets and pathways (Chandran et al., 2015). The explanation for the range of potential connections between these phytometabolites with their targets facilitates exploring mechanistic pharmacology. It also maps any potential adverse effects before clinical trials. Subsequently, hazard is limited to speed up the medication improvement pipeline (Kitano, 2007).

In ethnopharmacology-driven drug discovery, TCM has set a good effort by advancing its research by "omic" strategies utilized as a part of systems science. Researchers of TCM have strategized their research paradigm to accomplish the system pharmacology objective. The critical initial step is to make a database of herbs and their related target space. There are a number of helpful databases showing target information accessible on the web. After making the database of phytometabolites, the procedure for target screening follows. Target screening includes utilization of in silico systems, such as virtual screening (VS) and cheminformatics procedures, including pharmacophore research, docking, reverse docking, and QSAR methods for descriptors and fingerprints. These by and large give us target scores of the compounds, such as docking score, comparability, and fitness. These scores speak to the edge property, which interfaces two nodes. Reconciliation of this information with pathway data from different open source databases can create organized pharmacology models. The total system is assembled by means of Cytoscape, an easy to use free system for network model development. These systems are essentially portrayals of complex information utilizing graph theory. Scientists have discovered a powerful apparatus for predicting the targets involved in the bioactivity of the compounds present in herbs and formulations. The concepts of network pharmacology are illustrated in Fig. 2.3.

2.13 REVERSE PHARMACOLOGY

Reverse pharmacology is an exploration technique based on observations of customary medications that is a transdisciplinary approach coordinating traditional information, experimental observations, and clinical encounters with the point of reversing from the established lab-to-clinic procedure to a clinic-to-lab approach (Simoes-Pires et al., 2014). Ayurveda-based medication discovery utilizes this reverse pharmacology approach, in which drugs are first identified in light of large-scale use in the populace and approved in clinical trials. Time and cost are reduced for drug discovery from traditional medicine by reverse pharmacology (Patwardhan, 2007). The Ayurvedic information database permits drug researchers to begin from a thoroughly tried and safe natural material. By using this knowledge, the ordinary medication discovery starts from patients rather than laboratories. The reverse pharmacology approach initially affirms the activity of a medication (such as an Ayurvedic drug), after which additional studies should connect the action to bioactive parts (Mukherjee et al., 2011). This technique stresses security and viability, and is an alternative method for drug discovery. Medications, such as reserpine, obtained from *Rauvolfia serpentina*, arose simply following 20 years of work, despite the fact that its antihypertensive property was shown long beforehand. There is a need to record obscure, unintended, and novel prophylactic and positive effects in observational investigations. Because very few new molecules are being created, the extent of utilizing this approach for traditional approval is colossal and numerous studies are being prepared for the near future (Mukherjee et al., 2009a) Different aspects of reverse pharmacology are shown in Fig. 2.4.

2.14 PHYTOEQUIVALENCE

Phytoequivalence is the term being used to deal with the bioequivalence of a phytomedicine compared with another product that may be the subject of extensive research. In this concept, pharmaceutically equivalent drug products are formulated to contain the same amount of active ingredient in the same dosage form and to meet the same compendia or other applicable standards, that is, strength, quality, purity and identity, but may differ in characteristics, such as shape, scoring configuration, release mechanisms, packaging, excipients (including colors, flavors, preservatives), expiration time, and, within certain limits, labeling. The concept of phytoequivalence was developed in Germany in the mid 1990s, which means that one herbal extract matches, or is equivalent to, another herbal extract, more specifically, one of the clinically proven extracts.

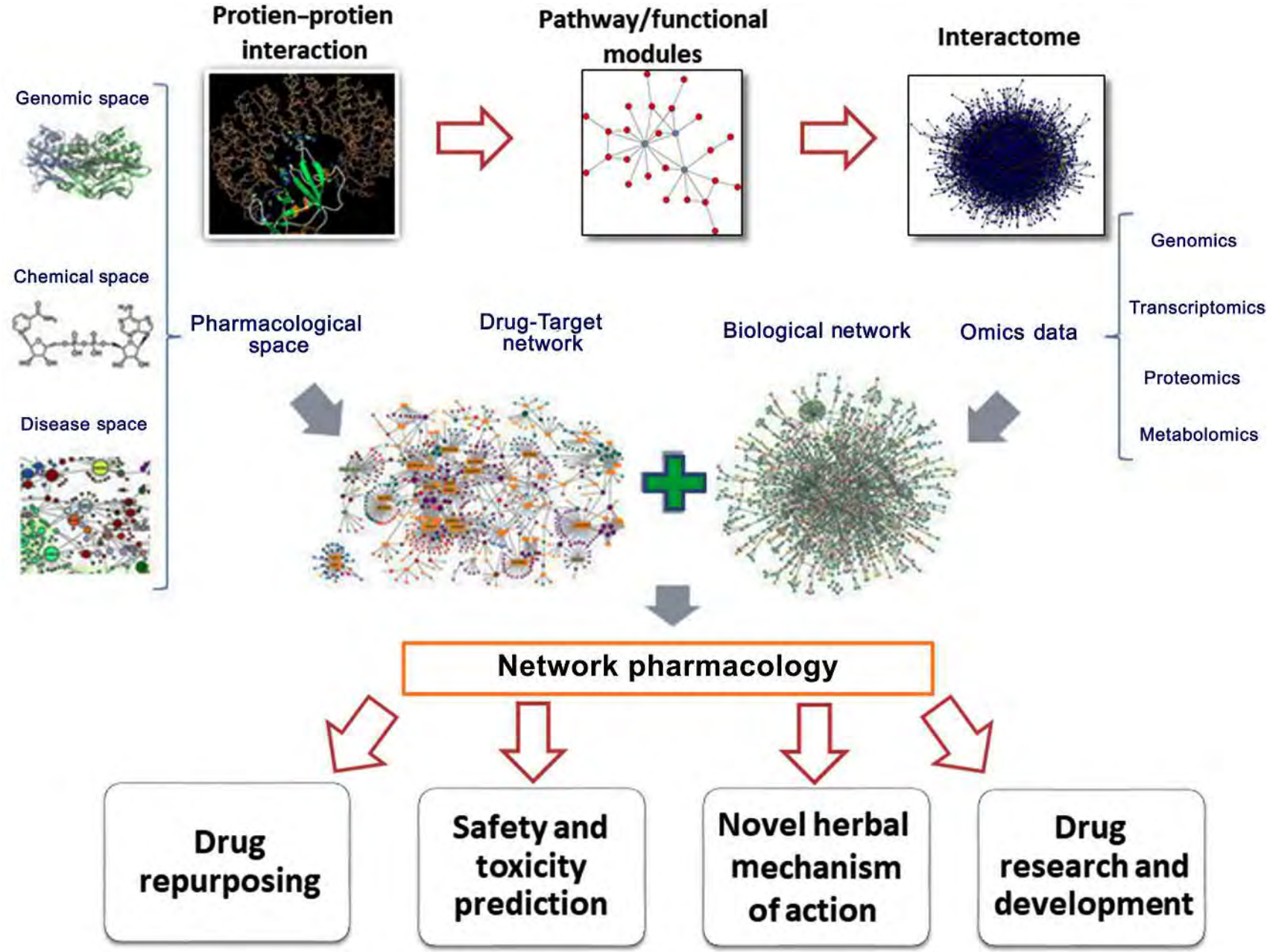

FIG. 2.3 Network pharmacology concepts and applications.

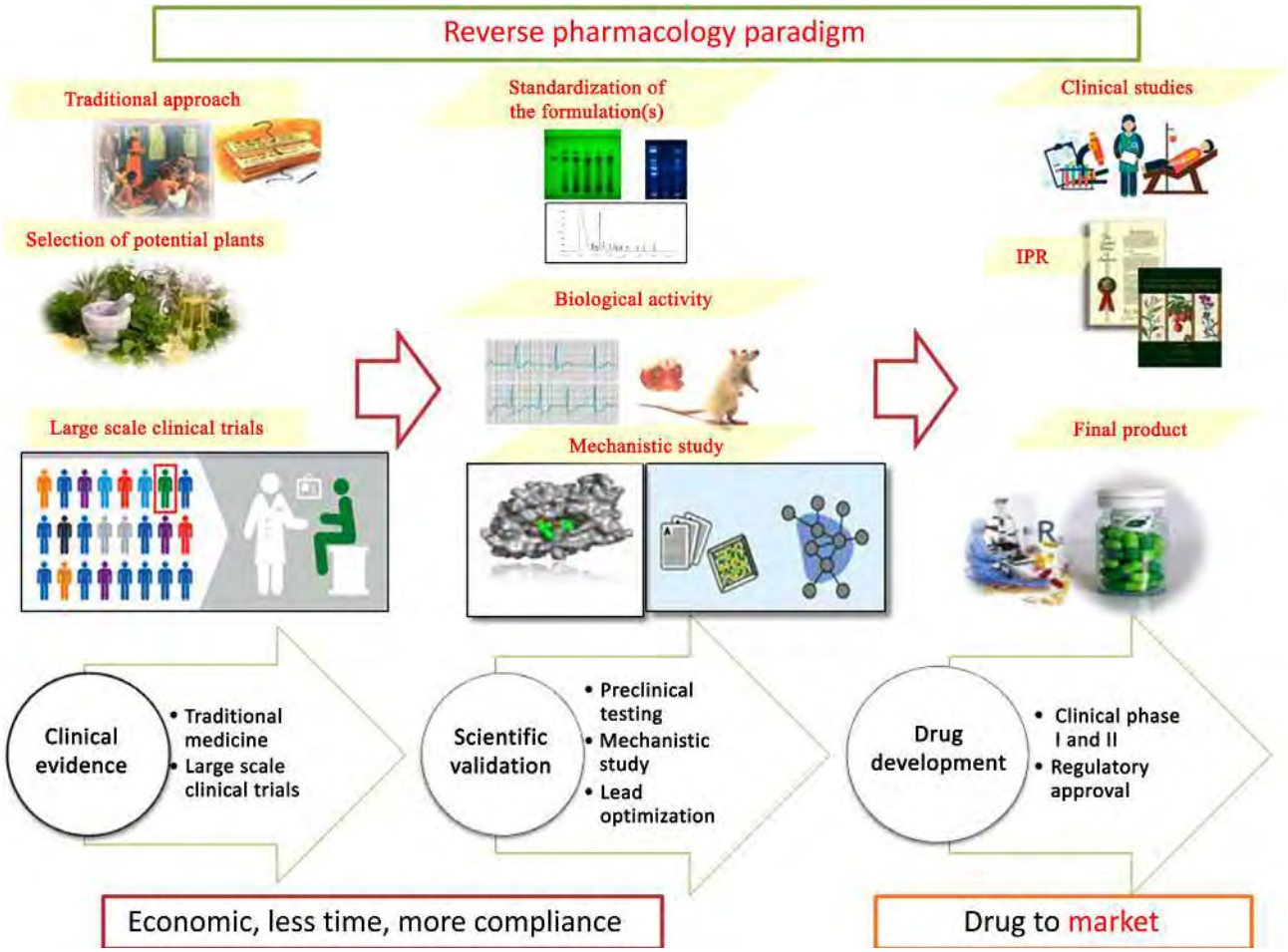

FIG. 2.4 Reverse pharmacology in drug development.

The history of phytoequivalence in Germany was marked by previous versions of the German Pharmacopeia (DAB), which specified exact conditions for plant preparations, including extraction methods, time, and specific solvents. Several comparative clinical trials showed that phytopharmaceuticals had full therapeutic equivalence with chemotherapeutics and had the simultaneous advantage of being devoid of any adverse effects. The mechanism of action of herbal drugs and their extract preparations, which differ in many respects from that of synthetic drugs or mono substances, can be characterized as a polyvalent action and interpreted as additive or, in some cases, potentiating. Phytoequivalence basically means chemical equivalence. A chemical fingerprint profile of an herbal product of proven efficacy should be constructed, which may serve as the reference for quality control at commercial scale. Standardization based on a single or a small number of chemical markers or classes of compounds serve to promote quality control and batch-to-batch consistency. A number of variables are important in the formulation of natural products, such as the percentage of alcohol in a hydroalcoholic solvent, extract concentration, uniformity of plant material, and the time and temperature of extraction. Phytomedicines must be compared and tested directly to synthetic drugs. Pharmaceutical companies should set aside 15% of gross sales revenues to research in order to research areas of phytoequivalence. The need of the hour is to evolve a systematic approach and to develop well-designed methodologies for the standardization of herbal raw materials and herbal formulations (Mukherjee, and Houghton, 2009).

2.15 PHYTOPHARMACEUTICALS

Ethnobotany/ethnopharmacology and medicine discovery utilizing natural products have remained important issues as of late. Ethnobotany is the investigation of plant-human connections installed in powerful environments of common and social parts. At the end of the day, ethnobotany is the investigation of contextualized plant use. Plant use and plant-human connections are formed by history, by physical and social conditions, and by the innate characteristics of the plants themselves.

Phytobiotechnology incorporates plants and plant-based innovation for the nourishment and control of diseases (nutritherapy) and phytotherapy implies treatment of infections by the use of medicinal plants as well as therapeutic plant extracts. The plant or part thereof can be made in at least one of the following forms: decoction, concoction, mixture, galenical, tincture, or a tisane, or taken with food or as a enema or as a salve for topical application. Phytobiotechnology requires that traditional knowledge on the uses of a medicinal plant be demonstrated scientifically and afterward detailed into a product using basic pharmaceutical information in its common form and that some type of standardization (fundamental investigation of phytochemical constituents) and quality control be used. Phytobiotechnology is an environmental way to deal with drug development from mother earth without artificial interference (Yongabi, 2004).

An ethnobotanical idea spins around the use and administration of plants by a human group. This investigation of traditional medicine is not intended to advocate an arrival to the use of these cures in their native form or to exploit traditional solutions. The goals of ethnopharmacology are to safeguard and record an important social legacy before it is lost and to research and assess the specialists used. In this manner, it assumes a large part in the assessment of natural products and all the more especially the natural medications from traditional and fabrication assets. Field observations and descriptions of the use and impacts of traditional cures, herbal identification, and phytochemical and pharmacological studies, all fall within the extent of ethnopharmacology (Mukherjee et al., 2007a, b).

Ethnopharmacology has effectively assumed a critical part in the advancement of traditional medicine and is probably going to assume a larger part in the years to come. Cooperation among ethnobotanists, ethnopharmacologists, doctors, and phytochemists is basic for productive results in therapeutic research on plants. While ethnopharmacologists have a more prominent part to play in the legitimization of a combination of activities, the phytochemist's role will marginally shift toward the standardization of botanicals.

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Chapter 3

Quality Evaluation of Herbal Medicines: Challenges and Opportunities

Chapter Outline

3.1 FACTORS AFFECTING THE QUALITY OF HERBS

The increasing demand for herbal medicines in both developing and developed countries inevitably leads to maintaining the quality and purity of herbal raw materials and finished products. Standardization relating to herbal drugs arises from the complex composition of drugs that are used in the form of whole plant, plant parts, or extracts obtained therefrom. To ensure the reproducible quality of any herbal remedy, proper control of the starting material is most essential. To control the quality of the starting material, several factors need to be considered, as discussed in subsequent sections.

3.1.1 Authentication and Reproducibility of Herbal Ingredients

Herbal ingredients must be accurately identified by macroscopical and microscopical characteristics, and compared with authentic material or an accurate description of authentic herbs. It is essential that herbal ingredients be referred to by their binomial Latin names of genus and species. Only permitted synonyms should be used. Even when correctly authenticated, it is important to realize that different batches of the same herbal ingredient may differ in quality due to a number of factors.

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Details on the identification and authentication of herbs and their morphological/histological evaluation will be described further in Chapters 4 and 5.

3.1.2 Inter/Intra Species Variation in Plants

There are considerable inter- and intraspecies variations in different plants, for which the primary and secondary metabolite also varies considerably. This results in variation of the individual constituents and thereby causes difficulties in standardization. All of these variations are genetically controlled and are related to the country of origin for that particular species.

3.1.3 Environmental Factors

Many factors, such as climate, altitude, rainfall, and other conditions responsible for the growth of plants, affect the quality of herbal ingredients present in a particular species, even a species found in the same country. This results in major variations in the herbal ingredients present in some specific species of plants.

There are several environmental factors controlling the variation in the hundreds of compounds in a particular herbal preparation, topical cosmetics, or oral formulations. So, being cognizant of these factors is very important for obtaining a uniform quality of plant. Plants are constantly exposed to different environmental factors, many of which produce stress on the plant. The plant gradually adapts itself to its changing environment and the production of these metabolites changes. The changes take place at the genetic and the protein level, which alters the expression of several enzymes participating in the biosynthetic pathways of the secondary metabolites. As a result, production of those metabolites also changes.

The biosynthesis of certain secondary metabolites of plants is generally controlled by several factors. Secondary metabolites are generally localized in some specific plant parts, tissues, and growth stages. Production of these compounds is affected by some environmental factors (Bandaranayake et al., 2006).

Over the years, plants have undergone evolution and adaptation to cope with the extremes of climatic conditions, such as drought, excessive rainfall, extremely high or extremely low temperature, extremely sultry weather throughout the year, flood, and so on. During the course of these evolutionary changes, plants have been changing their pattern of production of secondary metabolites. The production is altered in such a manner as to compensate for the environmental stress, which exerts a negative effect on the plant's fitness and survival. Several factors influence the growth and development of plants as well as the secondary metabolites present therein, as discussed in the subsequent sections.

3.1.3.1 Solar Radiation

Solar radiation, especially in the ultraviolet region, is essential for the photosynthesis of plants. Interestingly, a particular region of the ultraviolet range is harmful to the body of the plant (UV-B region 280–315 nm). The plant needs to protect itself from these rays. So, in order to do so, it produces phenylpropanoids, that is, flavonoids, anthocyanins, hydroxycinnamic acids and their esters, anthocyanidins, and tannins in increased quantities. Flavonoids have different roles in plants. Depending on their role, they have diverse chemical structures. These compounds provide protection to the plant against pathogenic and herbivorous attacks and ultraviolet radiation. The special attribute of these compounds is that they absorb strongly in that particular range. This is very important from the perspective of natural products scientists as these compounds have pronounced antifungal, antibacterial, antiviral, antiinflammatory, antioxidant, antitumor, antihepatotoxic, antilipolytic, vasodilator, immunostimulant, and antiallergic activities.

So, the degree of exposure of plants to solar radiation is an important clue for the metabolome determination of the plant, as well as for the type of activities of the particular plant. Other than the polyphenolic compounds, there are other classes of compounds for which production is enhanced due to environmental stresses. These include the glycol-alkaloids, such as α-solanine and α-chalconine, which accumulate in potato tubers and, as a result of this, accumulation of phytosterols also takes place in the plant tissues. The former is detrimental for the human health, as it causes gastro-intestinal or neurological disorders in humans. On the other hand, the phytosterols are good for human health as they help to keep cardiovascular disorders at bay.

3.1.3.2 Soil Nutrients

The nutrient supply to the plant is an important factor for controlling the secondary metabolite profile. It is obvious that the production of primary metabolites, rather than secondary metabolites, is more important for the plant. If the soil is nutrient rich or has been made nutrient rich, then the production of nitrogen-containing metabolites, such as alkaloids or cyanogenetic glycosides, is possible. If the soil is deficient in nutrients, then the plant won't be able to synthesize these

constituents. A limited phosphate supply in the soil enhances the production of anthocyanidin and an inadequate iron supply results in enhanced production of phenolics. According to the carbon nitrogen balance hypothesis, a plant growing in soil that is deficient in nitrogen will produce more carbohydrate-rich metabolites. The amino acid, phenyl alanine, is used as a precursor in the biosynthesis of different alkaloids. Phenyl alanine is incorporated into the structure of the alkaloids via the phenylalanine ammonia-lyase enzyme.

The production of phenolic compounds and of proteins is interdependent. An increase in the production of proteins results in a decrease in the production of phenolics. It is well established that the production of secondary metabolites is dependent on the plant's genetic makeup and on external factors. In the roots of the plant *Rodiola sachaliensis*, the effects of pH and nutrients have been well observed. A close relationship has been established between the nitrogen content of the soil and the production of the cyano-genetic glycosides of medicinal plants.

3.1.3.3 Stress

During ontogeny, plants interact with the surrounding environment; they come in contact with different abiotic components, such as water, light, temperature, soil, and chemicals (minerals/fertilizers). Plants require an appropriate amount of these components for their developmental growth and survival. However, either more or less of these abiotic components cause stress to the plant, ultimately leading to variation in the production or accumulation of metabolites (Verma and Murmu, 2015) (Table 3.1).

3.1.3.3.1 Water Stress

Water is a key molecule in the physiological activities of a plant as it works as a medium for the transportation of metabolites and nutrients to all parts of the plant. When the supply of water becomes limited or transpiration rates increase in plants, it causes water stress, that is, drought stress and salinity stress.

TABLE 3.1 Metabolites Present in Some Frequently Prescribed Medicinal Plants

Name of the Plant	Main Productive Plant Part	Name and Type of Metabolite
Senna (*Cassia angustifolia*)	Leaf	Sennoside A and B (anthraquinone glycoside)
Cinchona (*Cinchona officinalis*)	Bark	Quinine and Quinidine (quinoline alkaloid)
Opium (*P. somniferum*)	Dried latex	Morphine, Papaverine (isoquinoline alkaloids)
Ispaghula (*Plantago ovata*)	Dried husk	Mucillages (carbohydrate)
Pterocarpus (*Pterocarpus marsupium*)	Wood exudates	Pterostilbene (unsaturated stilbene type glycoside)
Vinca (*C. roseus*)	Whole plant	Vincristine, Vinblastine (indole alkaloid)
Nux-vomica (*Strychnos nuxvomica*)	Seeds	Strychnine, Brucine (indole alkaloid)
Fennel (*Foeniculum vulgare*)	Dried fruit	Fenchone, Anethole (monoterpenoid)
Clove (*Eugenia caryophyllus*)	Flower bud	Eugenol (monoterpene)
Fenugreek (*Trigonella foenum-graecum*)	Seeds	Trigonelline (pyridine alkaloid)
Digitalis (*Digitalis purpurea*)	Leaf	Digoxin, Digitoxin (steroidal glycosides)
Squill (*Urginea maritima*)	Dried bulb	Scillarens (steroidal glycosides)
Belladonna (*Atropa belladonna*)	Leaves and flowering and fruiting tops	Tropane alkaloids (Atropine, Hyoscyamine)
Ma-Huang (*Ephedra* sp.)	Dried whole plant	Ephedrine (proto-alkaloid)

3.1.3.3.2 Drought Stress

In drought conditions, water deprivation brings about different histological changes in the plant body, such as stomatal and membrane damage and osmotic stress, accompanied by disrupted enzyme activities, which are responsible for changes in photosynthesis. The effect of drought stress varies from plant to plant and from metabolite to metabolite. In the case of plants containing volatile oil, such as Chamomilla (*Matricaria chamomilla*), the volatile oil content has been found to decrease with an increase in the drought stress. In the case of some plants, such as *Artemisia annua, Hypericum perforatum*, and *Catharanthus roseus*, secondary metabolite production has been found to increase due to drought stress. The hyperforin content has been found to increase in *H. perforatum* in response to drought stress. Artemisinin in *Artemisia* and betulinic acid, quercetin, and rutin in *Hypericum brasiliense*, have been found to increase in response to drought stress. In the plant *G. longituba*, water stress has been found to decrease the total flavonoid content. It has been found that 75% field water capacity is optimum for the production of essential oils and other secondary metabolites in *Ocimum basilicum* and *Ocimum americanum*. It has been observed in the case of *Achnatherum inebrians* that alkaloid levels go up in the presence of salt and drought stress. In these cases, the level of Ergonovine becomes higher than Ergine. Phenolic compounds have been found to play a protective role during moisture-deprived seasons, such as during winter in certain regions.

3.1.3.3.3 Salinity Stress

It has been observed that the salinity of the soil is an agent that causes production of secondary metabolites in a particular plant. The concentration of the Na^+ content in the soil is an important trigger in this respect. Reserpine and vincristine production in the plants *Rauwolfia serpentina* and *C. roseus* increases in response to increased salinity stress. *Ricinus communis* increases the production of Ricinine in the shoots but decreases it in the roots in response to salinity stress. *Achillea fragrantissima* shows an increase in the phenolic content. Protocatechuic, chlorogenic, and caffeic acids production increases in the plant *M. chamomilla*. *Mentha piperita*, *Thymus maroccanus*, *Origanum vulgare*, *Majorana hortensis*, *M. chamomilla*, *Salvia officinalis*, *Mentha pulegium*, and *Mentha suaveolens* show decreases in the content of essential oil.

3.1.3.3.4 Chemical Stress

Different chemical factors influence the production of metabolites in the plant. They are minerals, heavy metals, fertilizers, pollutants, gaseous toxins (CO_2 and ozone), pesticides, growth regulators and elicitors, $FeSO_4$, $ZnSO_4$, Miczink, $CuSO_4$, and others. Nitrogen and phosphorous, two key ingredients for plant growth and development, supplied through fertilizers, have a significant effect on the production of Naphtho dianthrones (such as the hypericin, hyperforin) in St. John's Wort. Nitrogen and phosphorous are also responsible for manipulation at the genetic level. This has been revealed through genomic and bioinformatics studies. Phosphorous has also been found to significantly affect the growth and production of the secondary metabolites of the plant. It has been found to have a lesser correlation with the essential oil content of the plant.

3.1.3.3.5 Temperature

Temperature stress has been shown to influence enzymatic activities in the plant body. The most important enzymes in this context are the native antioxidant enzymes, such as the superoxide dismutase, catalase, peroxidase, and several antioxidants. Hypericin, pseudohypericin, and hyperforin concentrations in the shoot tissues of St. John's Wort have been found to increase in response to a temperature in the region of 35 °C. Different volatile compounds have also been found to be affected by temperature stress. Polyphenolic compounds have proven to be a miraculous class of compounds as they possess important temperature-protective activities. Anthocyanins have been shown to possess this particular activity in plants. Herbivore stress is an important factor in the production of different types of secondary metabolites. Mechanical damage inflicted by herbivore insects is very effective in putting stress on the plant for the production of secondary metabolites. Ozone has also been found to influence the secondary metabolite production.

3.1.3.3.6 Ecological Factors

In the era of "set-aside" land, growing herbs as an organic crop offers a new opportunity for farmers who find that their usual crops are no longer economical to grow. This is one of the major factors that support the increased use of medicinal herbs. However, the rise in popularity of herbal medicines directly threatens the survival of some wild species. For example, demand for *Panax quinquefolium* (American ginseng) has become so great that it now fetches around US$ 1100 per kg. About 2 centuries ago, it was a common plant in the woodlands of Northern and Eastern America. The extinction of plant species as a result of over intensive collection is nothing new. The herb Silphion, a member of the carrot family, was used extensively as a contraceptive by the women of ancient Rome. Silphion proved to be difficult to cultivate and was gathered from the wild

in such large quantities that it became extinct during the 3rd century AD. Today, if herbal medicine is to grow at its present rate, it is imperative that manufacturers proceed with the production of herbal drugs in an ecologically sensitive manner.

3.1.3.4 Seasonal Variation

The content of secondary metabolites in plants can vary due to seasonal variation as well as intra- and interplant and interspecies peculiarities, in spite of the existence of genetic control, gene expression, genotypes, and biological and environmental factors, as well as biochemical, physiological, ecological, and evolutionary processes. Opium poppy (*Papaver somniferum*) is a rich source of more than 80 alkaloids, of which morphine, codeine, thebaine, narcotine, and papaverine are the major ones, and is extensively used in the pharmaceutical industry. These alkaloids have seasonal variation in their content at different developmental stages. The optimum harvesting time of the alkaloid and diurnal variation should be taken into consideration simultaneously, otherwise the principle compound may be transformed or degraded. This might be due to an increase or decrease in the enzyme activity in the alkaloid biosynthetic pathway following an irreversible path, so once converted into another compound, the previous compound becomes absent from the latex.

3.1.4 Geographical Location

This factor is nothing but an ensemble of the different environmental factors discussed so far. Due to regional effects, the climatic conditions also change. Locations and environmental conditions definitely have an impact on the biosynthesis of PSMs. In some locations or regions or in certain climatic conditions, plants adapt better than in other conditions. Productive climatic conditions favor the growth and development of plants and also play an important role in determining the concentration of secondary metabolites. So, the concentration of secondary metabolites can vary in the same plants in different regions, depending on the availability of favorable or unfavorable climatic conditions. The accumulation or production of secondary plant metabolites also depends upon the physiological needs of the plants according to whether climactic conditions are favorable or unfavorable. For example, the content of Camptothecin (a mono-terpeneindole alkaloid) in *Nothapodytes nimmoniana* is influenced by the location and climatic conditions. The effect of different locations on the antibacterial activity in *Mentha spicata* from different regions results in different yields. Plants from a higher altitude have a higher antibacterial activity (Verma and Murmu, 2015). Environmental factors, such as light, temperature, soil nutrients, and moisture, influence the production of secondary metabolites, such as terpenoids, alkaloids, flavonoids, flavonol glycosides, hydroxycinnamic acids, tannins, phytosterols, glycoalkaloids, luteolin, apigenin, phenolic compounds, isoprene, anthocyanins, and cyanogenetic glycosides.

3.1.5 Plant Parts Used

Usually, the active constituents vary between the different parts of a plant. It is not uncommon for an herbal ingredient to be adulterated with parts of the plant that are not normally utilized. The same situation arises when exhausted plant parts of the same physical appearance are mixed to increase the weight of the supplied herbal ingredient, causing adulteration. The plant body is composed of different parts. Specific plant species possess specific type(s) of metabolites in some specific parts only. This takes place due to different factors. The adaptive needs of the plant trigger storage of secondary metabolites in some specific plant parts. Some preliminary examples are given in Table 3.1:

So, what we notice here is that the main therapeutic constituents in certain plant species are located in specific parts. Secondary metabolites are those chemical substances that are not directly involved in the growth and development of plants. They lead to the biosynthesis of simple to complex metabolites, utilizing the intermediates from the primary metabolites through specific pathways. Important secondary metabolites include alkaloids, terpenes, phenolics, and vitamins, among others. Most of these metabolites have no direct role in plant growth and development and are important to those plants in which their biosynthesis occurs. Most secondary metabolites are toxic and therefore play a defensive role against biotic factors, such as protecting against pathogens and herbivores, allelopathy, and other factors.

Example 3.1
Many researchers have reported that the concentration of secondary metabolites varies from plant species to plant species and even among different parts of the same species (Table 3.2).

- *Lespedeza capitata* exhibits a higher concentration of phenolics in the leaflets compared with the roots.
- The total phenolics value is higher in sunny regions compared with shady regions.
- Eight plant species belonging to seven different families have a level of secondary metabolites in the leaves that is comparatively greater than the stem of the same plant species.

TABLE 3.2 Effect of Plant Parts on the Total Phenolic Content

Plant Species	Plant Part	Total Phenolic (mg/g)
Silphium perfoliatum	Leaves	20.9 mg/100 g
	Inflorescence	23.7 mg/100 g
	Rhizome	19.0 mg/100 g
Larrea tridentate	Leaves	36.2 mg/100 g
	Inflorescence	40.8 mg/100 g
	Rhizome	28.6 mg/100 g
Nerium Oleander	Young leaves	120 µg/g
Rhododendron sp.	Young leaves	340 µg/g

- Young plant parts contained a greater level of alkaloids compared with old parts.
- The levels of saponin are found to be comparatively greater in the stem over the leaves.
- Old leaves contain a high level of phenolic content as compared with old stems, while young leaves and stems show a mixed trend toward the total phenolic content (Achakzai et al., 2009).

Example 3.2

- A number of studies have shown that cinnamaldehyde makes up between 33.95% and 87.23% of the essential oils in *Cinnamomum cassia*.
- In this study, the chemical fingerprints of cinnamon bark, cinnamon twigs, and shaved cinnamon bark were established using liquid chromatography (LC) quadruple time-of-flight (TOF) mass spectrometry (MS) in conjunction with principal component analysis (PCA).
- From 125 samples of cinnamon, the following eight compounds and their detection ratios were identified: coumarin, cinnamaldehyde, cinnamyl alcohol, cinnamic acid, 2-hydroxycinnamaldehyde, 2-hydroxycinnamic acid, 2-methoxycinnamaldehyde, and 4-methoxycinnamaldehyde.

The quantity of cinnamyl alcohol, coumarin, and cinnamaldehyde also varied between the three parts of the plant. Cinnamon twig was also shown to depart from the other clusters. The proposed method provides a fast and efficient means of identifying cinnamon herbs for quality control purposes (Chen et al., 2016) as depicted in Table 3.3.

3.1.6 Time of Harvesting

While collecting a particular herbal ingredient, the optimum time for harvesting should be specified. The constituents, such as various concentrates obtained from the secondary metabolites, vary considerably during the growing cycle. Thus, harvesting at the proper time plays an important role in obtaining the maximum concentration of the desired constituent.

3.1.7 Post Harvesting Factors

The treatment of the collected herbal raw materials, such as storage and transport, can greatly affect the quality of an herbal ingredient. Improper storage after collection may result in microbial contamination and processes, such as drying, may result in a loss of thermolabile active constituents.

TABLE 3.3 Cinnamaldehyde Content Variation in Different Parts of *Cinnamomum* sp.

Cinnamaldehyde Content Variation	
Cinnamon Bark	64.0%
Cinnamon twig	97.4%
Shaved cinnamon bark	50.0%

3.1.8 Contaminants in Herbal Ingredients

Herbal materials should be free from insects, other animal matter, and excreta. As it is not possible to make herbal materials completely free from all these contaminants, specifications have been set up to limit them. This includes the determination of the ash value, which constitutes the inorganic matter after incineration of the particular herbal ingredient. Treatment with hydrochloric acid results in acid insoluble ash, which consists mainly of silica. This may be used as a measure of the soil present. The case for the presence of foreign organic matter is the same. Foreign organic matter is usually present in almost all herbal ingredients, consisting of related parts of the plant or other plants. Standards should be set in order to fix the limit for these unwanted plant contaminants. Sometimes, aerobic bacteria and fungi may be present in plant material due to faulty growing, harvesting, and storage or processing. It is not uncommon for herbal ingredient to have aerobic bacteria present at 10^2 to 10^8 colony-forming units per gram. Some pathogenic organisms, including *Enterobacter*, *Entrococus*, *Clostridium*, *Pseudomonas*, *Shigella*, and *Streptococcus*, have been shown to contaminate different herbal ingredients. Herbal drugs with high starch content are prone to increase microbial growth, so a limit should be set to control all of these contaminants.

3.1.8.1 Environmental Contaminants

Medicinal plants are obtained from natural sources, which may be wild or cultivated sources. Thereby, they are exposed to environmental contaminants, such as microbial species, pesticides, fertilizers, and heavy metals. As a result, these contaminants accumulate in the plant tissues or in the secondary metabolite reservoirs in the plant body, which may be leaf, bark, wood, seeds, fruit pulp, rhizomes, juices, resin ducts, and others. There is a common, well-accepted, but wrong notion that "natural" or "herbal" means the material is safe for use and one can easily count on it. The reality is something different. Microbial contamination in the herbal product can not only deteriorate the active constituents, but there have been clinical case studies exhibiting serious infections due to consumption of contaminated herbs. Pharmacopoeia limit tests for microbial load in crude drug set for the maximum allowable microbial load in a certain amount of the crude drug. Microbial contamination may be bacterial or fungal. Again, from them, chemical contamination by different toxic metabolites, known as endotoxins (Bacteria), Aflatoxins, and Ochratoxins (Fungi) take place. A bacterial species not only may remain in a viable form, but it may also remain in the form of a spore, which under favorable circumstances may be converted to a viable form. Chemical contaminants may occur as agrochemical residues: pesticides, fertilizers, fumigants, mycotoxins, and residues of solvents. Other than these two types of contaminants, there may be radioactive contaminants, such as Cs-134 and Cs-137. Another important source of inferior plant quality is adulteration, which means the intentional or unintentional admixture into the medicinal plant product of toxic plants, inaccurate herbal species, synthetic products, and other substances, such as corticosteroids, NSAIDs, and benzodiazepines.

3.1.8.2 Pesticides, Fumigants, and Other Toxic Metals

Herbal materials growing as cultivated crops may be contaminated by DDT or other chlorinated hydrocarbons, organophosphates, carbamates, or polychlorinated biphenyls. Ethylene oxide, methyl bromide, phosphine, and other fumigants are sometimes used to control pests that contaminate herbal ingredients. Lead, cadmium, mercury, thallium, and arsenic have been shown to be contaminants of some herbal ingredients. Limit tests for acceptable levels of all these pesticides, fumigants, and toxic metals are of the utmost necessity to control the quality of plant materials. To increase the quality of herbal ingredients, similar limits should be placed on other contaminants as well, including endotoxins, mycotoxins, and radionuclides. Such limits are essential to ensure the high quality of therapeutically potent medicinal plants.

3.1.9 Chemical Variation in Medicinal Plants

The chemical composition of a medicinal plant varies depending on various environmental factors, such as humidity, rainfall, and altitude, and also factors that are indigenous to the plant. Various steps used to process the plant material affect the chemical composition of the plant. This necessitates batch-to-batch standardization of plant materials (Mukherjee et al., 2015). Herbal materials, in contrast to synthetic drugs, are quite complex mixtures of several compounds. They often work synergistically. So, as the popularity of herbal medicines increases worldwide, the need for examining the quality of herbal medicine from the perspective of chemical constituent variation is important (Xie et al., 2009). Administration of a single isolated component, which is approved as a drug, is not equivalent to the administration of the whole plant extract. As an example, we take Artemisinin and *A. annua* Linn. The administration of the whole plant extract has been shown to have 45-times higher bioavailability. Temperature, light, and solvents often cause degradation and/or transformation of purified components; isomers and conformations may also cause changes in the markers. The chemical constituents in a medicinal plant vary depending on various factors, such as environmental determinants, geographical variants, inter and intraspecies

variation, and variation among the different parts of the plant. Also, the metabolite content varies depending on various factors. This section will focus on the metabolite variation among plants of the same species and their determination. This will include factors, such as the extraction conditions, storage, and genetic factors of the plant, that are responsible for the variation (Mukherjee et al., 2015).

3.2 ADULTERATIONS OF HERBAL DRUGS

Adulteration or debasement of an article refers to a set of deliberate or accidental admixtures in which undesirable materials (free from or inferior in therapeutic and chemical properties) are admixed with an herbal medicinal product. They may be classified into several categories.

Spoilage is one type of adulteration, in which, microbes or pests infest a product. As a result, a product becomes unfit for use. In order to address this problem, the drying and storage conditions of the product need to be improved.

In the case of *Deterioration*, the original herbal medicinal product is rendered inactive or less active through maltreatment, aging, or extraction, followed by marketing of the exhausted residue.

Admixture may be defined as the act of adding one substance to an herbal medicinal product accidentally due to ignorance or carelessness.

Sophistication refers to the case of a spurious or inferior material being mixed into a drug in order to defraud. One example that may be cited is the addition of starch into ginger, followed by the addition of a little bit of coloring matter, which results in a perfect shade of yellow color.

In some cases, an herbal medicinal product is entirely replaced with a different substance. This is known as substitution. One example is the supply of cheap cottonseed oil in place of olive oil (Evans, 2009).

Herbs are the major components in all traditional systems of medicine. Adulteration and substitution are found most frequently in the raw material trade of medicinal plants. Adulteration has become a common malpractice in the herbal raw material trade. Deforestation and the extinction of several plant species has resulted in the unavailability of authentic plant species. Due to this, adulteration and substitution of many herbal medicinal products are encouraged. In the case of an adulterated herb, it has often been noticed that several adverse reactions take place due to the incorporation of some unintended herbs. Scientific studies have been able to discover several adulteration techniques that are difficult to detect without employing proper microscopic studies (Sarin, 1996).

3.2.1 Various Means of Adulterations in Herbal Medicine

Adulteration is performed by deliberately admixing foreign matter (usually with wrong, inferior, or cheaper plant materials, synthetic drugs, etc.) to herbal materials or preparations to maximize profits. Inorganic matter or unofficial plant parts may also be incorporated into the drug (Pferschy-Wenzig and Bauer, 2015). Table 3.4 demonstrates several cases in which synthetic products are substituted for herbal drugs. Some herbal weight-loss products are regularly substituted with conventional allopathic medicines, such as stimulants, anorexics, benzodiazepines, antidepressants, diuretics, and laxatives (Vaclavik et al., 2014).

Different types of adulterations will be illustrated in the following sections.

3.2.1.1 Substitution With Inferior Commercial Varieties

Owing to a macroscopic similarity to the original drugs, various inferior varieties of the same species are used as an adulterant. Some examples are:

1. Indian Senna (*Cassia angustifolia*) has been adulterated with Arabian Senna, *C. obovate* (dog Senna), and Provence Senna (*C. auriculata*).
2. Original ginger (*Zingiber officinale*) is adulterated with Cochin and with African and Japanese ginger.
3. Tragacanth (a natural gum) is adulterated with Smyrna and hog tragacanth.
4. Fruits of *Capsicum minimum* are regularly substituted with *Capsicum annuum* fruits and Japanese chillies.
5. *Strychnos nuxvomica* seeds are adulterated with *S. nux-blanda* or *S. potatorum* seeds.

3.2.1.2 Adulteration by Artificially Manufactured Substitutes

To maintain the expected morphology and texture of various herbal medicines, some substances are manufactured artificially and are incorporated as substitution of the original ones. Examples include compressed chicory adulteration in coffee, inverted sugar (artificial) in honey, and yellow paraffin wax for beeswax.

TABLE 3.4 Commonly Used Adulterants in Several Indian Medicinal Plants

Main Drug	Adulterant
Gum of Gugglu (*Commiphora wightii*)	Gum of Shallaki (*Boswellia serrata*)
Leaf of Araluka (*Ailanthus excels*)	Leaf of Vasaka (*Adhatoda vesica*)
Arimeda (*Acacia fernaciana*)	Aragvadha (*Cassia fistula*)
Kuchala seed (*Strychnos nuxvomica*)	Katak seed (*Strychnos potatorum*)
Manjistha (*Rubia cordifolia*)	Kiratikta (*Swertia chirayta*)
Pattanga (*Caesalpinia sappan*)	Raktachandan (*Pterocarpus santalinus*)
Kampillaka (*Mallotus phillipenensis*)	Istica churna (brick powder)
Yastimadhu (*Glycyrrhiza glabra*)	Gunjamool (*Abrus precatorius*)
Pippali (*Piper longum*)	Chavya (*Piper retrofactum*) and Tambula (*Piper betle*)
Guduchi satva (*Tinospora cordifolia*)	Powder or flour of potato, sweet potato
Erandkarkati seed (*Caryca papaya*)	Maricha (*Piper nigrum*)
Vidanga (*Embelia ribes*)	Sp. of Vidanga (*Myrsine Africana*)
Arjuna (*Terminalia arjuna*)	Jarula (*Lagerstroemia speciosa*)
Ashoka (*Saraca asoca*)	Kasthadaru (*Polyalthia longifolia*)
Talish patra (*Abies webbiana*)	Sthaunyak (*Taxus baccata*)

3.2.1.3 Substitution by Exhausted Drugs

This may be defined as the admixture of a certain species (such as clove, coriander, or fennel) with the same species, but one that has been exhausted. This may be done by extracting the active constituents out (volatile oils, in this case). Manipulation of the color and taste is done through incorporation of additives.

3.2.1.4 Substitution by Superficially Similar but Cheaper Natural Substances

This type of adulteration is observed in the case of costly drugs. For example, paraffin wax is tinged yellow and adulterated with yellow bees wax, while artificial inverted sugar is mixed with honey. In this case, the adulterated herbal drug is in no way related to the authentic drug. This mixture may or may not possess the required medicinal or chemical constituents to the extent desired. Some examples may elucidate this fact.

Ailanthus leaves are substituted for Belladonna, Senna, and Mint. Belladonna leaves are also substituted with Phytolacca and Scopolia leaves. Xanthium leaves are substituted for Stramonium and leaves of Dandelion are substituted for Henbane. Indian Dill is adulterated with European Dill or Caraway.

3.2.1.5 Adulteration by Addition of Worthless Heavy Materials

Several cases have been observed in which a significant quantity of stone, lead shot, or limestone have been mixed with Licorice root, Asafoetida, or pieces of opium.

3.2.1.6 Addition of Synthetic Principles

Some synthetic products are also added to natural products, such as citral oil in Lemon and Benzyl benzoate in Balsam of Peru, among others.

3.2.1.7 Adulteration With Inferior Drugs

Generally, this is done by mixing adventitious materials or naturally occurring substances with an herbal medicinal product in excessive quantities or with components of the plant other than the part of interest. One example is liverworts and epiphytes growing in the bark of plants that are adulterated with *Cascara*, *Cinchona*, or buchu stems (Evans, 2009).

Inferior drugs may even lack any therapeutic value. Due to their resemblance to the original drug, they are used as adulterants. Some other such examples include mother clove and clove stalks in clove buds.

3.2.1.8 Harmful Adulterants

Besides the techniques mentioned above, some other techniques are also being used for adulterating herbal medicines. For example, harmful adulterants are used, which are mixed with the authentic drug, for example, limestone in asafetida and rodent fecal matter to cardamom seed. Crude drugs in powder form also are being adulterated, for example, Dextrin in ipecacuanha, exhausted powder ginger in colocynth and ginger (see Table 3.5).

3.2.2 Reasons of Adulteration

Adulterations have caused a major problem in the research of commercial natural products. Due to the failure of researchers to recognize the importance of identifying the correct source of test materials, the results of studies on unidentifiable, adulterated commercial herbal products, for example, "Ginseng capsule" or "*Aloe vera*" are not reproducible. Irrespective of claims by individual suppliers, manufacturers, and associated trade groups on quality, no meaningful assay procedures or quality assurance methods have been introduced to guarantee the purity and quality of many natural ingredients.

Different procedures are available for the detection of adulteration and it is better to obtain confirmatory evidence by using as many different means of detection as possible to establish: (i) the identity of the adulterant and (ii) determine the quality of the drug. Ideally, the varied geographical sources of herbal medicine, including the various environmental and soil conditions under which it was grown, harvested, dried and stored, should be known. Even the chemical treatments that were made, if any, as well as the pesticides or fumigants, if any, that were used, should be studied. The appropriate level of testing must therefore be carefully assessed before using the raw materials based on the monographs available in different official books as well as various regulatory guidelines, including the WHO (1992).

3.2.2.1 Confusion in Vernacular Names

Medicinal plants have existed in different traditional systems of medicines, such as Ayurveda, Siddha, Unani, and Traditional Chinese Medicines (TCM). Now, the problem is that the prescribed names of different medicinal plants sometimes resemble each other, although they are not same species. One such example is Parpata (an Ayurvedic term, suggesting *Fumaria parviflora*) and Parpadagam (Siddha, suggesting *Mollugo pentaphylla*). It has been practically observed that in the South Indian states, where the Siddha system of medicine is quite popular, *M. pentaphylla* is more commonly used, whereas in North India, *F. parviflora* is more easily available. Differentiating between them may be accomplished by identifying their leaves and stems. *M. pentaphylla* possesses pale yellow or mild brown colored stems and small, simple leaves, whereas *F. parviflora* possesses dark brownish and digitate leaves.

TABLE 3.5 Some Examples of the Preferred Time of Collection of Some Herbs

Name of the Plant	Preferred Time of Collection	Rationale
Rhubarb (*Rhamnus purshiana*)	Summer	Anthranols are converted to anthraquinones during summer only
Peppermint (*Mentha* sp.)	At a particular age of the plant	High proportion of Pulegone in young plants of peppermint will be replaced by Menthone and Menthol
Datura (*Datura metel, D. innoxia*)	At a particular age of the plant	Reduction in the percentage of alkaloids in datura
Vasaka (*Adhatoda vasica*)	Just before the flowering season	The whole plant has come to a healthy state and contains an optimum quantity of metabolites
Digitalis (*Digitalis purpurea*)	Just before the flowering season	
Clove (*Eugenia caryophyllus*)	Before full expansion	Contains an optimum quantity of metabolites
Saffron (*Crocus sativus*)		
Barks	Spring	Ease in separation

3.2.2.2 Lack of Knowledge About Authentic Sources

Nagakesar (*Mesua ferrea*) is widely prescribed in the Ayurvedic system of medicine in India. Samples of *M. ferrea* are often adulterated with *Calophyllum inophyllum* flowers. *M. ferrea* is collected from the Himalayas and the Western Ghats. However, due to some restrictions in the collection of plant samples from the forest, *C. inophyllum*, available in the plains, is adulterated to Nagakesar. Authentication of the genuine drug may be done through microscopic studies, in which the presence of a two-celled ovary represents the original flowers and a single-celled ovary represents *C. inophyllum*.

3.2.2.3 Similarity in Morphology

Mucuna pruriens is a plant belonging to the Papilionaceae family, whose seeds are used as medicines. Seeds from other plants, such as *M. utilis* and *M. deeringiana* (Papilionaceae), are often admixed with the seeds of *M. pruriens*. The authentic seeds of *M. pruriens* may be identified using microscopic techniques. The authentic seeds of *M. pruriens* are about 1 cm in length, whereas the seeds of *M. deeringiana* and *M. utilis* are slightly elongated (1.5–2 cm), and buff colored.

3.2.2.4 Similarity in Color

One of the important morphological characteristics used for identification of drugs is the color of the drug. Drugs having almost the same color are sometimes misidentified and adulterated. The original source of "Ratanjot" is *Ventilago madraspatana* roots, and its adulterant is *Arnebia euchroma var euchroma*. Both of these are capable of producing a red dye. Presently, the former one is not available in the market, and by the name of Ratanjot, the latter one is most commonly used.

3.2.2.5 Careless Collections

Some adulterations also occur due to the carelessness of the collector. *Parmelia perlata*, commonly known as Shaileya, is a commonly prescribed drug in the Ayurveda, Siddha, and Unani systems of medicine. Market samples of this drug are routinely adulterated with *P. perforata*, *P. cirrhata*, and *Usnea* sp. In this case, the authentication of the plant is performed by the thallus nature of the plant.

3.2.2.6 Other Unknown Reasons

Vidari is an authentic plant obtained from *Pueraria tuberosa*, which is substituted with *Ipomea digitata*. The gymnosperm, *Cycas circinalis* (originating from Kerala, India) is sold under the name of Vidari. Thus, it has become another source of adulteration.

3.3 DETERIORATION OF HERBAL DRUGS

Deterioration of herbal drugs in storage is another factor affecting the equality of herbal medicinal products. The storage conditions (light, humidity, oxygen, temperature) affect the stability of the herbs and the secondary metabolites present therein. Also, microbial growth in the phytopharmaceuticals tells upon the quality of the products. The primary factors leading to the deterioration of herbal medicines can be summarized as follows.

3.3.1 Light

Wormseed (*Dysphania ambrosioides*) contains Santonin as the principal constituent. When wormseed is exposed to light, it undergoes photodecomposition and becomes black. Similarly, Rhubarb powder undergoes decomposition in clear glass jars and becomes reddish. In order to prevent such phenomena, it is recommended that drugs should be stored in amber colored bottles or with lightproof wrapping. Similarly, the glycosides of digitalis seem to be photosensitive, particularly with respect to polarized and reflected light.

With an eye to these detrimental effects, the World Health Organization has recommended that photosensitive herbal drugs should be stored in light-resistant containers. If that is not possible, then the bottle should be stored in light-resistant boxes.

3.3.2 Moisture

The percent relative humidity (%RH) of the storage environment of the herbal medicinal product determines the moisture content, a factor affecting the stability of the herbal medicinal product. Some drugs do not tend to absorb moisture from the

atmosphere and are thus called air-dry. These drugs do not need to be kept in air-tight containers. Depending on the %RH, these drugs contain 10%–12% moisture. The presence of moisture in drugs results in reactivation of enzymes in the drug, resulting in decomposition of the constituents. Moisture-sensitive drugs need to be stored in the presence of siccatives, such as calcium chloride, calcium sulfate, or sodium sulfate. Squill powder picks up moisture from the atmosphere quickly and soon becomes a sticky mass. Also, a strict control of the humidity of the storage region is necessary for controlling the moisture content of the herbal medicinal product.

3.3.3 Temperature

Temperature has a significant influence on the stability of herbal drugs, which is very often unsuspected. Several enzymatic breakdowns of the secondary metabolites of the plants take place faster in response to temperature elevation up to 45 °C. Due to an elevation in the temperature, essential oil-bearing plants lose volatile constituents. Absorbent cotton wools, when subjected to elevated temperature, undergo degradation and may become nonabsorbent due to the effect of temperature. Herbal medicinal products that require storage conditions that are different from normal room temperature should be labeled accordingly (cold temperature, cool temperature, freezer, etc.).

3.3.4 Airic Oxidation

Atmospheric oxygen sometimes causes direct oxidation of the secondary metabolites of herbal medicinal products. Linseed oil, turpentine oil, and lemon oils become resins in this manner. Cannabinol, the principal component of Indian hemp, Cannabis indica, rapidly becomes a resin, similar to oil of turpentine and oil of lemon. The resinous deposits observed on the walls of the storage container indicate such deterioration. In addition to this, the rancidification of fixed oils (such as cod-liver oil or mustard oil) results in the formation of unstable peroxides.

3.3.5 Bacteria and Molds

Crude drugs are particularly vulnerable to contamination by the spores of bacteria and mold that are ubiquitous in air. Under proper storage conditions, their presence does not cause any problem, but it is generally accepted that the viable count permissible for herbal medicinal products should be the same as that for foodstuff. The impact of microscopic organisms is not generally exceptionally noticeable except for some chromogenic types of microbes, for example, *Bacillus immense*, which creates red pigments in bland materials (Evans, 2009). Nonetheless, bacterial development is normally accompanied by the development of molds, whose proximity is rapidly apparent by the trademark smell and by the mass of sticking particles entangled in the mycelial hyphae. Dusty cotton wool is shaped by bacterial attack, causing the trichomes to break into short lengths and rendering it extremely weak. Keeping in mind that the end goal is to recognize a specific shape or microscopic organism that is multiplying in a stored item, it is important to culture it in an appropriate medium with a view to acquire fruiting bodies for examination. In any case, if the drug to be inspected is invaded quickly, at that point it might be possible to make microscopic specimen directly from the sample. Generally, the molds that develop in improperly stored drugs incorporate the genera Mucor (e.g., dimorphic shape, *Mucor circinelloides*), Rhizopus (e.g., dark shape, *R. nigricans*), Penicillium (e.g., blue form, *P. glaucum*), Aspergillus (e.g., green form, *A. repens*), and Saccharomyces.

3.3.6 Mites and Nematode Worms

In the case that mites are found in stored medications, they are normally present in very large numbers up to 1.0 mm long. Various mites that are found include *Tyroglyphus siro* (Cheese mite), *Aleurobius farinae* (Flour mite), and *Glycyphagus spinipes* (Canthrides mite). Every one of these mites can be inspected microscopically by clearing the sample of powder containing them with a chloral hydrate reagent. The best known cases of nematode worms are "Vinegar eel"—*Turbatrix aceti*, *Anguillula aceti*, *Anguina tritici*, which are found in wheat flour or in an herbal restorative item containing dull materials. These worms are noticeable to the unaided eye as moving strings, consistently twisting and curving in the medium they possess.

3.3.7 Insects and Moths

Different types of Lepidoptera damage stored herbs and cause harm at the larval stage, when the invasion can spread quickly. The moths are unspectacular in appearance, 22–30 mm long with grayish wings, for example, *Ephestia kuehniella* (Flour moth) and *E. ellutella* (Cocoa Moth). Other insects, such as cockroaches and ants, may also damage stored herbs.

3.3.8 Coleoptera or Beetles

These insects constitute the largest domain of the animal kingdom, containing around 250,000 types of which about 600 have been observed to be related with stored herbal drugs. *Stegobium paniceum* is one such creepy crawly, which is found in many medications, including gentian, licorice, and rhubarb, in addition to verdant medications and seeds. Having a place with a similar family is *Lasioderma serricorne* (tobacco or stogie insect) which is ruddy dark colored in shading, 2–2.5 mm long, and found in many stored herbal restorative items, including ginger and licorice.

3.3.9 Control Measures for Deterioration

The container utilized for storage and its enclosure must not communicate physically or synthetically with the material inside in any way that would adjust its arrangement. A firmly closed compartment must shield the substance from extraneous issues or from loss of the material while handling and a firmly shut holder must shield the material from blooming, deliquescence, or dissipation under handling at typical capacities. The holding area ought to be kept clean and spillage must not be permitted to enter breaks or through holes. Occasionally showering the premises with insecticides will halt the spread of infestation. The standards that apply to the control of invasion in warehouses are also appropriate to small-scale stockpiling. Good housekeeping is the most extreme basic. Each stock ought to be assessed routinely and tainted material is best destroyed by burning. In this regard, a quick swing over to wipe out the impacts of disintegration because of both the essential and auxiliary factors, as noted above, is desirable. Cool, dry conditions are the most reasonable to hinder living organisms. As every single living organism requires water, extremely dry medications ought to be resistant to auxiliary decay. In some cases, herbal therapeutic items obtained by the herbalist may now have been cleaned, which is usually accomplished by treatment of the mass transfer with ethylene oxide or methyl bromide under controlled conditions. Medications so treated should be subject to a satisfactory limit for harmful buildups, for example, for Senna cases 50 ppm of ethylene oxide is the limit. Various assessment procedures for herbal products, from the point of view of cost control and for deciding substituents, will be specified in later sections.

3.4 SUBSTITUTION OF HERBS

At some point, a first herb is substituted with another herb for comparable helpful effects when two distinctive plant species are utilized. These two distinctive plant species are regularly substituted with each other, such as Bharangi (*Clerodendrum indicum*) and Kantakari. They have glycosides—Verbascoside and Solasoninie, solamargin, solasurine separately. Both *C. indicum* and *S. xanthocarpam* have indicated antihistaminic properties. Both *C. indicum* and *S. xanthocarpam* are usually utilized in infections identified with the respiratory framework, which are normally connected with the arrival of histamines and different autacoids.

3.4.1 Substitution of the Species Belonging to the Same Family

Plant species belonging to the same plant family have also been observed in the substitution process as a substitution for the original plant species. This type of substitution is done usually with plants from the same family. *Datura metel* and *Datura stramonium* can be considered here. The chemical constituents are alkaloids—scopolamine, atropine, hyoscyamine, and hyoscine, among others. The alkaloids are found to be bronchodilators, relaxants, and inhibitors of the emissions from the mucous membrane. The alcoholic concentrate of *D. metel* shows anthelmintic effects. The alkaloids introduced in both species are well-demonstrated bronchodilators. They likewise hinder the emissions of the mucous membrane of the respiratory tract. Both *D. metel* and *D. stramonium* are beneficial for maladies of the respiratory tract, as they are demonstrated anthelmintics.

3.4.2 Substitution of Different Species

At times, it has been observed that in the customary framework solution, one plant has a few equivalent words and in different cases the regular name is the same for two unique types of plant. Two kinds of Gokshura are found: *Tribulus terrestris* (Zygophylaceae) and *Pedalium murex* (Pedaliaceae). *T. terrestris* has chemical constituents, such as chlorogenin, diosgenin, rutin, rhamnose, and alkaloid. While *P. murex* has sitosterol, ursolic corrosive, vanillin, flavonoids, and alkaloids, both species have been demonstrated for nephroprotective, lithotriptic, diuretic, and hepatoprotective functions. If we analyze the clinical conditions for which Gokshura is shown, that is, Mutrakrrcha, Mutraghata, Ashmari, Prameha, and so on, both *T. terrestris* and *P. murex* seem, by all accounts, to be proper.

3.4.3 Substitution of Different Parts of the Same Plant

This type of substitution is observed in traditional medicines when the part of use is not mentioned in the codified text. This generally happens for the herbs and shrubs used in the traditional system. The foundation of *Sida cordifolia* and the entire plant of *S. cordifolia* can be considered. The root has synthetic constituents, for example, sitoindoside and acylsteryglycoside. While the entire plant has alkaloid, hydrocarbons, unsaturated fats, and ephedrine, different concentrates of the entire plant demonstrate antibacterial, antioxidant, hypoglycemic, hepatoprotective, and cardiotonic properties. In spite of the fact that it is the root which is specified as the official piece of *S. cordifolia* in the works of art, such as Balya, Brimhana, Shothahara, and so forth, present day inquiries demonstrate that even the ethereal parts are also similarly successful (Table 3.5).

3.4.4 Substitution Due to Same Action

Sometimes plant species demonstrating comparable activities are utilized as a substitute for other plants having the same pharmacological activity, such as *Embelica officinalis*, which has antioxidant, hepatoprotective, antimicrobial, hypoglycemic, and hypolipidemic properties. Semecarpus demonstrates antitumor, hypotensive, anticytotoxic, and anticancer properties. Both Amalaki and Bhallataka are Rasayana (rejuvenator) drugs. In several cases, the Rasayana plants are used as an adjuvant treatment in chronic and also malignant illnesses. Amalaki can be utilized as Rasayana in chronic weakening infections, such as bronchial asthma, diabetes, and so on, while Bhallataka would be a better decision in malignant conditions, both in strong tumors and in leukemia.

3.5 COUNTERFEITING OF HERBAL MEDICINE

Counterfeiting of herbal drugs, herbal dietary supplements, and polyherbal formulations are major issues, in which the identity of the source is deliberately and fraudulently mislabeled in a way that suggests it as an authentic approved product. The development of authentic analytical methods that can reliably profile the phyto-chemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a major challenge to scientists. Counterfeit products may include products without the active ingredient, with an insufficient quantity of the active ingredient, with the wrong active ingredient, or with fake packaging. The World Health Organization (WHO) has defined counterfeit drugs as those that are deliberately mislabeled with respect to identity and/or source. Counterfeiting can apply to both branded and generic products with counterfeit products, including drugs with the correct ingredients or with the wrong ingredients, without active ingredients, without sufficient active ingredients, or with fake packaging.

3.5.1 Types of Counterfeit Drugs and Their Consequences

There are four possibilities for counterfeit in herbal medicines: herbal medicines adulterated with undeclared synthetic pharmaceuticals, with undeclared heavy metals, herbal medicines without active ingredients, and herbal medicines with the wrong active ingredient.

Various types of counterfeit drugs are:

- Counterfeit drugs containing the same dose of the active ingredient
- Mislabeled medications
- Counterfeit drugs containing an incorrect dose of the active ingredient
- Counterfeit drugs that do not contain the active ingredient
- Counterfeit drugs containing a potentially harmful substance
- Counterfeit drugs containing an unlisted active ingredient
- Genuine medicines marketed for incorrect or recreational use

In a study, ElAgouri et al. (2015) investigated five different products sold in the Middle East market claiming to be 100% natural for male enhancement (Tiger King, Hercules, Herbal Viagra, Plant Viagra, and Natural Viagra). The products were proven to contain undeclared sildenafil citrate with variable heterogeneous amounts ranging from zero to more than 180 mg in a single dose and with poor pharmaceutical quality and consistency. These results were confirmed by high-performance liquid chromatography (HPLC), high-performance thin-layer chromatography (HPTLC), NIR, content uniformity, weight variation, dissolution, friability, and SeDeM diagram analysis as a quality control and assurance tool. Moreover, the heterogeneity of the adulterant active pharmaceutical ingredient within the products was assured by in vivo

and clinical studies (ElAmrawy et al., 2016). Kerner et al. found in a study that 80% of pills sold online are "Counterfeit Viagra" containing 30%–50% of the active ingredient sildenafil. They also found that some counterfeit products were contaminated with pesticides, paint, and printer ink (Kerner, 2013).

3.5.2 Preventive Measures

To combat counterfeiting of approved or authenticated herbal medicines, an anticounterfeiting strategy and the associated technology should be developed and implemented by every country. Similar to combating the counterfeit of unapproved traditional herbal medicines, there are four ways to deal with. The most important approach is the complete regulation of traditional herbal medicine, which will be achieved by implementing regulatory harmonization (Mullaicharam, 2011).

3.5.3 Methods of Detection

Several methods have been developed to detect drugs that may be suspect, while researchers are working assiduously to develop other rapid detection schemes. They range from simple thin-layer chromatography (TLC) to modern sophisticated instruments, such as LC–MS, gas chromatography, HPLC, HPTLC, and others. Chromatography and MS have been widely used for the detection of counterfeit herbal products. Analysis of bioactive marker compounds is necessary to determine the impurities that may be present in herbal products. This can be used for the determination of the source of the raw materials used, as well as the processing route employed. MS techniques have also been widely used to characterize pharmaceutical products. Emphasis has been on TOF approaches with electrospray ionization detection being commonly employed in herbal drug profiling (Twohig et al., 2010).

The use of holograms and security print features as a means of confirming genuine products has grown rapidly over the last decade. Holograms are generated from the interference patterns obtained through the interaction of laser beams. The complexity of origination varies from the traditional 3D image to computer-generated 2D diffraction patterns. Currently available security holograms produce 2D–3D designs, in which different planes of 2D artwork are visible at different angles. Electronic tracking systems, such as radio-frequency chips that make use of the tagging of products by manufacturers, are being developed to track products through the distribution chain. Such methods are able to transmit a large volume of specific information about the product and will allow distributors and retailers to track their product when necessary.

The most essential criteria for substitution are the pharmacological activities rather than the morphology or phytoconstituents. Substitution is the rational replacement of herbal medicine to get similar therapeutic action. Substitution and adulteration can be understood in two ways: legally (official substitutes) and illegally (commercial aspect). The legal substitute of a drug is scientifically supported, in which the properties of substituted drugs are the same as the original drug. Illegal means using a different drug instead of the original one and is not scientifically supported but it is commercially beneficial to the adulterator or drug dealer.

3.6 COLLECTION OF HERBS

The first step in the process for the production of an herbal drug is the collection of the herb. This should be carried out with care, with the help of skilled labor. During collection, proper identification and rejection of unsuitable plants and plant parts is an important issue that requires delicate handling. Though some plants are harvested mechanically (Digitalis, Tea, Vinca, and Senna leaves, underground drugs, such as roots, rhizomes, and tubers), still many plants require manual collection. Drugs should be collected in a highly scientific manner when they contain the maximum quantity of constituents. The season at which each drug is collected is so important, as the amount, and sometimes the nature, of the active constituents may change throughout the year (Table 3.5).

3.6.1 Methods of Bark Collection

Bark is generally collected in the spring or early summer because the cambium is very active and it is thin walled and gets detached easily. Following are the methods for collecting bark:

3.6.1.1 Felling Method

The fully grown tree is cut down near ground level by an axe. The bark is removed by making suitable longitudinal and transverse cuts on the stem and branches for fully grown tree. The disadvantages of this method are that the plant is fully destroyed and the root bark is not utilized.

3.6.1.2 Uprooting Method

In this method, stems of definite age and diameter are cut down, the root is dug up, and bark is collected from the roots, stems, and branches. In Java, cinchona bark is collected by this method.

3.6.1.3 Coppicing Method

The plant is allowed to grow up to a certain age and diameter. The stems are cut at a certain distance from ground level. Bark is collected from the stem and branches. The stumps that remain in the ground are allowed to grow up to a certain level; again, the shoots are cut to collect the bark in the same manner. Cascara bark and Ceylon cinnamon bark are collected by this method.

3.6.2 Illustrative Examples With Discussion

Example 3.3

It is well known that the chemical composition of secondary plant metabolites depends strongly on factors like climatic conditions, harvesting time, and plant genotype. The results of this research (Donno et al., 2013) confirm this hypothesis: different species and varieties present different compositions and concentrations of polyphenolic compounds, but it is also important to consider that the pedoclimatic characteristics of sampling sites strongly influence the presence of these molecules when comparing the results of commercial bud preparations. The results are highly variable depending on the genera: in *Ribes nigrum*, the sampling year influences the TPC of the analyzed varieties, while in *Rubus ulmifolius* there are no differences between 2 years for each cultivar (Donno et al., 2013).

Example 3.4

Sage (*Salvia oficinalis* L.) is an important herb, used as a flavoring in meat, poultry, and cheese dishes, and in vinegar. It is also significant as an essential oil in pharmacology. The plants are collected from wild populations, air dried, and then processed. Plants are collected once a year, during the summer. Having bright leaves (almost white), 1.5% essential oil on the basis of dried leaves, and a special aroma, the leaf is of high quality. The essential oil, including a high percentage of thujones (50%) and a low percentage of camphor (*20%*), is considered to be of a superior grade. The harvesting of *S. officinalis* in the spring and autumn are more favorable for the optimum quality of the metabolites, rather than the summer (Putievsky et al., 1986).

Example 3.5

Hypericin and other naphthodianthrones in the plant *H. perforatum* have been analyzed by HPLC. Studies of the accumulation dynamics and between-accession variation of the contents of these secondary metabolites were carried out by high-performance liquid chromatography (HPLC). The data were statistically processed with ANOVA and PCA. A significant difference between pseudohypericin and hypericin content in floral budding and in the full flowering stages was detected. The highest amount of secondary metabolites was observed in the flowering stage. The study revealed variations in *H. perforatum* that were evident within the population. Mean concentrations of pseudohypericin and hypericin among accessions varied from 3.45 to 6.82 mg/g and from 1.17 to 2.59 mg/g, respectively. Accessions of *H. perforatum* showed remarkable differences in chemical composition depending on the provenance of the plants (Bagdonaitė et al., 2010).

3.6.3 Good Agricultural Practices

Depending on the method of cultivation, different Standard Operating Procedures for cultivation should be followed by cultivators. A suitable area for the cultivation and a standard operating procedure for the cultivation should be developed, depending upon the needs of the plants. Medicinal and aromatic plants should not be grown in soils that are contaminated by sludge and heavy metals, residues of plant protection products, and any other unnatural chemicals. So, the chemical products (pesticide and herbicide) used should impart as minimum a negative effect as possible and human feces should be avoided. Depending upon the soil fertility and the nutritional requirement of the medicinal plants, the type of the fertilizer and the amount of the fertilizer to be used is determined. Proper irrigation and drainage should be carried out according to the climatic conditions and soil moisture. The soil used for cultivation should be well aerated. The use of pesticides and herbicides has to be documented. Irrigation should be minimized as much as possible and only be applied according to the needs of the plant. Water used for irrigation should be free from all possible forms of contaminants.

The area for cultivation should be strictly isolated from contaminants, such as house garbage, industrial waste, hospital refuse, and feces. Field management should be strengthened and proper measures, such as pruning and shading, should be provided for increasing the yield of the active constituent and maintaining the consistency of the yield. The pesticides used

should give high efficacy, hypotoxicity, and low residue at the minimum effective input so that the residues of pesticides are also reduced and protected from the ecological environment. Application and storage of plant protection products have to be in conformity with the regulations of manufacturers and the respective national authorities. The application should only be carried out by qualified staff using approved equipment. Though several countries in the world have a rich heritage in herbal drugs, very few have a claim for their procurement of crude drugs only from cultivated species. Our reliance on wild sources of crude drugs, and the lack of information on the sound cultivation and maintenance technology of crude drugs, has resulted in the gradual depletion of raw material from wild sources. Though the cultivation of medicinal plants offers a wide range of advantages over wild sources, it can be an uneconomical process for some crude drugs that occur abundantly in nature, such as nux vomica and acacia. On the other hand, crude drugs, such as cardamom, clove, poppy, tea, cinchona, ginger, linseed, isabgol, saffron, peppermint, and fennel, are obtained mostly from cultivated plants. The cultivation of crude drugs involves a keen knowledge of various factors from the agricultural and pharmaceutical sphere, such as soil, climate, rainfall, irrigation, altitude, temperature, use of fertilizers and pesticides, genetic manipulation, and the biochemical aspects of natural drugs.

3.7 IDENTIFICATION AND AUTHENTICATION OF HERBS

Identification and authentication are two major steps in the quality evaluation of herbal medicines. These methods are very important in herbal drug authentication and quality control, and they continue to provide the main methods used in pharmacopeia worldwide. A sufficient number of tests should be performed on representative samples as necessary to establish the identity of the ingredients. Although the focus here is on tests that establish identity, the unique properties of the material are also considered in selecting tests. The botanical identity—scientific name (genus, species, subspecies/variety, author, and family)—of each medicinal plant under cultivation should be verified and recorded. If available, the local and English common names should also be recorded. Other relevant information, such as the cultivar name, ecotype, and the chemotype or phenotype, may also be provided, as appropriate. For commercially available cultivars, the name of the cultivar and of the supplier should be provided. In the case of landraces collected, propagated, disseminated, and grown in a specific region, records should be kept of the locally named line, including the origin of the source seeds, plants, or propagation materials.

The authenticity of botanicals is a matter of paramount concern. Botanical specimens and the dietary supplements derived from them can vary in quality and in chemical constituent profiles because of a number of factors. Subtle variations in botanical specimens are known to have profound effects on the quality, efficacy, and safety of botanical dietary supplements and can potentially alter the results of clinical studies that rely on these materials. A complete array of authentication and evaluation tools can be utilized to provide a well-rounded scientific approach to the authentication of botanical products. It is vital that the authenticity of botanical supplements be established using appropriate analysis tools regardless of whether the end products are being considered for evaluation in clinical studies or are being developed for the consumer market. For the proper, correct identification, the collection of herbs, preparations of an herbarium, and taxonomical identification are very necessary.

The authentication of herbal drugs and the identification of adulterants from genuine medicinal herbs are essential for both pharmaceutical companies as well as for public health and to ensure the reproducible quality of herbal medicine. The standardization of herbal raw drugs includes the authorization of data on raw plant drugs, botanical authentication, microscopic and molecular examination, identification of the chemical composition by various chromatographic techniques, and the biological activity of the whole plant. Macroscopic and microscopic evaluation and chemical profiling of herbal materials for quality control and standardization are vital (Mukherjee and Wahile, 2006). The macroscopic identification of medicinal plant materials is based on sensory evaluation parameters, such as shape, size, color, texture, odor, and taste, while microscopy involves comparative microscopic inspection of the powdered herbal drug. Further, various advanced methods, such as chromatography, spectrophotometry, and a combination of these methods, as well as electrophoresis, polarography, and the use of molecular biomarkers in fingerprints are currently employed in the standardization of herbal drugs. The standardization of herbal medicine involves the determination of the content of a chemical constituent or a group of substances. Botanical extracts made directly from crude plant material show substantial variation in the composition, quality, and therapeutic effects (Rai et al., 2005). Standardized extracts are high-quality extracts containing consistent levels of specified compounds and they are subjected to rigorous quality controls during all phases of the growing, harvesting, and manufacturing processes (Mukherjee et al., 2012).

Plants have different vernaculars, that is, local names in different languages or dialects. As a result, misidentification during the collection, drying, and storage of the plant materials becomes a challenging task. Such examples are seen particularly in the TCM, in which, for example, a plant, named *Stephania tetrandra* S. Moore (Menispermaceae), is known as Fangji in some regions of the country, whereas in some other regions of the country, it is known as Hafangii or Guangfangji, thus resulting in ambiguity. Instead of counting on the vernaculars, the first thing to be done for authentication

of a plant species is to have the following things clarified: (i) the Latin binomial name, clearly mentioned in standard books as GRIN taxonomy and scientific literature, (ii) the common name of the plant, (iii) a photograph of the plant and its parts, (iv) indication of the collected plant parts, with Herbarium samples, (v) geographical indication of the plant, and (vi) information on postharvest processing. For authentication, we first collect voucher specimens of the plant from different phonological stages, such as flowering, fruiting, etc. These are stored as herbarium samples. They are identified using macroscopic studies, microscopy, or DNA-based methods.

3.8 TAXONOMY OF HERBS

The identification of a species is the most critical task, which necessitates application of instinct, knowledge, and skill. A correctly identified species is given recognition with the help of an appropriate name. The nomenclature of organisms is thus a means to afford communication about them and to standardize and organize an unambiguous reference system about them. Inadequacy and inaccuracy of baseline information regarding delimitation of natural biotic entities and their recognition are likely to magnify small gross errors of correlation and extrapolation to large dimensions while digitizing data for species distribution maps and their utilitarian perspectives. The misidentification of a plant species, or the inadvertent use of totally unrelated species or by closely related species of inferior quality, can hinder their medicinal use, the adverse effects may even kill a consumer. Medicinally useful plants and plant products must have their specific identity correctly ascertained. The main emphasis of vouchering is to correctly identify herbal medicines, that is, to link herbal material directly to a plant species. The most ideal approach to guarantee the correct identification is to acquire and send duplicates of herbal materials to authorities, preferably at a larger botanical investigative foundation that holds accumulations from the area where the material was gathered, to enable comparisons. Such accumulations are, in the botanical field, called herbaria (solitary: herbarium). Legitimate herbaria are recorded in the Index Herbarium, a worldwide rundown of botanical accumulations.

Plants are classified as herbs, shrubs, and trees based on height and stem/trunk dimensions. A plant image is recognized in two ways, either the full plant, including the canopy and stem parts, or by the individual parts. The classification of plants also depends on how well these parameters are grouped. Among the plant features, the plant body and height are the most important and effective visual features for classifying plants per the plant taxonomy. The height of the plant is an identifying characteristic that defines the variety of the plant within a species. Many methods exist for measuring the plant height, such as the angle of elevation and the distance from the viewer. The clinometer is a device used to record the height of any object from the ground level. But these methods do not give an accurate height value and are not suitable for the development of image processing applications (Fig. 3.1).

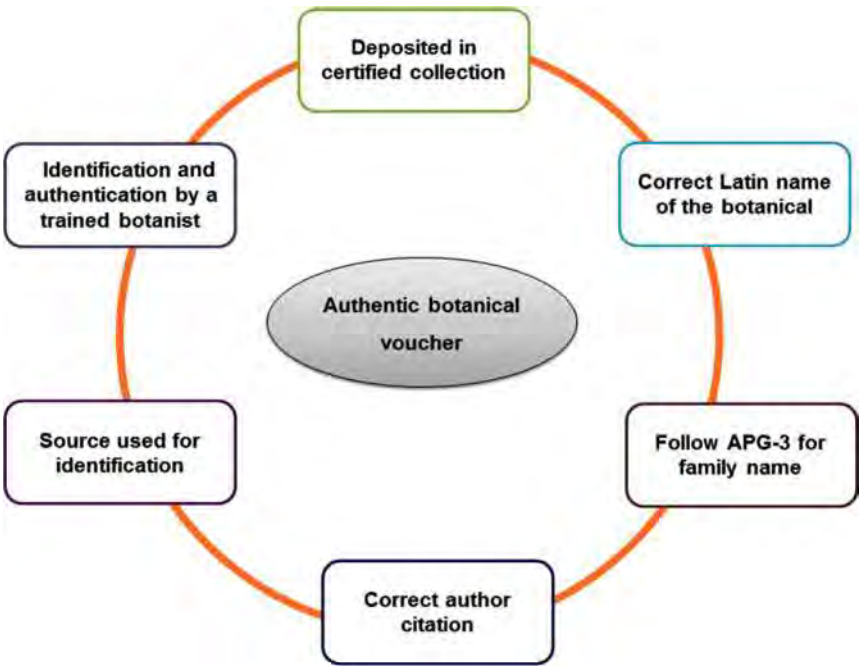

FIG. 3.1 Authentication of medicinal plants.

3.8.1 Nomenclature of Medicinal Plants

Before 1753, plants and animals were called by vernacular names, or by long, cumbersome descriptions. The problem of this is self-evident: it was never precisely clear if two scientists (or professionals) were referring to the same species with a similar name, especially if no material was accessible for joint comparison. In 1753, Carolus Linnaeus, a Swedish researcher, proposed a binomial naming framework. Because Latin was the language of science at that time, all names were built as Latin binomials. Since then, researchers have utilized the same framework for naming species, the International Code of Nomenclature for green growth, organisms, and plants which states that just a single correct name can refer to one taxon (e.g., type of plant). This universal use of one name permits an unambiguous classification. In any publication, the whole name, including the creator, ought to be referenced. The generic name may be abbreviated once the full name has been referenced. Finished plant names can be found in writing and on the internet.

The rules pertaining to plant nomenclature are divided into two major systems, namely, The International Code of Nomenclature for Cultivated Plants and The International Code for Botanical Nomenclature. Plants are divided into families in which similarly related plants are grouped together based on a clear similarity of morphological characteristics. Families may contain one genus or a large number. A genus may similarly contain one species or a large number of related individuals, for example, the Rosmarinus genus contains just two species, *Rosmarinus eriocalix* Jord. and Fourr. and *R. officinalis* L. Variations occur within a species and these are accommodated in the following manner: a subspecies (ssp.) is a distinct variant often arising because of the evolution of a plant from geographic factors, varieties (var.) have small differences in morphology, and the form (forma) has very minor differences, for example, the leaf or fruit color. Cultivars offer further evidence of diversity and according to The International Code of Nomenclature for Cultivated Plants (1980), cultivars named since 1959 should be given vernacular names, which should be in roman type within quotes, for example, "Rosa."

3.8.2 Author Names and Synonyms

Author names are given to indicate who exactly described any given species, and at which time. Based on the International Code of Nomenclature for algae, fungi, and plants, the correct plant name is the one that was first published. Any different names given to a species later are called synonyms. The earliest names are, of course, the ones put out by Linnaeus, for example, Arnica Montana L., because Linnaeus developed the binomial system. Authors in parentheses, for example, Schisandra chinensis (Turcz.) Baill., indicate that the author in parentheses (Turczaninow) initially described the species Kadzur chinensis Turcz. in 1837, yet Baillon (Baill.) correctly placed the species in 1868. The use of author names has regularly been condemned, yet it is fundamental in case a similar name has accidentally been given to two different species. Synonyms are names other than the right and accepted names that have been given to a species. In the above illustration, *Kadzura chinensis* would be a synonym of *Schisandra chinensis*. Synonyms can originate either because similar species were identified by different writers under different names or because the correct developmental relationship of a species was only recognized after it had been described. The same species may be known by a wide assortment of synonyms, because the species was initially gathered and described by many specialists who had no knowledge of the others. Quinine, for instance, was originally derived from an assortment of types of the family Cinchona. One of the most important sources, *Cinchona* calisaya Wedd., is additionally known under 47 different synonyms. The significance of this becomes evident when trying to run a search for publications about the utilization or compounds of specific species. "*Cinchona calisaya*" may yield only a portion of the possible results, and only the inclusion of all names would give an entire review of available references. An outstanding case in the United States is dark cohosh: dark cohosh is correctly recognized as *Actaea racemosa* L. (the species was described first by Linnaeus in 1753), however, *Cimici fugaracemosa* (L.) Nutt. (described by Nuttall in 1818) is widely utilized. A search on PubMed for *A. racemosa* yields 549 outcomes, while a scan for *Cimicifuga racemosa* yields 540 outcomes. The blend of the two names plus the vernacular "dark cohosh," nonetheless, yields 661 results. Careful utilization of binomial names is therefore important for any exploration of herbal prescriptions.

3.8.3 Classification Systems

The current plant scientific categorization framework is known as Angiosperm Phylogeny Group (APG)-3. This system updates the conventional ordered framework of morphology with current genetic information. APG-3 has two important advantages for herbal solution investigations. First of all, by giving a united and ordered framework, it allows for a superior examination of family diagrams in herbal medicine, that is, it can clarify plant groups with comparable genetic qualities, and in this manner perhaps similar phytochemistry. A good case is the Mauve family (Malvaceae): Before the execution of

APG-3, Malvaceae, Tiliaceae, Sterculiaceae, and Bombacaceae were counted as free families, despite the fact that in traditional medicine many species having a place with these families had similar restorative applications. With APG-3, all species of these families have a place with Malvaceae, which provides a much better fit and a superior apparatus for screening. Similarly, APG-3 has prompted the split of bump families, such as Scrophulariaceae, into smaller units, thus also allowing for a less demanding choice of screening targets. The genus Veronica, before in Scrophulariaceae, was found to belong to Plantaginaceae, which, from a medicinal perspective, bodes well. Sambucus, before being classified in Caprifoliaceae, ended up belonging genetically to Adoxaceae. Phyllanthus, surely understood for species like *Phyllanthus stipulatus* L. and *Phyllanthus niuriri* L. (chanca piedra, for urinary issues), and *Phyllanthus emblica* L. (amla, Indian gooseberry, known for anticancer and antiinflammatory properties) belonged formerly to the Euphorbiaceae (Spurge family, mostly with lethal latex) and now forms its own particular family (Phyllanthaceae), which again, from a restorative perspective, makes sense. Valeriana (some time ago Valerianaceae) belongs presently to the Caprifoliaceae.

3.9 HERBARIUM DOCUMENTATION FOR MEDICINAL HERBS

The worldwide stock of plants in assorted varieties comprises at present around 350,000 species, and it is anticipated that around 420,000 plant species exist. This huge assorted variety represents an extensive variety of phytochemicals, and a wide variety of compound arrangements even inside one single category, contingent upon the development conditions, including soil, atmosphere, supplement status, and so on. The most dangerous event in the herbal medicine trade is, in any case, connected to the buying and utilization, either in drugs or research, of botanicals that are either accidentally or deliberately wrongly distinguished, or are simply gathered under a vernacular name with no resulting ordered treatment, and regularly without having any vouchered material that could later be used to check the plant's identity. A significantly more frequent event is the contamination of botanicals with more common and less expensive species, which, despite the fact that they are generally not harmful, may totally lack efficacy. Crude botanicals are also frequently given in ground or powdered form, which makes morphological identification extremely troublesome or practically impossible. While minute and organoleptic strategies do sometimes allow isolating the right species from adulterants, if the material is pulverized or exceptionally coarsely ground, such a recognizable proof of powdered material or extracts is impossible. Therefore, the main possibility to later recognize a specific botanical securely is to rely on botanical voucher specimens that can be specifically connected to the material in question. This is where plant scientific classification and trained taxonomists play a fundamental role in the herbal supplement industry.

Voucher specimens are quite essential for botanicals as recognizable proof, as well as serving as the repository for the chemical compounds of a plant. Vouchers gathered at different times and in various areas can hence clearly reflect a compound as affected by edaphic and collection conditions. Aside from giving an unmistakable reference for a specific batch of material, voucher specimens also permit a follow-up. Vouchers are, in actuality, more important than just the beginning identification. A voucher example needs to incorporate all plant parts essential for recognizable proof, that is, flowers, fruits, and a segment of the stem with the leaves attached. Without fruitful parts, distinguishing proof may be impossible. Particularly if utilized as a part of an herbal prescription, the voucher must contain all the plant parts (e.g., bark, roots, seeds) that are really utilized for identification.

3.9.1 Voucher Specimen

It is acceptable to make a skimpy specimen if that is all the material available. But if sufficient material is available, it requires little additional effort to make ample sheets. If only skimpy fertile material is available, a voucher can often be improved by adding extra sterile material. Because the objective of a good specimen is to provide an adequate representation of a plant in a convenient form, one should always include the full range of characters exhibited by the plant, including, for example, the largest and smallest leaves, young leaves to show pubescence, and stipules. Specimens should always be improved by adding extra flowers or fruits and inflorescences. There is no reason to include only one inflorescence or one flower per specimen when there is an abundance of material at hand. It is very important to collect fertile material if at all possible. If possible, flowers and fruits should always be collected for each specimen. It is important to not ignore the vegetative characters. If there are different types of leaves, they should be included. Mature and immature twigs, especially in vines, should also be included, as should sap shoots or stump sprouts and saplings, which often have very different characters than the mature material, and can be very useful. Field pressing is usually less efficient than collection in plastic bags. Fragile material can be placed in a field press and stronger material held in a plastic bag for pressing later. It is useful to always carry small plastic bags or newspaper for wrapping smaller or more fragile plants. These can then be put into a larger bag. As an alternative to using small bags, small samples can be wrapped in any large leaf. When collecting in plastic bags, the specimens should be carefully folded to the correct length for a herbarium sheet and placed firmly, but carefully,

into the bag. They should never just be dropped in the bag. This way, separate collections will not become entangled and there will be less damage. Later, when emptying the bag, it can be turned upside down and carefully emptied. One should never try to pull material out of the bag. This usually breaks up the specimens. It is preferable to use large bags rather than small ones, as there will be less damage to the plants. For large, heavy plants, it is best to put them into a separate bag as they may damage other, more delicate plants in the bag. This is especially true for palms and large aroids. To prevent significant wilting, plants may be wrapped in moist newspaper and placed in a plastic bag, which should be kept shaded if at all possible. Plants shrink on drying, which is especially true for more succulent plants. Keep this in mind when collecting and pressing. What may appear to be ample material when fresh may be skimpy once it is dried. When collecting material, it is important to actually look at the plant to estimate the height or to note other characters. Many collectors who have trouble remembering this information never closely examined the plant in the first place. If having trouble remembering details, then one should always carry a small notebook or a marking pen to write directly on the leaves.

3.9.2 Preparation of Herbarium

The method of herbarium preparation and storage depends on the type of plant being processed. Most specimens are mounted on standard herbarium sheets. They include reproductive and vegetative organs, features critical to identification. Plant parts that cannot be easily pressed, such as large flowers, bulbs, fruits, cones, bark, or large-diameter woody stems, can be dried in boxes or paper bags. Lichens and bryophytes, which include mosses, liverworts, and hornworts, are usually dried in packets, paper bags, or boxes, as pressing irreversibly distorts material for adequate identification.

3.9.2.1 Collection of Plant Material

Botanicals should be collected from national forests, state parks, etc., and permission should be obtained before going on private property. You may need something for digging and cutting (knife or herbarium clippers). A vasculum, which is a lightweight metal container with a hinged lid and handle on its side, can be used to store specimens collected from the field, but most collectors use large plastic bags for carrying and storing specimens. Bags should be closed, folded, or tied so that plants do not hang down excessively. Collect those specimens which have flowers and fruits; no sterile specimens should be collected. For herbaceous plants, collect the entire plant, including the roots. Fragments of plants sometimes lack the key features for identification. Most fibrous roots may be trimmed and all soil should be washed or shaken from the specimens. If the specimen is large, some leaves or even sections of stem may be discarded. For woody plants, branches that include several mature leaves and the flower and/or fruit should be clipped. After collection, plant material should be kept in airtight bags.

3.9.2.2 Pressing and Drying

Most plants will fall apart after 2 or 3 days in the event that they are not dried or preserved in some form. If they are refrigerated, they can be kept for a maximum of a day or two longer. Material intended to serve for vouchering phytochemical profiles should be dried as quickly as reasonably possible, and should not be protected synthetically so as to not modify the compound structure. The plant ought to be keep inside a collapsed daily paper. The plant ought not stretch out past the paper and overlap the stem into a “V” or “W” shape if required. The accumulation number is written on the edge of the daily paper in which the example is squeezed as this guarantees coordinating the example with the best possible region, date, and so on, regardless of whether the ID and marking is done many months after the collection. The newspaper is placed between two blotters, which in turn are sandwiched between two corrugates. The blotters draw moisture from the plants, and the corrugate allows the moist air to pass from the press, which expedites drying. The straps are tightened to ensure the specimens will dry flat, without wrinkling. If the sample is bulky, for example, thick woody stems or large fruits, a foam pad, such as that used under carpeting, can be placed between the newspaper and the blotter. This will “wrap around” the thicker plant parts and ensure a nice, flat press job. If the plant is a thin-leaved aquatic plant or has delicate flowers, it may be pressed better between pieces of waxed paper within the newspaper.

3.9.2.3 Labeling of Specimen

After the plant has been dried, a label is prepared for attachment to the specimen. The identity of the sample is determined and the remainder of the label data are obtained from the field book. If the plant size is greater than the pressed sample, its height can be given on the label. The label should always include the scientific name (binomial) of the plant, the location (state, county, precise area), collector’s name, and collection number. Information about the abundance, habitat, and plant associations should be also included.

3.9.2.4 Storage of Specimens

Once specimens have been identified, mounted, and labeled they should be kept in a refrigerator at −20 °C for 48 h to kill insects, and then stored in cabinets in a specially equipped room called an herbarium. Specimens may also be frozen if they have been sitting out for a while during any of the stages listed above. Plant specimens properly stored and handled can be kept for a 100 years or more. Most herbaria store specimens on shelves in sealed metal cabinets or wooden cabinets. Proper storage involves the organization, handling, and maintenance of the collection.

Once the collection is stored, periodic checks must be made to ensure that the plants are stable and not affected by insects or excessive moisture. Insects can seriously damage an herbarium collection. Even with the most meticulous care and the best equipment, insects are certain to attack the specimens at some time. Over the years, various methods have been tried to prevent insect damage, such as dipping the specimens in insecticide or fumigating the plants, cabinets, or the entire room.

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Chapter 4

Qualitative Analysis for Evaluation of Herbal Drugs

Chapter Outline

Quality Control and Evaluation of Herbal Drugs. https://doi.org/10.1016/B978-0-12-813374-3.00004-1

4.1 IMPORTANCE OF QUALITATIVE ANALYSIS

Qualitative analysis highlights several aspects of the detection of the physical and physicochemical characteristics of herbal drugs due to the presence of different constituents present therein. This type of analysis not only helps in primary identification of the herbs but also gives an impression about the quality of the herbs used in phytopharmaceutical formulations.

The starting materials usually consist of fresh plants or their parts, which are subjected to other operations, such as drying and preservation, as described in other chapters. The processing of a fresh plant or its parts into a dried crude drug has been described in numerous technical publications and monographs and is considered under the province of pharmaceutical phytology. Phytomolecules are available in the cell fluid of living plant cells by formation in the protoplast; then, they migrate to the tonoplast through the plasma membrane and appear in the vacuoles. Some phytomolecules (e.g., cellulose) that include lipophilic constituents (e.g., oils, balsams, resins) are subjected to expulsion through the plasmalemma to the exterior and forming the cell membrane. In the case of rose oil (rose petal), oil cells (Lauraceae and Zingiberaceae family) are distributed in the cell fluid and turned into ethereal oil, which further fills the cell space. In the case of the Rutaceae family, lysigenic excretion of vacuoles is followed by the dissolution of the cell wall, whereas in the Umbelliferae family, Schizogenic excretions of vacuoles are formed through expansion of intercellular spaces resulting in the formation of ethereal oil. Generally, hydrophilic constituents are stored in the aqueous cell fluid but the lipophilic constituents are rarely present in it.

Postharvesting treatment is important to reduce the bad effects of the preserved plant materials (e.g., tubers, seeds, and certain dried fruit). Knowledge about the postharvesting physiology of crude drugs is important when the consequence of rapid or gradual loss of water in medicinal plants (during the processing of harvested plants to form drugs by drying and related aspects) may affect the chemical constituents. The modifying processes may proceed slowly because of the low water content in the structure of the fruit skin or the seed case.

The physical and physicochemical state of the interior of the cell undergoes enormous changes through loss of water. On drying, the enzymes predominantly localized in the plasma of the living plant cell, come into contact with the active substances dissolved in the cell fluid. Depending on the type of the enzyme and the substrate, this may lead to hydrolysis, oxidation, polymerization and other changes. In some plants, the active substances and the enzymes exist in different (separated) cells, resulting in reactions of enzymes and active constituents during harvesting due to tissue disruption (e.g., in Cruciferae containing mustard oil, in which the enzyme complex myrosin is present in the cells free of glycoside).

The rationale behind drying crude drugs is that dry, moisture-free drugs are less vulnerable to decomposition reactions to achieve a higher degree of storage and stability. Many of the enzymes responsible for decomposition survive during the drying process and they can be activated in the presence of moisture. So, the moisture content should be reduced below the prescribed limits of the pharmacopeia. A number of enzymes are responsible for the production of plant secondary metabolites, such as oxidases/peroxidases. These are mainly responsible for the oxidation of phenols, unsaturated fatty acids, and terpenes. The breakdown of esters, glycosides, and polysaccharides is accomplished by hydrolases. For the biosynthesis of enantiomeric secondary metabolites, such as the ergot alkaloids, morphine, quinine, and isomerases play key roles.

In order to protect the drugs from the action of these enzymes, they should be dried as rapidly as possible and the enzymes have to be denatured. This is known as stabilization of drugs and it is hardly ever practiced on a large scale. Enzymatic processes are not always undesirable. In many cases, active and aromatic substances and other useful compounds are released only through such processes. The fermentation of tea, the retting or steeping (fermentation) of fresh cocoa beans, which produces the color and aroma of the cocoa, and the release of coumarin from melilot (Melilotus) (or white clover, *Trifolium repens*) and woodruff (*Asperula odorata*, *Gallium ododratum*) can be mentioned as examples. There is no applicable rule in general for the type of drying and storage processes to be carried out after harvesting.

One of the major challenges for optimal quality in the manufacturing of botanicals includes the poor quality of the raw materials, which plays a major role in the production of phytopharmaceuticals of standard quality.

Very little specification of standards is mentioned in the official monographs considering the huge resources of botanicals.

- Even when the standard is specified, a range of variations occur which do not correspond to those as stated in the pharmacopeia.
- Due to geographical variations, there are variations in the content of metabolites. The requirements of the herbal raw material cannot be fulfilled, due to the large geographical variations.
- The crude drug rendered may be unfit to use through infestation or microbial contamination. Pesticide or preservative residue exceeds the permitted levels.
- The large demand for herbal drugs of pharmacopoeial quality make it difficult to maintain the quality and purity.

The purity of botanicals depends upon the absence of foreign matter, whether organic or inorganic, while quality refers to the concentration of the active constituents present in the drugs. By virtue of these constituents or components, the product is used and its economic and commercial value is estimated. Based on the concentration and nature of the constituents, a crude drug may conform to all the official standards of purity and be of good quality.

It is virtually impossible to avoid some naturally occurring inorganic or organic contaminants while collecting crude drugs from the field. The principal inorganic matter found in crude drugs is sand or other inorganic constituents in the soil. Less frequently, bits of metal, glass, or stone may also occur. The most common foreign organic material consists of different parts of the plant species that are not officially included in the description of the drugs, or it may be the parts of the neighboring plants of another species. Beside these, there are some other contaminants, such as insects, molds, and animal excreta. These affect the purity of any crude drugs and they require proper assessment and detection based on different pharmacognostical and phytochemical parameters, as described in the subsequent sections.

The evaluation of these qualitative parameters gives a clear idea about the specific characteristics of the crude drugs under examination, besides their macromorphological or cytomorphological characters and microscopical nature in both full and powder form. While these diagnostic features enable the analyst to know the nature and characteristics of crude drugs, further evaluation of different parameters indicates their acceptability by criteria other than the morphological characteristics. The procedures normally adopted to acquire qualitative information about the purity and standard of a crude drug include the determination of various parameters as described in later sections.

4.2 MOISTURE CONTENT

4.2.1 Importance and Significance of Moisture Content

An inevitable component of crude drugs is moisture, which must be eliminated as far as practicable. The preparation of crude drugs from the harvested plant drug involves cleaning or garbling to remove soil or other extraneous material, followed by drying, which plays a very important role in the quality as well as the purity of the material. The objectives of drying fresh material are:

- to aid in preservation;
- to "fix" the constituents, that is, to check enzymatic or hydrolytic reactions that might alter the chemical composition of the drug;
- to facilitate subsequent comminution (grinding into a powder); and
- to reduce their weight and bulk.

Insufficient drying favors spoilage by molds and bacteria and makes possible the enzymatic destruction of active principles. The moisture requirements for the active growth of some of the common molds and bacteria that may be found in or on drugs are relatively low. Therefore, the drying process should reduce the moisture content of the drug below this critical, or threshold level. Because the moisture requirements for enzymatic activity and that which microorganisms demand vary not only with the species, but also with other environmental factors (e.g., temperature, oxygen and carbon dioxide tension, light), it is difficult to state a precise upper limit of moisture that can be permitted in crude drugs. The USP and the NF make no commitment in this regard in most cases. However, most drugs may be stored safely if the moisture content is reduced to 6% or less. A notable exception is agar, for which USP permits as much as 20% moisture. Not only is the ultimate dryness of the drug important, the rate at which the moisture is removed and the conditions under which it is removed are equally important. If the rate is too slow, much spoilage may occur before the drying process is completed. Therefore, in general, drying should be accomplished as rapidly as possible with good practice. The duration of the drying process varies from a few hours to several weeks, depending on the water content and other features of the drugs.

Consideration of the time during which an elevated drying temperature is maintained is important because destructive enzymatic reactions are accelerated by increasing the temperature, although the net effect of most such reactions commonly encountered in the preparation of crude drugs is accelerated only up to approximately 45°C. However, higher temperatures shorten the time required for drying, and thus the time during which destructive reactions can occur is also shortened. The methods employed for drying are variable in detail, but they may be classified as spontaneous or as artificial. Artificial methods may be physical, which involves the use of elevated temperature and/or decreased pressure (vacuum), or the use of radiation of infrared or radio-frequency wavelengths; or they may be chemical, which involves the use of desiccants.

The crude plant materials are generally dried and stored as voucher specimens, as well as for extraction and isolation purposes. They are not usually stored as such, though, according to the API (Ayurvedic Pharmacopeia of India), many

quality control parameters have been determined using the raw plant material, without drying it. The moisture content of a plant material is important from a stability perspective. The moisture content affects the following parameters:

- Microbial growth, which may be bacterial or fungal growth and is deleterious to the drug.
- Enzymatic activity inside the plant cells may be a threat to the stability of the compounds.
- On drying, the bulk of the material also gets reduced, which helps in the storage of the plant material.
- After drying, further comminution and size reduction becomes easier for the drug.

The official compendia, such as the Indian Pharmacopeia and the Ayurvedic Pharmacopeia of India, have given guidelines on the optimum moisture content for official crude drugs. The moisture content in a crude drug may be determined by different methods, such as the Karl Fischer (KF) titration method, the toluene distillation method (Azeotropic distillation method), and the determination of the loss on drying. It has been found that the enzymatic activity in a plant varies with the moisture content and temperature. An increase in the temperature will initially result in an increase in the enzymatic activity and, after the temperature crosses 45°C, the enzymatic activity gradually decreases. The drying of the plant material is to be carried out quite rapidly. Otherwise, a slow drying process may allow enzymatic activity and microbial growth. Another important aspect of the moisture content is the ash values, extractive values, and other important parameters of the crude drug, which should be measured on a dry basis. In the following section, the method for calculating the LOD (loss on drying) and discuss the toluene distillation method and the KF method for moisture content determination (Mukherjee, 2002).

The general formula for moisture content determination is

$$w = \frac{M_w}{M_s} \times 100$$

where W = moisture content of the crude drug in percent, M_w = mass of water in the sample, and M_s = mass of bone-dry solid in the sample.

4.2.2 Determination of the Moisture Content of a Crude Drug

The presence of excess water in raw material facilitates the growth of microorganisms that can reduce the quality of the raw material. The moisture content of raw material is determined by the following methods, as stated by WHO.

An excess of water in herbal materials will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. Limits for water content should therefore be set for every given herbal material. This is especially important for materials that absorb moisture easily or deteriorate quickly in the presence of water. The moisture content is determined by several methods. Among them, the WHO has prescribed two methods.

4.2.2.1 The Azeotropic Or Toluene Distillation Method

This method is used to measure the presence of water in the crude drug. In this method, the crude drug is distilled with an immiscible solvent (e.g., toluene R or xylene R) so that the water content of the sample is absorbed by the solvent. Then, the total systems are subjected to distillation and are separated in a receiving tube. In the case of anhydrous solvent, water may remain in the sample and produce false results. For this, the saturation of the solvent with water is essential. A schematic diagram of the toluene distillation method is presented in Fig. 4.1.

The toluene distillation apparatus, also known as the Dean-Stark apparatus (Fig. 4.2), consists of several units. The first unit is a 500-mL round-bottom flask (1). The sample and the toluene are kept in this unit and heating is carried out with a heating unit with a rheostat to control the temperature, such as a heating mantle. The vapor is carried to the condenser by a tube (2) and the azeotropic mixture of toluene and water is condensed in the condenser (3) by a continuous flow of cold water. The condensate is collected in the graduated collecting tube (4), which has graduations of 0.1 mL.

The moisture content may be calculated using the following formula after the experiment:

$$\text{Moisture content} = \frac{100\left(n_1 - n\right)}{w}$$

where w is the weight in g of the material being examined; n the number of mL of water obtained in the first distillation; and n_1 the total number of mL of water obtained in both distillations.

4.2.2.2 Determination of the Loss on Drying

This method is used to determine the loss of water and volatile matter through heating of the sample (100–105°C) or keeping it in a desiccator along with P_2O_5 for a specific time period at room temperature.

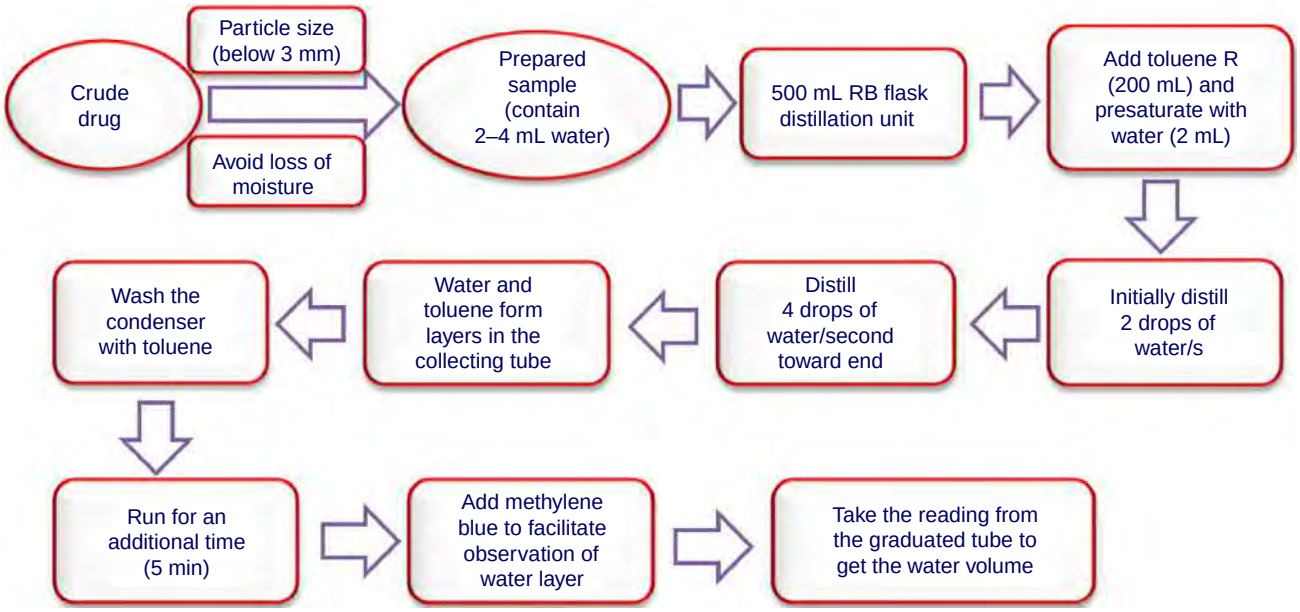

FIG. 4.1 Schematic representation of the toluene distillation method.

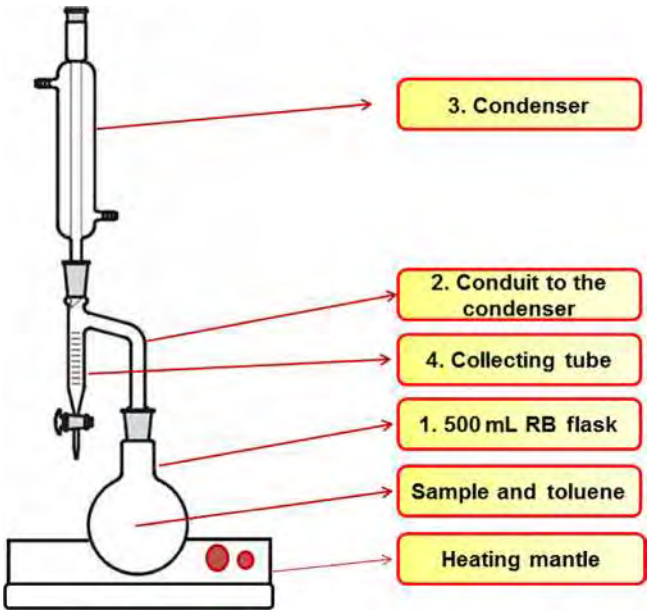

FIG. 4.2 Instrumentation of the Dean-Stark apparatus.

With the help of the test for loss on drying, both the water and volatile matter contents are determined. The drying of crude drugs may be performed by heating the crude drug at 100–105°C or by placing in a desiccator over P_2O_5 under atmospheric or reduced pressure. The latter loss on drying method is presented in the schematic diagram Fig. 4.3.

4.2.2.3 Titrimetric Determination of Moisture Content

The moisture content in plant samples is determined by titrimetric methods with KF (Karl Fischer) titration. The KF titration is based on the principle that a solution of iodine and sulfur dioxide in methanol liberates sulfuric acid and hydroiodic acid in the medium. This liberated acid is thereafter titrated with pyridine. This gives an estimate of the water content in the sample.

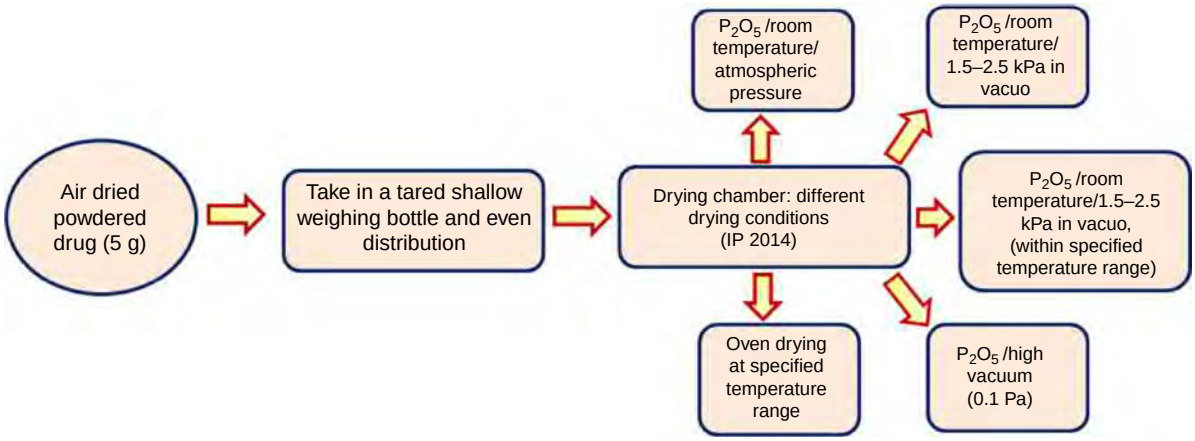

FIG. 4.3 Schematic representation of the loss on drying method.

The chemical equation of the procedure is written below:

$$SO_2 + I_2 + 2H_2O \rightarrow H_2SO_4 + 2HI$$

Most of the water is enclosed in cells and can only be extracted slowly. The complete water content can never be found by using a drying oven method. This is why the KF method has also proved to be an accurate and rapid absolute method for such samples. In order to achieve the correct result quickly, the titration is often carried out at an elevated temperature. The addition of formamide normally accelerates both the extraction of the water and the KF reaction. However, the formamide fraction must not exceed 50% as otherwise the stoichiometry of the KF reaction changes. The procedure for the determination of the water content of dried plant material is presented in Fig. 4.4. The moisture content determination method for fresh, undried plant material is depicted in Fig. 4.5.

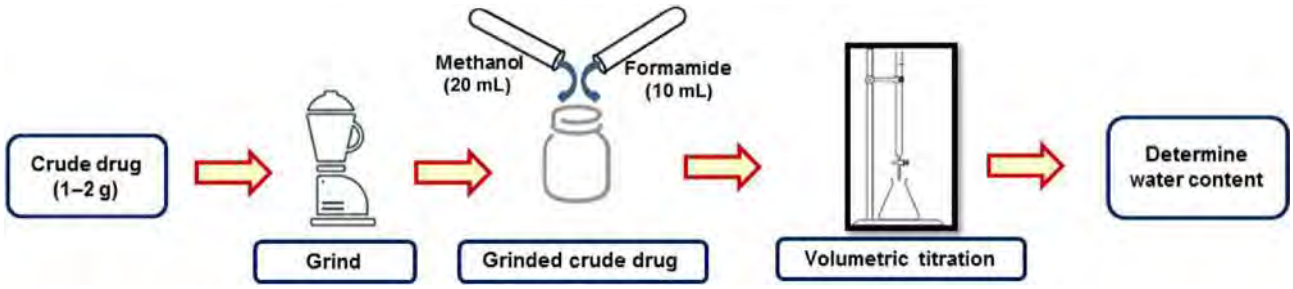

FIG. 4.4 Determination of the moisture content of a dried crude drug.

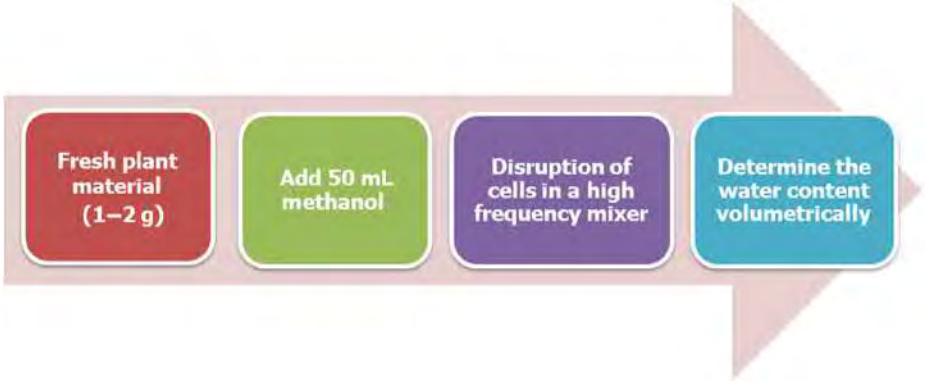

FIG. 4.5 Determination of the moisture content of a fresh crude drug.

TABLE 4.1 The Moisture Content of Several Plant Species as Specified in the Indian Pharmacopoeia (2014)

Name of the Drug	Loss on Drying
Ajwain (*Trachyspermum ammi* Mill.), Family Apiaceae, dried fruits	Not more than 10%, determined on 5 g by drying in an oven at 105°C
Amalaki (*Emblica officinalis* Gaertn.), Family Euphorbiaceae, dried fruit pericarp	Not more than 12%, determined on 5 g by drying in an oven at 105°C
Amaltas (*Cassia fistula* Linn.), Family Caesalpiniaceae, dried fruit pulp	NMT 6.2%, determined on 5 g by drying in an oven at 105°C
Arjuna (*Terminalia arjuna* Roxb.), Family Combretaceae, dried stem bark	NMT 10%, determined on 5 g by drying in an oven at 105°C
Ashwagandha (*Withania somnifera* Linn., Dunal), Family Solanaceae	NMT 12%, determined on 5 g by drying in an oven at 105°C
Bibhitaki (*Terminalia belerica* Gaertn. Roxb.), Family Combretaceae	NMT 12%, determined on 5 g by drying in an oven at 105°C
Bhringaraj (*Eclipta alba* Linn.), Hassk. (Family Asteraceae), aerial part	NMT 15%, determined on 5 g by drying in an oven at 105°C
Gudmar (*Gymnema sylvestre* R.Br. Family Asclepiadaceae), dried leaves	NMT 14%, determined on 5 g by drying in an oven at 105°C
Kalmegh (*Andrographis paniculata* Nees) Family Acanthaceae	NMT 12%, determined on 5 g by drying in an oven at 105°C
Manjistha (*Rubia cordifolia*, Linn., sensu Hook) Family Rubiaceae root	NMT 5%, determined on 5 g by drying in an oven at 105°C
Daruharidra (*Berberis aristata* Family, Berberidaceae) dried bark	NMT 10%, determined on 5 g by drying in an oven at 105°C
Mandukaparni (*Centella asiatica* Linn.), Family Umbelliferae	NMT 12%, determined on 5 g by drying in an oven at 105°C
Maricha (*Piper nigrum* Linn.) Family Piperaceae	NMT12%, determined on 5 g by drying in an oven at 105°C
Nagakesar (*Messua ferrea*) (Family Clusiaceae)	NMT 9%, determined on 5 g by drying in an oven at 105°C
Sunthi (*Zingiber officinale*), Zingiberaceae	NMT 12%, determined on 0.2 g
Vasaka (*Adhatoda vasica* Nees), Acanthaceae	NMT 12%, determined on 5 g by drying in an oven at 105°C
Haridra, Turmeric (*Curcuma longa*), Family Zingiberaceae	NMT 6.2%, determined on 5 g by drying in an oven at 105°C

There is a particular upper limit for the moisture content in the Indian Pharmacopoeia for each crude drug. If the moisture content of the drug is greater than this limit, it is not acceptable. The moisture content is to be determined mostly using the oven drying method according to the Indian Pharmacopeia.

The Indian Pharmacopeia has laid down guidelines and limits for the moisture content of the pharmacopoeial crude drugs as listed in Table 4.1.

4.3 ASH VALUES

The ash content of a crude drug is generally taken to be the residue remaining after incineration. It usually represents the inorganic salts naturally occurring in the drug and adhering to it, but it may also include inorganic matter added for the purpose of adulteration. There is a considerable difference within narrow limits in the case of some individual drugs. Hence, an ash determination furnishes a basis for judging the identity and cleanliness of a drug and gives information relative to its adulteration with inorganic matter. Ash standards have been established for a number of official drugs. Usually, these standards give a maximum limit on the total ash or on the acid-insoluble ash permitted. The total ash is the residue remaining after incineration, which includes "physiological ash" derived from the plant tissue itself and "nonphysiological ash," often derived from environmental contaminations, such as sand and soil. For example, sclereids in the pericarp of Colocynth and the cork of Licorice are examples of physiological ash. Contamination by sand or earth is an example of nonphysiological ash. The total ash chemically consists of carbonates, phosphates, silicates, and silica. The total ash content alone is not sufficient to reflect the quality of herbal medicines because plant materials often contain considerable levels of physiological ash, calcium oxalate in particular. Thus, the acid-insoluble ash content is another index to illustrate the quality of Chinese herbal medicine. The acid-insoluble ash is the part of the total ash that is insoluble in diluted hydrochloric acid. The ash or

residue yielded by an organic chemical compound is, as a rule, a measure of the amount of inorganic matter present as impurity. In most cases, the inorganic matter is present in small amounts that are difficult to remove in the purification process and are not objectionable if only traces are present. Ash values are helpful in determining the quality and purity of crude drugs in powder form. The water-soluble ash is used to estimate the quantity of inorganic compounds present in drugs. The acid-insoluble ash consists mainly of silica and indicates contamination with earthy material (Chanda, 2014).

The ash content of most fresh herbs is rarely greater than 5%. Pure oils and fats generally contain little or no ash; products, such as cured bacon, may contain 6% ash, and dried beef may be as high as 11.6% (wet weight basis). In fats, oils, and shortenings it varies from 0.0% to 4.1% ash, while in dairy products it varies from 0.5% to 5.1%. Fruits, fruit juice, and melons contain 0.2%–0.6% ash, while dried fruits are higher (2.4%–3.5%). Pure starch contains 0.3% and wheat germ 4.3% ash (Marshall, 2010).

4.3.1 Principles of Determination of Ash Value

Two major types of ashing are used: dry ashing, primarily for proximate composition and for some types of specific mineral analyses and wet ashing (oxidation), as a preparation for the analysis of certain minerals. Microwave systems are now available for both dry and wet ashing to speed the processes. Most dry samples (whole grain, cereals, and dried vegetables) need no preparation, while fresh vegetables need to be dried prior to ashing. High-fat products, such as meats, may need to be dried and the fat extracted before ashing. The ash content of foods can be expressed on either a wet weight (as is) or on a dry-weight basis.

Dry ashing refers to the use of a muffle furnace capable of maintaining temperatures of 500–600°C. Water and volatiles are vaporized, and organic substances are burned in the presence of oxygen in air to CO_2 and oxides of N_2. Most minerals are converted to oxides, sulfates, phosphates, chlorides, and silicates. Elements, such as Fe, Se, Pb, and Hg, may partially volatilize with this procedure, so other methods must be used because ashing is a preliminary step for specific elemental analysis. Wet ashing is a procedure for oxidizing organic substances by using acids and oxidizing agents or their combinations. Minerals are solubilized without volatilization. Wet ashing is often preferable to dry ashing as a preparation for specific elemental analysis. Wet ashing often uses a combination of acids and requires a special perchloric acid hood if that acid is used. Ash content represents the total mineral content in foods. Determining the ash content may be important for several reasons. It is a part of proximate analysis for nutritional evaluation. Ashing is the first step in preparing a food sample for specific elemental analysis. Because certain foods are high in particular minerals, ash content becomes important. One can usually expect a constant elemental content from the ash of animal products, but that from plant sources is variable (Marshall, 2010).

4.3.2 Methodology of Determination of Ash Value

The ash value of crude drugs is determined using the dry ashing method as per the Indian Pharmacopoeia (2014). Here, the finely powdered drug is first heated to around 675°C. The organic matter is oxidized to carbon dioxide and removed. It is expected to yield a carbon-free ash, which is identified by a dull grayish, white, pinkish, or red color. If not, it is further extracted with hot water, which is then filtered with an ashless filter paper and the residue, along with the filter paper, is burnt to a white mass. After that, the filtrate is added to the ash and again the temperature is raised to 675°C and the residue is collected. In the absence of a carbon-free mass, ethanol is added to the residue and burnt at the same conditions; this is considered to be the carbon-free ash. Practically, during heating at high temperatures, the carbonates of the inorganic compounds get converted to oxides due to heating at high temperatures. For estimation purpose, it may be further treated with a solution of ammonium carbonate to yield carbonated ash, with dilute sulfuric acid to yield sulfated ash, or with nitric acid to yield nitrated ash (Indian Pharmacopoeia, 2014).

4.3.2.1 Dry Ashing Technique for Total Ash Determination

First, 2–3 g of the powdered crude drug is placed in a platinum or silica crucible. Initially it is heated gently and then the temperature is increased up to 675 ± 25°C. After that, it is checked for complete removal of carbon. If the carbon has been removed completely, then the percentage of ash is calculated by directly weighing the ash. Otherwise, first extract the ash with water, filter with an ashless filter paper, and then ignite the ashless filter paper at 675 ± 25°C. After that, again the same decision needs to be taken. If a carbon-free residue is not obtained, wash the ash with ethanol and then carefully burn the whole thing at 675 ± 25°C so that it does not catch fire. Then, weigh the carbon-free ash for determination of the total ash. A schematic diagram below depicts the procedure well (Fig. 4.6) (Indian Pharmacopoeia, 2014).The percentage of ash is calculated on a dried basis of the crude drug.

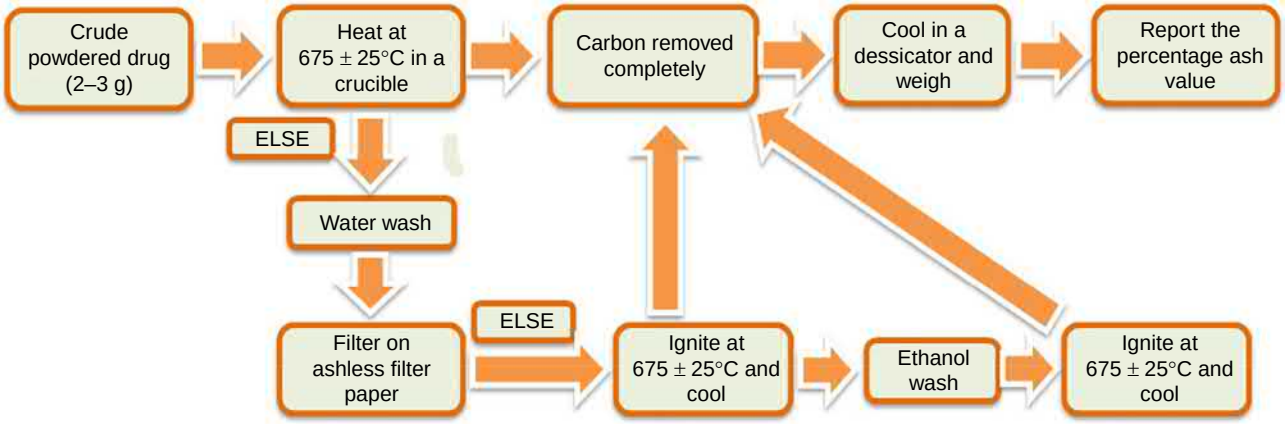

FIG. 4.6 Dry ashing technique for crude drugs.

4.3.2.2 *Wet Ashing Technique for Total Ash Determination*

This technique is useful both for total ash value determination and also for specific elemental analysis. The finely powdered crude drug is placed in an Erlenmeyer flask and then 3 mL concentrated sulfuric acid is added to it. This system is heated until brownish fumes disappear and white fumes start coming out. The flask is taken and 5 mL concentrated nitric acid is added. The heating is resumed. When the color of the solution becomes dark, it is filtered and placed in a volumetric flask and then diluted with ultrapure water. The solution is diluted as required and then subjected to further analysis, such as atomic absorption spectroscopy (Fig. 4.7). For estimation purposes, a blank solution consisting of 3 mL sulfuric acid and 5 mL nitric acid should be performed simultaneously. The percentage of ash is calculated on a dried basis of the crude drug (Marshall, 2010).

4.3.2.3 *Acid-Insoluble Ash*

Acid-insoluble ash is an indication of the portion of the ash that is insoluble in dilute hydrochloric acid. It indicates mainly the silica and sand-like substances present in the ash. The acid-insoluble ash is determined by washing the ash with 2 M HCl and then placing the ash on a Gooch crucible, washing with water, and filtering with ashless filter paper. Then, the filter paper is burnt off and the weight of the carbon-free mass is taken for estimation. The acid-insoluble ash, compared with the total ash, gives an estimate of the contaminating materials in the crude drug.

Determination of acid-insoluble ash

For this, the ash obtained by the dry ashing technique, or the sulfated ash obtained by treatment of the ash by sulfuric acid, is boiled with dilute hydrochloric acid. Then, the suspension is filtered by an ashless filter paper. After that, the filter paper and the filtrate are washed and then ignited until the residue takes on a dull red hue. It is cooled in a desiccator and then weighed to get the amount of ash that is equivalent to the acid-insoluble ash. The percentage of ash is calculated on a dried basis of the crude drug. The procedure is further explained in Fig. 4.8.

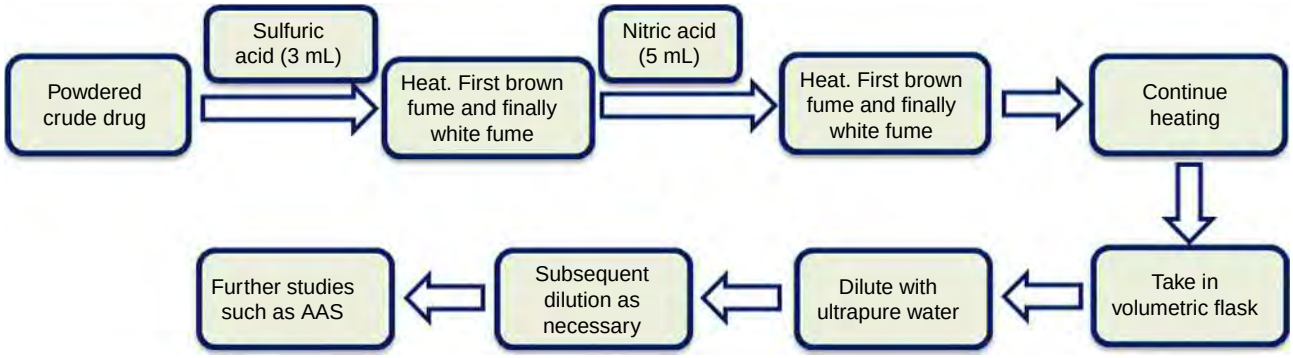

FIG. 4.7 Wet ashing technique for crude drugs.

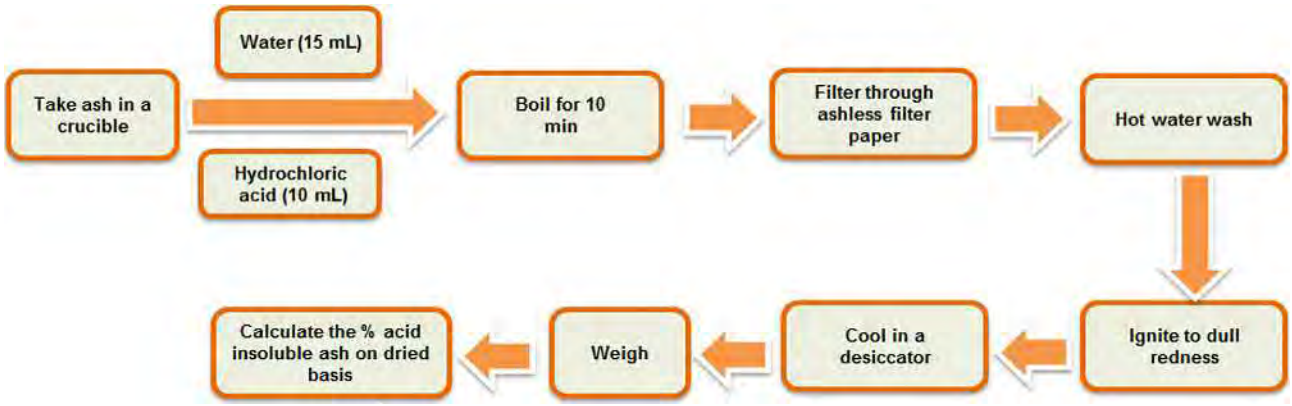

FIG. 4.8 Determination of acid-insoluble ash in a crude drug.

4.3.2.4 Water-Soluble Ash

Water-soluble ash is a determinant of whether the substance was previously extracted, that is, for detecting exhausted drugs. For doing this, first extract the ash by boiling it in water. After that, the residue is to be filtered by ashless filter paper. After burning, the ash, which is cooled and weighed. The difference between the weight of the total ash and the residue remaining after washing with water is known as the water-soluble ash.

Determination of water-soluble ash

The ash is boiled with water and filtered. Then, the filter paper, along with the residue, is ignited at 450°C. The ash is then weighed and the weight is subtracted from the weight of the total ash in order to get the percentage of the water-soluble ash.

4.3.2.5 Determination of Sulfated Ash

In a crucible, 1 g of the crude drug under examination is heated until it is charred. It is then moistened with sulfuric acid and heated at high temperature. This is performed to remove all the carbons and the oxides of the metals that are produced during heating. This is ensured by the removal of all the black particles. Again, the ash is moistened by a few drops of sulfuric acid and the heating is continued until constant weight is attained (Fig. 4.9).

4.3.2.6 The Microwave Ashing Technique

Instead of heating the samples on a hot plate or a Muffle furnace, they may be heated by microwave. In the case of wet ashing, the sample, along with an acid, such as nitric acid, is placed in a small vessel. The vessel can be of mainly two types, a closed vessel or an open system. The closed vessels are mounted on a carousel and placed in a microwave heating apparatus. In this type of apparatus, due to heating in a closed vessel at high pressure, the acid boils above its boiling point. As a result,

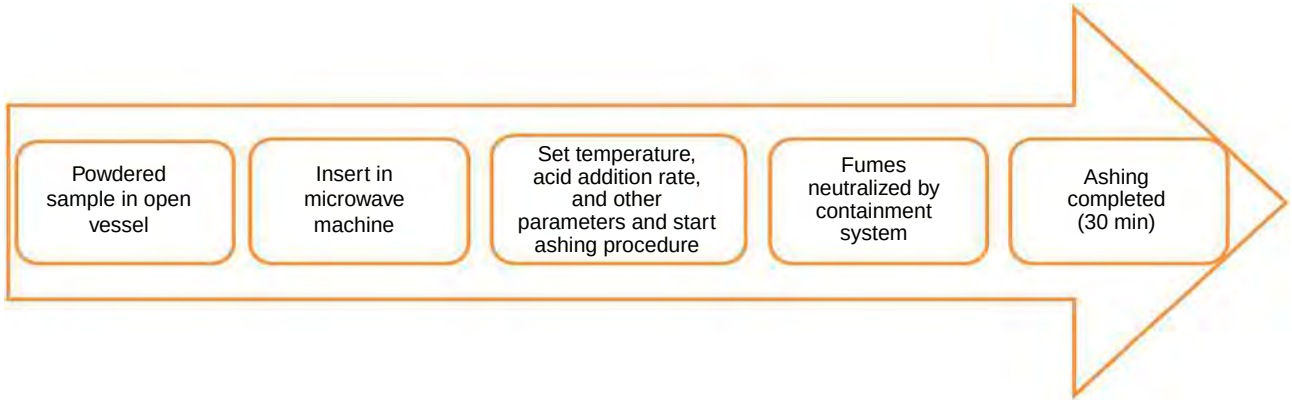

FIG. 4.9 Determination of sulfated ash in crude drugs.

the digestion may be carried out in less time. A heating program may also be set by a software-operated variation of the microwave frequency, thus effecting a temperature ramp. A schematic representation of wet digestion by microwave heating in a closed assembly is shown in Fig. 4.10.

Open vessel system

The same procedure may be conducted using an open-vessel system, which is shown in Fig. 4.11. Here, six samples may be used at a time. The sample and the acid are reacted in an open-vessel system and inserted in a microwave heater. These systems are suitable for large sample sizes and for those types of samples that give off a large amount of gas. Here, the gas or the fumes are neutralized using a vapor containment system. Sulfuric or nitric acids are mainly used for this type of digestion.

4.3.2.7 Dry Ashing Using a Microwave System

The conventional dry ashing technique is a time- and power-consuming process, in which the sample needs to be heated up to a high temperature for a long time, up to 4–6 h. Instead, in the case of microwave heating, this timeline is reduced to only 35–40 min. So, it is quite a time-saving and economical process. Here, the crucible used for heating may be made up of porcelain, quartz, quartz fiber, or platinum. The method for dry ashing using a microwave muffle furnace is shown in Fig. 4.12.

4.3.3 Comparative Evaluation of Different Ashing Techniques

The two major types of ashing, dry ashing and wet oxidation (ashing), can be performed by conventional means or using microwave systems. The procedure of choice depends upon the use of ash following its determination, and limitations based on cost, time, and sample numbers (Marshall, 2010).

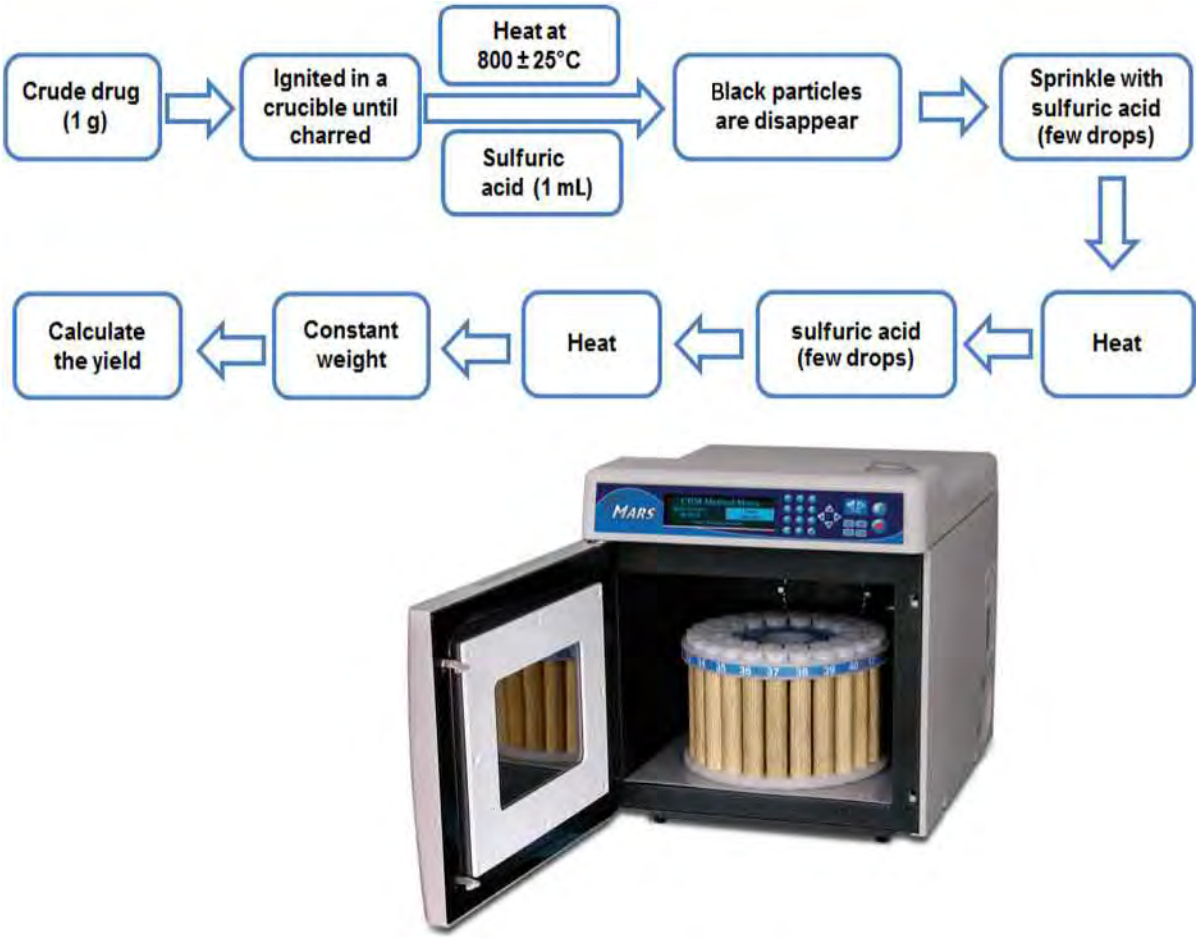

FIG. 4.10 Wet digestion by microwave heating in a closed assembly.

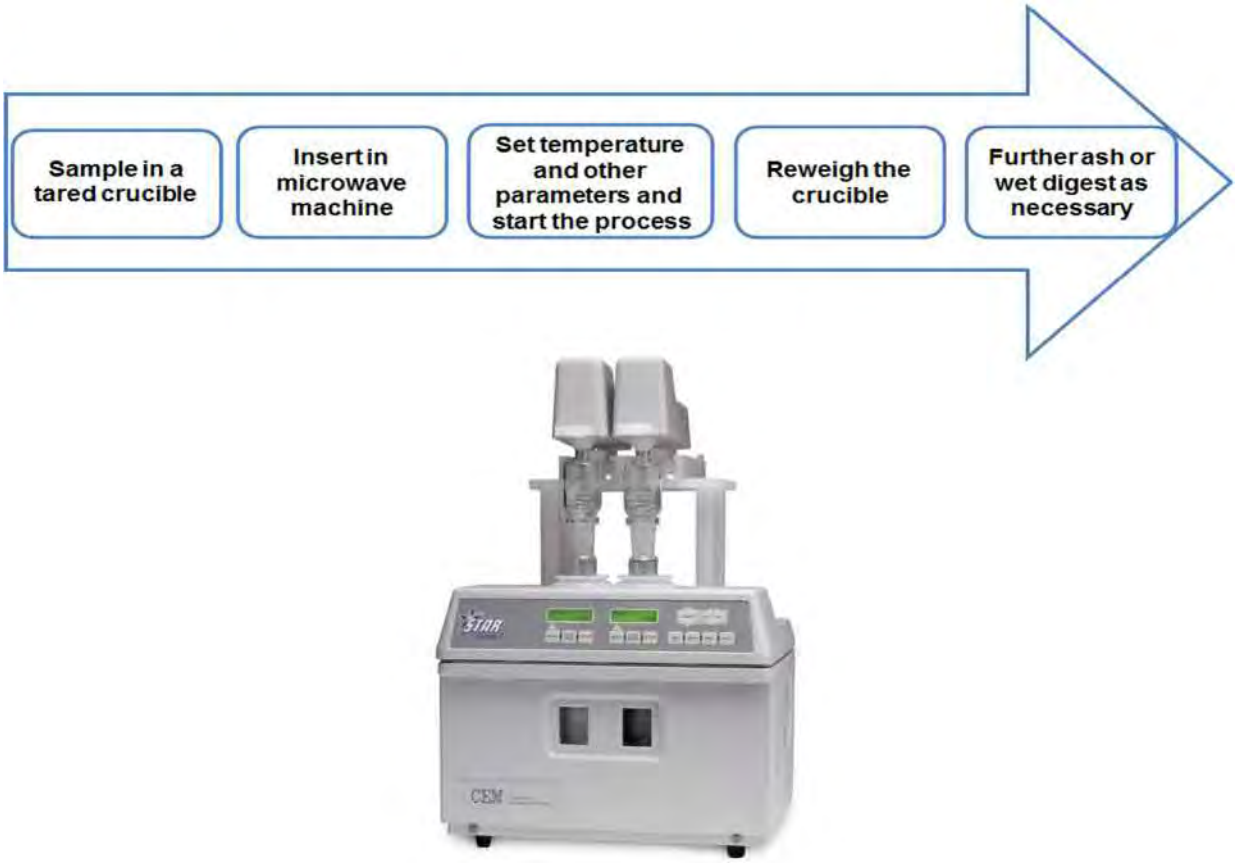

FIG. 4.11 Open vessel digestion system.

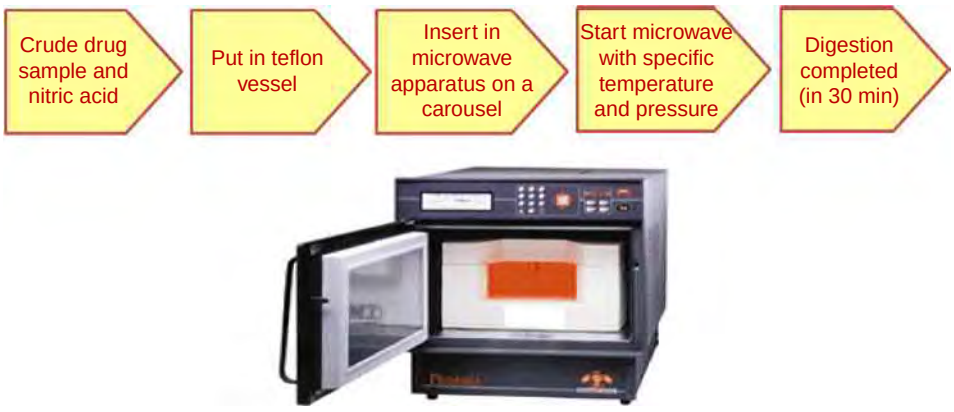

FIG. 4.12 Dry ashing using a microwave muffle furnace.

- The dry ashing technique is safer compared with the wet ashing technique. It may also be considered simpler and is the most widely used as a preliminary standardization tool for the determination of the ash value of crude vegetable drugs.
- One drawback of the dry ashing technique is that it causes a loss of volatile materials due to heating at high temperatures. Also, the heating setups required are a heat room along with drying ovens requiring a 220-V outlet. It is important to make sure that large furnaces of that type are equipped with a double-pole, single-throw switch. Heating coils are generally exposed, and care must be taken when taking samples in and out with metal tongs (Marshall, 2010).

- The wet ashing technique involves lower temperature, and thus prevents loss of volatile materials, such as arsenic, selenium, mercury, potassium, phosphorous, and sulfur from the crude drug. It is useful for the determination of heavy metals by atomic absorption spectroscopy or flame photometry later.
- The drawback of this technique is that it needs constant operator attention, expensive setups for proper management of the fumes, and even after that carries the risk of explosion hazards.
- The advanced technologies include microwave-assisted technologies, which save both time and power and are also safer. These involve expensive microwave setups, which are used for heating up the sample. They have the advantage that the fumes produced are effectively removed or neutralized by an air-containment system.

There is an upper limit on the ash value, the acid-insoluble ash value, and, in some cases, water-soluble ash values for almost every crude drug. Several such limits are summarized in Table 4.2 (Indian Pharmacopoeia, 2014).

From the above discussion, it is apparent that the official crude drugs in the Pharmacopeia should have an ash value below the one specified in the pharmacopeia. This is to limit the external contamination or the physiological unwanted tissues in the crude drug. This limit may vary from plant to plant. For example, the permissible limit of the total ash is as high as 30% in the case of Arjuna. Also, the limit is as low as 3% in the case of Vijayasara. The same circumstance follows in the case of the acid-insoluble ash values of the plants.

Ash values of a particular crude drug are subject to seasonal variation. Also, it has been seen that the ash value of a particular drug varies in the different plant parts. The ash value is also subject to interspecies variation. A study involving species of the *Sesbania*, *Sesbania rostrata*, *Sesbania exaltata* and *Sesbania sesban*, *Sesbania grandiflora*, *Sesbania bispinosa*, and *Sesbania cannabina*, can be found in Kadam et al. (2013) (Table 4.3).

4.3.4 Near-Infrared Spectroscopy for the Determination of the Ash Value

A novel technique for the determination of Ash values is near-infrared spectroscopy. This method has been used by Rao and Xiang (2009). Here, the plant powder is scanned in the reflectance mode by a near-infrared light beam. The spectra recorded were subjected to chemometric analysis using the OPUS software. The first and second derivative plots were

TABLE 4.2 Limits of Different Ash Values of Some Crude Drugs Specified in the Indian Pharmacopeia

Name of the Drug	Total Ash	Acid-Insoluble Ash
Ajwain (*Trachyspermum ammi* Mill.), Family Apiaceae	Not more than 15%	Not more than 7%
Amalaki (*Emblica officinalis* Gaertn.), Family Euphorbiaceae, dried fruit pericarp	Not more than 5%	Not more than 2%
Amaltas (*Cassia fistula* Linn.), Family Caesalpiniaceae dried fruit pulp	Not more than 18%	Not more than 1.3%
Arjuna (*Terminalia arjuna* Roxb), Family Combretaceae, dried stem bark	NMT 30%	NMT 2%
Ashwagandha (*Withania somnifera* Linn., Dunal), Family Solanaceae	NMT 7%	NMT 1.2%
Bibhitaki, *Terminalia belerica* (Gaertn. Roxb.), Family Combretaceae	NMT 8%	NMT 2%
Bhringaraj, *Eclipta alba* (Linn.), Hassk. (Family Asteraceae)	NMT 22%	NMT 11%
Gudmar, *Gymnema sylvestre* R.Br. (Family Asclepiadaceae), dried mature leaves	NMT 15%	NMT 6%
Kalmegh, *Andrographis paniculata* Nees (Family Acanthaceae)	NMT 15%	NMT 3%
Manjistha, *Rubia cordifolia*, Linn., sensu Hook (Family Rubiaceae)	NMT 10%	NMT 0.5%
Opium, *Papaver sominferum* Linn. (Family Papavraceae)	NMT 6%	—
Pippali, Large, *Piper retrofactum* Vahl. (Family Piperaceae)	NMT 8%	NMT 3%
Sarpagandha powder, *Rauwolfia serpentine* Bentham ex Kurtz (Family Apocynaceae)	NMT 8%	NMT 2%
Vasaka, *Adhatoda vasica* (Family Acanthaceae)	NMT 21%	NMT 2%
Vijayasara, *Pterocarpus marsupium* (Family Fabaceae)	NMT 3%	NMT 0.4%

TABLE 4.3 Comparative Ash Values of Plants Collected in Different Seasons

Plant Parts	Season	Total Ash (%)			Water Soluble (%)			Water Insoluble (%)		
		Plant 1	Plant 2	Plant 3	Plant 1	Plant 2	Plant 3	Plant 1	Plant 2	Plant 3
Leaves	Summer	7.54	6.02	5.67	3.22	2.53	2.30	4.34	3.49	3.37
	Monsoon	6.88	5.53	5.23	2.80	2.23	2.04	4.08	3.30	3.19
	Winter	7.34	5.80	5.43	3.12	2.40	2.17	4.22	3.40	3.26
Wood	Summer	4.20	2.70	2.12	1.80	0.94	0.73	2.40	1.76	1.39
	Monsoon	3.67	2.33	1.87	1.55	0.78	0.59	2.12	1.55	1.28
	Winter	3.97	2.54	2.02	1.75	0.86	0.67	2.22	1.68	1.35
Bark	Summer	9.52	7.89	7.40	3.98	2.97	2.42	5.54	4.92	4.98
	Monsoon	8.96	7.34	7.06	3.64	2.61	2.36	5.32	4.73	4.70
	Winter	9.29	7.66	7.27	3.88	2.82	2.40	5.41	4.84	4.87

Plant 1, Sesbania grandiflora; Plant 2, Sesbania bispinosa; Plant 3, Sesbania cannabina.

plotted. After that, a curve was plotted with the percent-ash value predicted versus the reference total ash value. This was done by partial least square analysis. This was found to be quite in keeping with the practical value obtained. This method was successfully applied for the determination of ash values of plants under Chinese traditional medicine. The advantage of this method is that it is both a high-throughput and nondestructive process, in comparison with the general muffle furnace methods for the determination of the ash value. Theoretically, it is predicted that the near-infrared spectroscopic technology is not efficient in detecting and quantifying inorganic materials present in the sample, but, practically, it has been found to be quite handy in predicting the ash value.

The ash value remains one of the most important pharmacognostical parameters to be tested in the quality control of herbal drugs. Herbal drugs are naturally obtained, so, there remains a chance of contamination of the drug with different heavy metals, pesticides, sand, soil, etc. Also, certain unwanted tissues, such as sclereids, may be intentionally or unintentionally admixed with the drug. So, for detecting them, at the preliminary stage, the determination of the ash value of the crude drug should be chosen and checked whether it exceeds the limit specified in the Pharmacopeia or other official books.

4.4 DETERMINATION OF EXTRACTIVE VALUE

The measurement of extractive values determines the amount of the active constituents in a given amount of plant material when extracted with a particular solvent. The extractions of any crude drug with a particular solvent yield a solution containing different phytoconstituents. The compositions of these phytoconstituents depend upon the nature of the drug and the solvent used. They also give an indication of whether the crude drug is exhausted or not. The standard extractive values monitored for medicinal plants include extractions with a series of solvents based on their polarity; these may include alcohol and water-soluble extractive values, the petroleum ether-soluble extractive value, the ethylacetate-soluble extractive value, and the acetone-soluble extractive value (Chanda, 2014).

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids, semisolids, or powders intended only for oral or external use. There are different types of extraction. They include maceration, percolation, digestion, decoction, continuous hot extraction using a Soxhlet apparatus, microwave extraction, and others. The method typically used for the determination of extractive values is aqueous or alcoholic decoction. After that, the extract is concentrated on a water bath. The concentrated extract is thereafter weighed and the extractive value is reported as a percentage of the dry weight of the initial crude drug. Extraction of a drug with a particular solvent extracts out the soluble constituents from that crude drug. It provides preliminary information about the quality of that particular drug (Mukherjee, 2002).

4.4.1 Principles and Methodology of the Determination of Extractive Values for Medicinal Plants

Extraction may be defined as a procedure by which a solvent permeates or ruptures the cell membranes of a particular crude drug, dissolves the soluble constituents, and brings them out. Thus, an extract is obtained, which is concentrated with the application of heat, vacuum, or both. The main consideration in this process is that crude drugs, being natural in source, can be degraded, deteriorated, or adulterated. Different pharmacopeias have prescribed limits for the extractive values for crude drugs.

4.4.2 Procedures for Determination of Extractive Values

4.4.2.1 Determination of Water-Soluble Extractive

The crude drug is kept with water in a stoppered flask at 80°C. After 10 min, 2 g of Kieselguhr is added to the suspension, filtered, and solvent evaporated on an evaporating dish in a water bath. Drying is performed for 30 min. The final drying is done in a steam oven. The percentage of the water-soluble extractive is calculated on the basis of the dried crude drug (Fig. 4.13).

4.4.2.2 Determination of the Ethanol-Soluble Extractive

This determination is performed by a cold maceration procedure and then by evaporation on a water bath. A quantity of 5 g of the air-dried crude drug is macerated by 100 mL ethanol. For the first 6 h, the mixture is shaken occasionally and the maceration is continued for 24 h. After that, the extract is filtered, evaporated on an evaporating dish quite meticulously, and then dried in an oven. Then, the percentage of the ethanol-soluble extractive value is calculated on the dried crude drug (Fig. 4.14). Table 4.4 gives the pharmacopoeial limits of the ethanol-soluble extractives of the pharmacopoeial drugs.

4.4.2.3 Determination of Hexane-Soluble Extractive

The hexane-soluble extractive value signifies the fatty portion of a crude drug. This includes the oils and fats present in the crude drug. The material is extracted using a Soxhlet apparatus, that is, taking resort of a continuous hot extraction. After concentration, the weight of the extractive is determined (Fig. 4.15).

4.4.2.4 Volatile Ether-Soluble Extractive

The crude drug is dried over phosphorous pentoxide for more than 12 h. Then, the drug is extracted with diethyl ether. The extract is first spontaneously evaporated on an evaporating dish and then dried over phosphorous pentoxide. After 18 h, the concentrate is heated at 105°C and the loss in the weight signifies the volatile portion of the extract. This is the volatile ether-soluble extractive method, which is shown in Fig. 4.16.

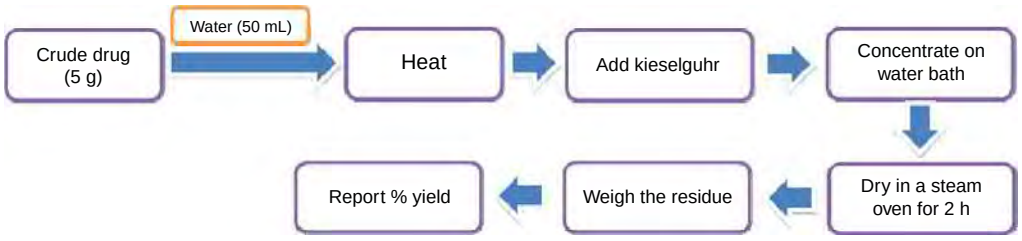

FIG. 4.13 Determination of water-soluble extractive.

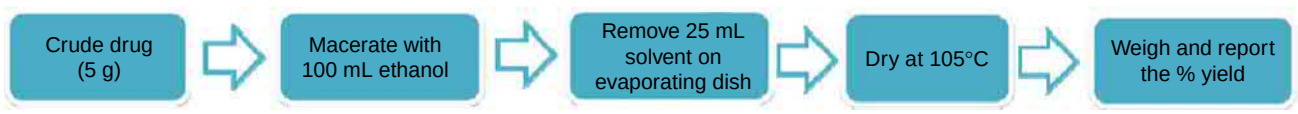

FIG. 4.14 Determination of ethanol-soluble extractive.

TABLE 4.4 Ethanol-Soluble Extractive Values of Some Crude Drugs Specified in the Indian Pharmacopoeia (2014)

Name of the Drug	Ethanol-Soluble Extractive Value
Ajwain (*Trachyspermum ammi*, Apiaceae)	Not less than 2%
Amalaki (*Phyllanthus emblica*, Phyllanthaceae)	Not less than 30%
Amaltas (*Cassia fistula*, Fabaceae)	Not less than 8%
Anantmula (*Hemidesmus indicus*, Apocynaceae)	Not less than 8%
Arjuna (*Terminalia arjuna*, Combretaceae)	Not less than 20%
Ashwagandha (*Withania somnifera*, Solanaceae)	Not less than 10%
Bhuiamla (*Phyllanthus niruri*, Phyllanthaceae)	Not less than 6%
Brahmi (*Bacopa monnieri*, Scrophulariaceae)	Not less than 6%
Daruharidra roots (*Berberis aristata*, Berberidaceae)	Not less than 2%
Daruharidra stems (*Berberis aristata*, Berberidaceae)	Not less than 2%
Haridra (*Curcuma longa*, Zingiberaceae)	Not less than 6%
Haritaki (*Terminalia chebula*, Combretaceae)	Not less than 35%
Kalmegh (*Andrographis paniculata*, Acanthaceae)	Not less than 3%
Sarpagandha (*Rauvolfia serpentine*, Apocynaceae)	Not less than 2%
Senna (*Cassia angustifolia*, Fabaceae)	Not less than 2%
Tulsi (*Ocimum sanctum*, Lamiaceae)	Not less than 3%
Vasaka (*Adhathoda vasica*, Acanthaceae)	Not less than 3%
Vidanga (*Embelia ribes*, Primulaceae)	Not less than 8.5%
Vijayasara (*Pterocarpus marsupium*, Fabaceae)	Not less than 14%
Punaranava (*Boerhavia diffusa*, Nyctaginaceae)	Not less than 0.5%

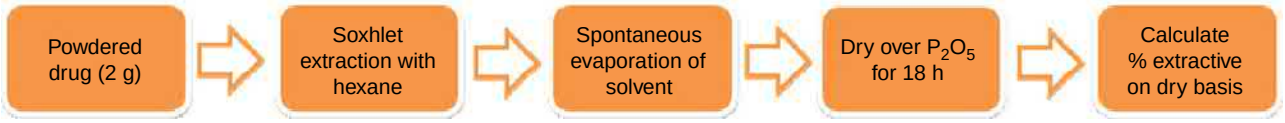

FIG. 4.15 Determination of hexane-soluble extractive.

FIG. 4.16 Determination of volatile ether-soluble extractive value.

4.4.2.5 Nonvolatile Ether-Soluble Extractive

This parameter is measured by the amount of the extract remaining after heating at 105°C. This is expressed as the nonvolatile ether-soluble extractive.

The extractive values for different parts of the plant *Enicostemma littorale* Blume in different solvents have been reported (Rathod and Dhale, 2013). About 5 g of the crude drug was coarsely powdered and extracted by a Soxhlet extractor using the solvents methanol, ethanol, Pet ether, and chloroform. The extracts were concentrated and the resulting extractive values are shown in Table 4.5.

TABLE 4.5 Extractive Values of *Enicostemma littorale* Blume in Different Solvents

	Extractive Value (%, w/w)		
Solvent	**Leaf**	**Stem**	**Root**
Ethanol	7.85	8.50	7.70
Methanol	6.55	6.70	6.20
Water	8.65	9.98	8.85
Pet ether	5.5	4.5	3.2

The determination of the extractive value remains a powerful tool for the quality control of herbal drugs even today. So, it may be concluded that the extractive value can serve as an indirect measure of the approximate estimation of a particular type of secondary metabolite, such as alkaloids, polyphenolics, terpenoids, and glycosides, prior to the assistance of highly sophisticated chromatographic marker profiling analyses. If the extractive value is found to be lower than the one specified in the official compendia, then poor quality materials may be detected at an early stage of herbal manufacturing. In combination with the organoleptic, microscopic, and powder analysis methods, this serves as a handy tool for controlling adulteration.

This test can be carried out to ascertain the quality of the plant material, that is, whether the plant material has been adulterated with exhausted plant material or not. The monographs on plant materials in the Indian Pharmacopeia or Ayurvedic Pharmacopoeia of India, has prescribed that, before carrying out thin-layer chromatography (TLC), HPTLC, or HPLC analysis for identification or assay of a plant material, the crude plant material is coarsely ground and extracted by the hot or cold extraction method with a suitable solvent, which is generally water, methanol, acetonitrile, chloroform, or any other suitable solvent. In some cases, the extract is further fractionated with a second solvent for a more accurate and precise result. Then, the extract is concentrated using particular evaporation conditions, generally a water bath. After reviewing the literature mentioned above, the Indian Pharmacopeia, and the book "Quality Control of Herbal drugs" by Mukherjee (2002), it is observed that the extractive values are also determined using almost the same methodology. In some cases, this method may prove to be misleading, as the compounds present in the plant extract, which are thermolabile but therapeutically important, may become degraded during the extraction and concentration process. At laboratory scale, practically, when we attempt isolation or purification of a plant extract for some specific activity, to prevent the thermolabile compounds from degrading, we generally first concentrate the extract on a water bath, or preferably in a rotavac, followed by lyophilization, to prevent charring of the thermolabile flavonoid-type compounds. So, after "concentrating to dryness," the extractive value that is obtained may be misleading from the perspective of the active constituents.

4.5 RESIDUAL SOLVENTS

In pharmaceutical terminology, residual solvents are the organic, especially volatile liquids, which are used or produced either during the manufacturing process or are the excipients in finished products. Using practical manufacturing techniques, complete solvent removal is not possible. Use of the proper solvents is done to enhance the yield and to determine the physicochemical characteristics (such as crystal form, purity, and solubility). For the synthesis of drugs, the solvents are considered to be important parameters. A limitation of this guideline is that it is restricted to the solvents used as excipients or solvates (such as Warfarin sodium clathrate). In order to meet the GMP specifications and to ascertain the safety, all of the residual solvents should be removed and their content should be reduced below the acceptable limit. For this, their contents should be evaluated and justified. Solvents that are toxic (class I solvents, such as benzene) must be avoided for preparation of pharmaceutical formulations. Class II solvents are to be used with precautions as they are associated with significant adverse effects. Class III solvents are known to produce less toxicity and should be preferred according to the requirements of pharmaceutical product design. Tables 4.7–4.9 provide the classifications of pharmaceutical solvents used for pharmaceutical preparations. Evaluations of the residual solvents in drug products need to be performed regularly. In order to control the residual solvent levels in a drug product, IPQC (in process quality control) is important, as the residual solvent levels in the ingredients and the different intermediate products need to be monitored. If it is found that the residual solvent level is below the prescribed limit, then there is no need to perform further checks. Otherwise, the formulation parameters should be evaluated for their efficacy toward reduction of the residual solvent level. If a solvent is used for preparation of a drug product, then the level of that solvent should also be evaluated.

4.5.1 Classification of Residual Solvents

The International Program on Chemical Safety (IPCS) and the WHO describe the "Tolerable Daily Intake" (TDI), as the exposure limits for toxic chemicals. The acceptable daily intake (ADI) is another term used to signify the exposure limits of toxic chemicals. Another new term, PDE (permitted daily exposure), describes the pharmaceutically acceptable intake of residual solvents. Based on their toxicity profiles, the residual solvents are classified into the classes described in the following sections.

4.5.1.1 Class 1 Solvents: Solvents to Be Avoided

Solvents belonging to this class are carcinogenic and highly hazardous. These solvents should be avoided for pharmaceutical products due to their toxicity and hazardous effects on the environment. However, if, these solvents are required to prepare any product having significant therapeutic effect, then the limit of the residual solvents should be kept within the permissible limit, for example, benzene (2 ppm), carbon tetrachloride (4 ppm), 1,2-dichloroethane (5 ppm), and 1,1-dichloroethane (8 ppm).

4.5.1.2 Class 2 Solvents: Solvents to Be Limited

Class 2 solvents are classified as nongenotoxic carcinogens. Also, they are referred to as teratogenic and genotoxic. For this reason, the use of these solvents should be restricted in the preparation of pharmaceutical formulations. The PDE values should be in the range of 0.1 mg/day. The range of the concentrations should be within 10 ppm. The precision should be determined by method validation.

4.5.1.3 Class 3 Solvents: Solvents With Low Toxic Potential

This category includes those solvents with low toxicity potential to human health and, hence, there is no permissible limit for the use of such solvents in pharmaceutical preparations. The Class 3 solvents, which have higher PDE values (50 mg/day or more), do not produce any health threats toward humans. There may not be scientific studies that evaluate the carcinogenicity after prolonged use. There are available scientific documents that suggest that short-term use of these solvents have less acute toxicity. The concentration of these solvents is acceptable at values less than 5000 ppm. Higher concentrations are also considered in the preparation of pharmaceutical formulations as per the capability of manufacturing of these solvents.

Chromatographic techniques (gas chromatography) are used to determine the residual solvents in a product. Any other procedure as described in pharmacopeias can also be used for this purpose. The pharmaceutical industries are free to select suitable and appropriate validated analytical procedures for the determination of residual solvents in herbal products.

4.6 TOTAL SOLIDS

Total solids are the combination of all solids. The "total solids" can be determined by evaporation of a water sample and drying of the residue (at 100–110°C). The mass of solids remaining to the volume of the water evaporated is measured. Shallow, flat-bottomed, flanged dishes, about 75 mm in diameter and about 25 mm deep, made of nickel or other suitable metals of high heat conductivity, and that are not affected by boiling water, are used (Mukherjee, 2002).

Accurately measure the quantity of the substances and place in a tared dish. Evaporate at as low a temperature as possible until the solvent is removed. Then, heat on a water bath until the residue is apparently dry. Transfer it to an oven and dry at 105°C. Then cool it in a desiccator and measure the weight.

4.6.1 Significance and Use

The results of the chemical analyses of biomass samples are typically reported on a 105°C dry-weight basis. The total solid content of a sample is used to convert the analytical results obtained to an oven dry-weight basis (Sluiter and National Renewable Energy Laboratory (U.S.), 2008). Another study by Gajera et al. (2017) revealed that the total solids of a blended juice of some cucurbitaceous plants varied on storage. The lowest value occurred at the beginning of storage (5.40%, w/w) and went to a maximum at 180 days (5.60%, w/w).

4.7 DETERMINATION OF CRUDE FIBER

It is necessary to assess the food used by humans or animals, specifically a determination of the extent of the ingestion of the food and its nutritional content absorption and maintenance in the body. A quantification of fecal output is a quick method for a digestibility study. Crude fiber determination is mainly performed to quantify the proportion of the food that cannot be digested by humans or animals (Morales et al., 1999). Crude fibers are generally those organic materials present in food or fodder that remain insoluble even when the material is treated with chemicals at a specific temperature and concentration. Because of their nature, they are considered to be adulterants. In order to find such unwanted materials in food and fodder, a crude fiber determination is performed. However, it is convenient to adulterate the drug powder rather than the whole herb or plant. For the purpose of adulteration, cheaper synthetic chemicals with the same nature as those found in natural products are added, for example, methyl salicylate to gaultheria oil and benzaldehyde to bitter almond oil. It is possible to determine the crude fiber content by measuring the cellulose content, lignin, and cork cell in the plant tissue; in this method, the material is defatted and boiled with dilute acid to eliminate the soluble material, washed, dried, and weighed (Mukherjee, 2002).

The crude fiber determination helps in identifying the quantity of adulterants present in any drug sample, food, or fodder. Determination of the crude fiber assists in distinguishing the soluble and insoluble parts, which are acquired from the parts of plants and plant cells. Although it has been observed that the crude fibers, other than ash, do not dissolve in water neither are they digested by boiling with H_2SO_4 and NaOH; thus, they represent the more resistant parts of plant cells, as well as some less resistant cell wall components, such as cellulose and pectin. The basic method of crude fiber determination includes chemicals, such as sulfuric acid and sodium hydroxide, with which the sample should be treated successively. The above-mentioned chemicals should be taken in specific concentrations and boiled. The residue should be separated by filtration on a sintered glass filter, washed, weighed, and ashed within a range of 475–500°C. The loss of weight resulting from ashing corresponds to the crude fiber present in the test sample (Mukherjee, 2002).

The USP XX has prescribed specific methods to determine crude fiber as follows. Take about 2 g of the drug sample, weigh accurately, and extract with ether. Then, add 200 mL of 1.25% sulfuric acid and boil the whole mixture for about 30 min in a 500-mL flask under reflux. Filter the mixture and wash the residue with water until it is acid free. Rinse the residue back to the flask and add 200 mL of boiling 1.25% sodium hydroxide solution and again boil under reflux for 30 min. Filter the liquid quickly and wash the residue with boiling water until neutral. Dry at 110°C to constant weight and incinerate to constant weight. The weight of the crude drug will be represented by the difference between the weight of the residue and weight of the incinerated residue. It will be expressed as a percentage of the original weight of the material. This is further explained in Fig. 4.17. The crude fiber is calculated on a percent-dry basis as described below.

$$\%\text{Crude fiber}\left(\text{dry basis}\right)=\frac{\text{dry residue}\left(\text{g}\right)-\text{ignited residue}\left(\text{g}\right)}{\text{Sample}\left(\text{g}\right)}\times 100$$

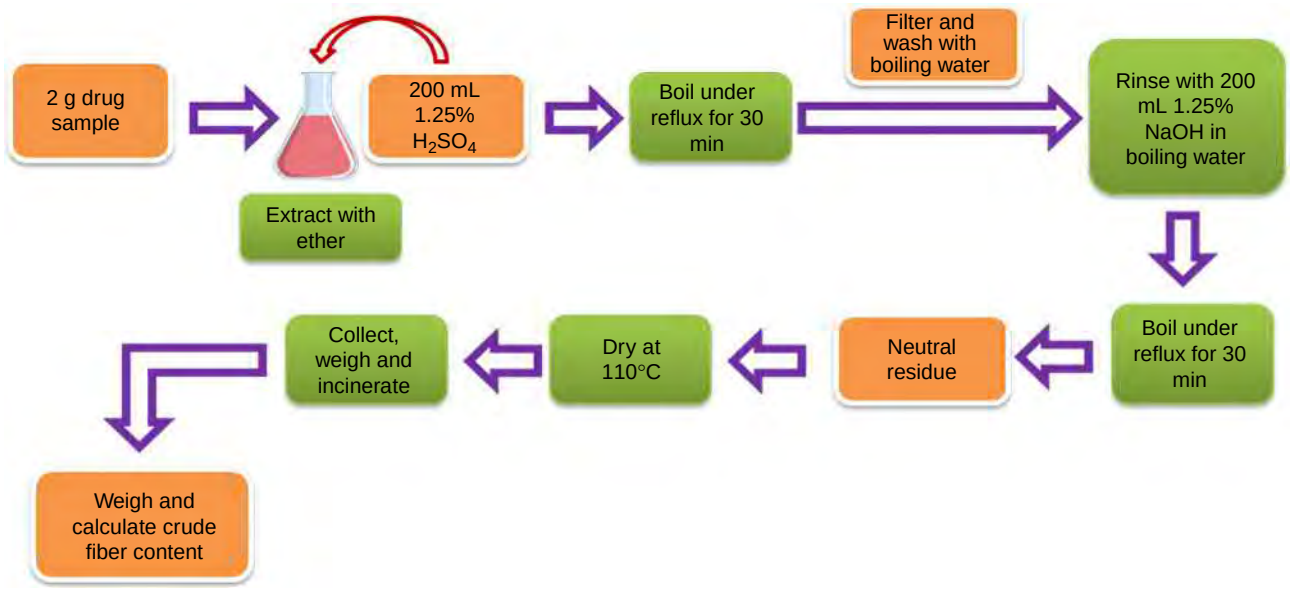

FIG. 4.17 Methodology for crude fiber determination.

4.8 BITTERNESS DETERMINATION

Testing for the determination of the bitterness value should not be carried out until the identity of the plant material has been confirmed.

4.8.1 Procedure for Determination of Bitterness

Botanicals having a strong bitter taste are used therapeutically as appetizing agents. The bitterness results from the stimulation of gastric juice in the gastrointestinal tract. As per WHO, the bitterness of a plant is evaluated by comparing the threshold bitter concentration of the plant material with quinine hydrochloride solution. Bitterness is believed to be responsible for the flow of saliva, gastric juices, and bile and, thus, enhances the function of the digestive system. Bitter extracts or substances are also known to act on the cardiovascular system by decreasing the heart rate and cardiac stroke volume (Olivier and van Wyk, 2013). As per WHO, the content of bitter substances can also be quantified chemically. However, the total bitterness of a plant material should be measured by taste. The bitterness value is presented in terms of unit equivalent of bitterness in a 2000-mL solution containing 1 g of quinine hydrochloride. For extraction of herbal material, potable drinking water is used and also mouthwash is required. The hardness of the potable water used has no significant influence on the bitter taste. For a determination of bitterness, training is required to perform the test (WHO, 1998).

The hardness of water rarely has any significant influence on bitterness. Sensitivity to bitterness varies from person to person, and even for the same person, it may be different at different times (because of fatigue, smoking, or after eating strongly flavored food). Therefore, the same person should taste both the material to be tested and the quinine hydrochloride solution within a short space of time. The bitter sensation is not felt by the whole surface of the tongue, but is limited to the middle section of the upper surface of the tongue. A certain amount of training is required to perform this test. For testing bitterness, a person should have the bitter sensation while testing a solution containing 0.058 mg of quinine hydrochloride in 10 mL of water. Each test procedure should be documented along with the stock solution of the herbal material used for the test. The test should be performed in the series of lower concentration to higher concentration to retain the bitter taste. The test concerning the determination of bitterness value should not be carried out until the identity of the plant material has been confirmed (WHO, 1998).

As described by the WHO (1998), the bitterness value of a specific sample can be analyzed as follows:

Quinine hydrochloride (0.1 g) is dissolved in sufficient potable drinking water to yield a final volume of solution of 100 mL. Further, the solutions (5 mL) are diluted with potable drinking water (500 mL). This solution contains 0.01 mg/mL of quinine hydrochloride. Nine test tubes are used for the serial dilution.

Safe drinking water is used to wash the mouth properly. After that, 10 cc of the most diluted solution is tested and remains in the mouth for 30 s. If the bitter sensation fades after 30 s, then the solution is spit out, followed by a waiting period of 1 min. After proper washing of the mouth, the next highest concentration of solution is tested for 10 min. After the first series of testing, the mouth is properly rinsed with potable drinking water and 10 min is allowed to pass until the next series is tested. The threshold of bitterness is described as the lowest dilution concentration at which the sample provokes a bitter taste after 30 s. It is assumed that if the solution in tube no. 5 (containing 5 mL of ST in 10 mL) gives a bitter sensation, then the threshold of bitter concentration should be tested using the solutions of tubes 1–4. If the solution of tube no. 5 does not give a bitter sensation, then the threshold bitter concentration should be determined by testing the solutions in tubes 6–10.

The bitterness value can be calculated as follows:

$$\text{Bitterness value} = 2000 \times \text{concentration of quinine hydrochloride R}\left(\text{in mg}\right) / \text{concentration of the stock solution}\left(\text{mg / mL}\right) \times \text{volume of stock solution}\left(\text{in mL}\right).$$

The bitterness value is expressed as unit per gram, expressing the mean and standard deviation (SD) values. The bitterness values of several important medicinal plants are presented in Table 4.6.

4.9 SWELLING INDEX

The swelling index is expressed as the volume of the solution (water or any other swelling agent) that is absorbed to swell 1 g of plant sample under specified conditions. The uses of solution are varied for individual plant samples (either whole, cut, or pulverized). The WHO Guideline 1998 has specified the swelling properties of several plant materials having specific therapeutic potential, including those plants containing gum and mucilage, pectin, or hemicelluloses. The dried ripe seeds of *Plantago ovata*, *Plantago psyllium*, *Plantago arenaria*, *Plantago indica*, and others contain mucilage in the

TABLE 4.6 Bitterness Values of Some Plant Species (Olivier and van Wyk, 2013)

Plant Name	Bitterness Value (Mean ± Standard Deviation)
Agathosma betulina	4859 ± 61
Aloe ferox	78,254 ± 34
Arctopus monacanthus	5407 ± 78
Artemisia afra	26,393 ± 30
Balanites maughamii	4211 ± 20
Dicoma anomala	8330 ± 15
Dicoma capensis	14,531 ± 21
Harpagophytum procumbens	4359 ± 94
Hypoxishemero callidea	2615 ± 50
Muraltia heisteria	5594 ± 58
Sutherlandia microphylla	14,123 ± 27
Vernonia oligocephala	10,032 ± 14
Warburgia salutaris	3425 ± 25
Withania somnifera	2456 ± 11
Ziziphus mucronata	6410 ± 13

epidermis of the testa. The seeds of this type of plant may be evaluated by measuring the volume of mucilage produced in 24 h from 1 g of the seeds. This evaluation procedure gives the swelling factor. A glass-stoppered measuring cylinder is used along with the plant material. The total system is then shaken properly for 1 h and kept for some time to measure the volume of the mixture (Mukherjee, 2002).

4.9.1 Determination of Swelling Index

Accurately weighed plant material is placed into a glass-stoppered measuring cylinder (25 mL). Then, 25 mL solution (water or any other swelling agent) is added and mixed by rigorous shaking for 1 h (at intervals of 10 min). The volume of water absorbed by the plant material (including mucilage) is measured. This test is carried out in triplicate and the mean values are calculated with relation to 1 g of plant material (WHO, 1998).

A plant material or drug (1 g) is placed in a ground-glass-stoppered cylinder and the drug is soaked with alcohol (1 mL) and water (25 mL). It is then mixed properly by shaking for 1 h at 10-min intervals and kept for 3 h. If any drug is floating on the surface, then the cylinder is rotated in the vertical axis to settle the plant material. The volume of solution absorbed by the drug (including mucilage) is measured. The swelling index for some plants is shown in Table 4.7 (WHO Guidelines for Herbal Drugs, 2011).

4.10 FOAMING INDEX

4.10.1 Determination of the Foaming Index

Several medicinal plants contain saponin and, therefore, they produce foam during shaking of an aqueous decoction. The saponins are high-molecular-weight-containing phytoconstituents, having detergent activity. Saponins are mostly characterized based on their frothing property. Medicinal plants of different groups, especially those derived from the families Caryophyllaceae, Araliaceae, Sapindaceae, Primulaceae, and Dioscoreaceae, contain saponins. As per WHO (1998), the foaming index is described as the ability to form foam from an aqueous decoction of a plant sample and its extracts. To get a reliable result for the foaming index, it is essential to standardize the experimental conditions. A drug containing saponins

TABLE 4.7 Determination of the Swelling Index of Some Plant Materials

Plant Name	Swelling Index
Agar agar	10
Cetraria glauca	4.5
Trigonella foenum-graecum	6
Fucus sp.	6
Ispaghula husk (*Plantago ovata*)	40
Ispaghula seed (*Plantago psyllium*)	9

has the capability to form froth, which depends upon the nature of the drug and/or the quantity of saponins present. This parameter also provides useful information and helps in the quality control of the drug (Chidambaram and Aruna, 2013).

4.10.1.1 Method 1

The plant materials and their extracts are measured in terms of a foaming index. Accurately weigh 1 g of coarsely powdered drug and transfer to a 500 mL conical flask containing 100 mL of boiling water. Then, cool and filter into a volumetric flask and add sufficient water through the filter to make the volume up to 100 mL (V1). Ten stoppered test tubes should be obtained and marked. Successive portions of 1, 2 mL up to 10 mL of the drug has to be put into separate tubes and the remaining volume adjusted with water up to 10 mL. After closing the tubes with stoppers, shaking is performed for 15 s and kept (15 min) followed by a height measurement of the foam in each tube. If the height of the foam is less than 1 cm, then it is considered that the foaming index of the tested plant sample is 100, which is not significant. However, if the height of the foam is more than 1 cm, then the foaming index of this sample is more than 1000. Details of the method are explained further in Fig. 4.18. The foaming index can be calculated by using this formula:

$$\text{Foaming index} = 1000 / a \text{ in case of V1}$$

$$\text{Foaming index} = 1000 \times 10 / a \text{ in case of V2}$$

where a = volume (mL) of decoction used for preparing the dilution in the tube in which exactly 1 cm or more of foam was observed.

4.10.1.2 Method 2

This method (see Fig. 4.19) is followed for determining the foaming index of saponin-containing medicinal plants, which produce persistent foam. The coarsely powdered herbal material (1 g) is placed in a conical flask (500 mL) with boiling water (10 mL) to boil at moderate temperature for 30 min. It is filtered and transfer to a volumetric flask (100 mL) after

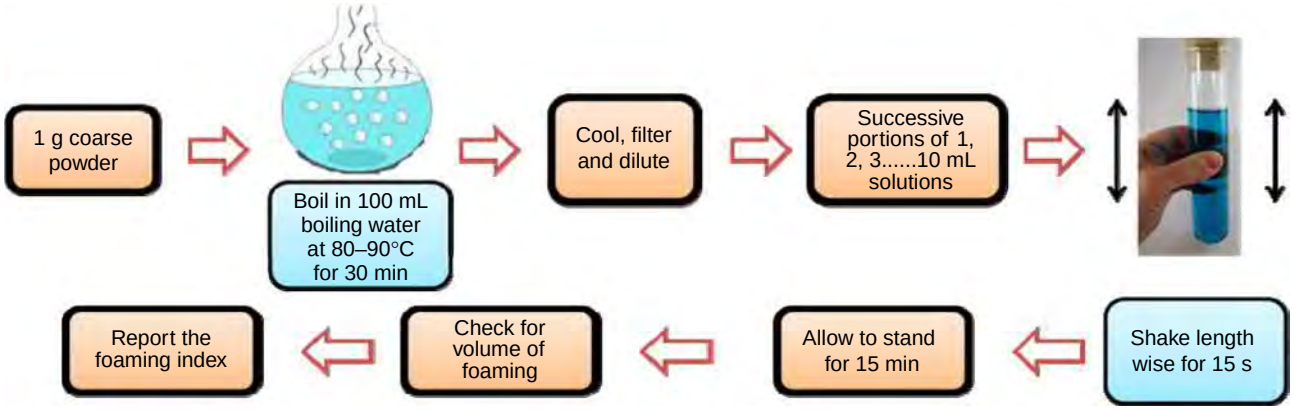

FIG. 4.18 Determination of foaming index (method 1)

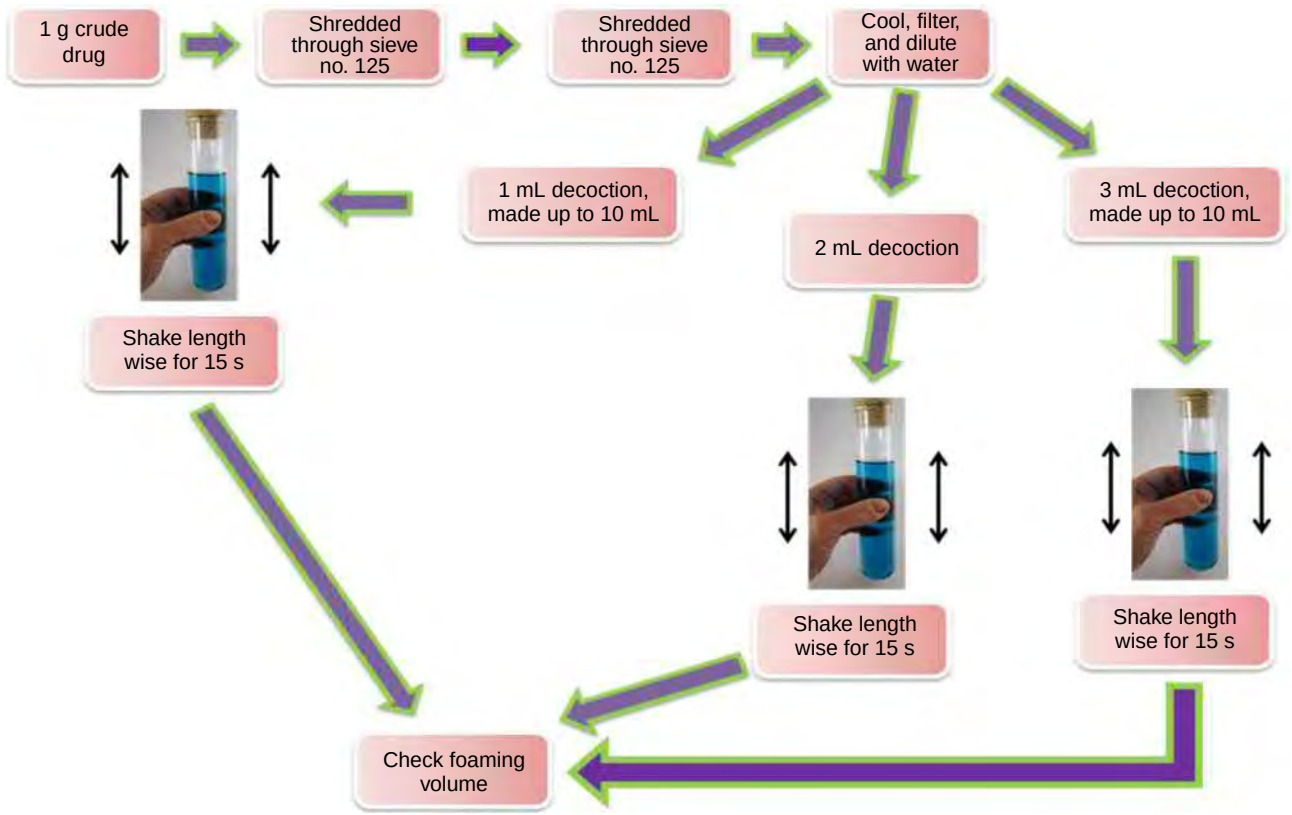

FIG. 4.19 Determination of foaming index (method 2).

cooling while water is added to make up the volume. Then, it is transferred to stoppered test tubes (quantity 10) in serial proportions (such as 1 mL, 2 mL, 3 mL, and so on) to make the final volume up to 10 mL. Lengthwise shaking of the tubes is performed for a time duration of 15 s (2 shakings/s) and allowed to stand for 15 min. The height of the foam is measured and interpreted as follows:

- The foaming index is <100 if the foam height is less than 1 cm in every tube.
- The volume of the herbal material is the foaming index if the height of foam is 1 cm.
- The foaming index is considered >1000 if the foam is 1 cm in height in each tube. Repeats of the samples should be carried out following method 2 (WHO, 1998).

4.10.1.3 Foam Test

The extracts were diluted to 20 mL with distilled water and shaken well in a graduated cylinder for 15 min. Formation of foam in the upper part of the test tube indicated the presence of saponin (Sayeed et al., 2013).

Foaming index

The foaming ability of an aqueous decoction of plant material and its extract is measured in terms of a foaming index. 1 g drug + 100 mL boiled water + heat 30 min + cool and filter into a 100-mL vol. flask + water to make up volume. Now pour this solution into 10 different stoppered test tubes of different volume (WHO, 1998) (Table 4.8).

4.11 HEMOLYTIC ACTIVITY

Many plants contain chemical substances, such as saponins, that have a hemolytic effect on human erythrocytes. It has been reported that erythrocytes obtained from different blood types have different stability. However, the effects of many plants on the red blood cell membrane are positive and even it may show some serious adverse effects, such as hemolytic anemia. However, to avoid unwanted reaction and side effects, many commonly used herbal plants are evaluated for their potential hemolytic activity (Zohra and Fawzia, 2014). Plants having medicinal value contain saponins as a key component,

TABLE 4.8 Foaming Index of Some Medicinal Plants (WHO, 1998)

Plant Name	Foaming Index (U)
Swertia chirata (Gentianaceae)	190
Hemidesmus indicus (Apocynaceae)	220
Trichodesma indicum (Boraginaceae)	100

imparting frothing and detergent properties. Saponins have the ability to produce changes in the erythrocyte membranes when added to a blood suspension, causing hemolysis and resulting in hemoglobin diffusion in the surrounding media (Mukherjee, 2002). Medicinal plants and their bioactive constituents can destroy the membrane of an erythrocyte. It has been observed that hemolytic activity has a relationship directly proportional to the chain length and the presence of branches or cyclic groups makes the surfactant less hemolytic in nature. Therefore, the hemolytic activity of surfactants with shorter chains is equal to the activity shown by CMCs. Surfactants with long hydrocarbon chains have good pharmaceutical applications (Söderlind and Karlsson, 2006).

4.11.1 Determination of Hemolytic Activity

The hemolytic activity of saponin-containing plant materials or drugs is evaluated by comparison with saponin R (reference standard). The hemolytic activity of saponin R is 1000 units/g. To determine the hemolytic activity, an equal volume of plant material (serial dilution) is mixed with a suspension containing erythrocytes. The lowest concentration at which complete hemolysis occurs is considered to be the hemolytic index (HI) of the plant material or drug. A similar test is performed simultaneously using saponin R. This procedure is applied for all medicinal plants containing saponin, but the results may vary (Mukherjee, 2002). The HI of a plant material is described as the 2% (v/v) of a blood sample containing erythrocytes that is hemolyzed by 1 g of saponin or tested plant sample. The HI is also determined by using digitonin (HI = 88,000) as a reference standard (Urbańska et al., 2009).

4.11.1.1 Method 1

The hemolytic activity of a plant should be determined by using human red blood cells (Fig. 4.20). Human erythrocytes from healthy individuals are collected in tubes containing EDTA (anticoagulant). The erythrocytes are harvested by centrifugation for 10 min at 2000× *g* at 20°C. The supernatant is discarded and the pellet is washed thrice with PBS. To the pellet, PBS should be added to yield a 10% (v/v) erythrocytes/PBS suspension. The 10% suspension is diluted in PBS (1:10). From each suspension, 0.1 mL is added in triplicate to 100 mL of a different dilution series of catechu dye

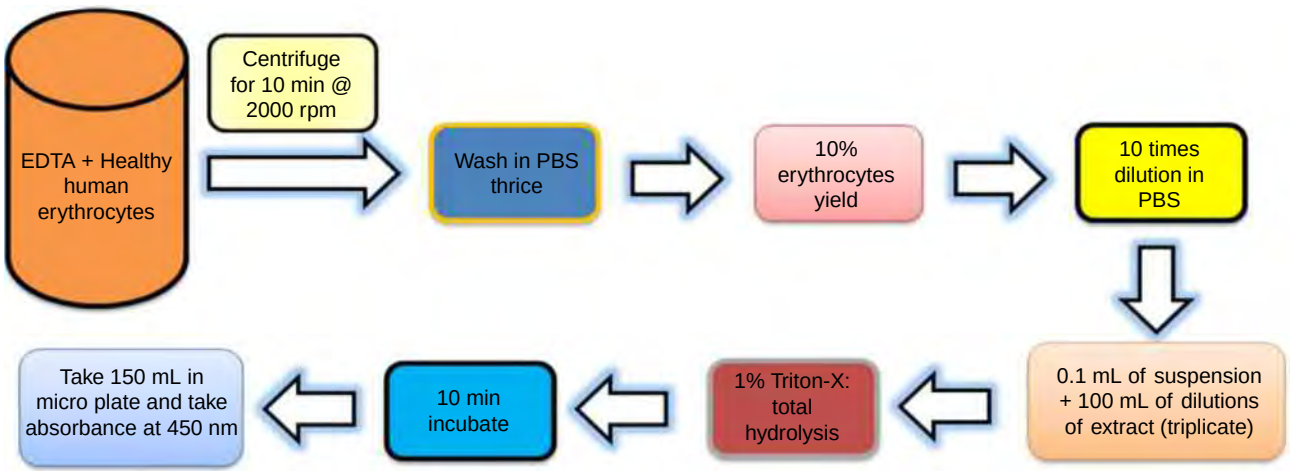

FIG. 4.20 Determination of hemolytic index (Method 1).

(or fluconazole and amphotericin B) in the same buffer in Eppendorf tubes. Total hemolysis should be achieved with 1% Triton X-100. The tubes should be incubated for 1 h at 37°C and then centrifuged for 10 min at 2000 × *g* at 20°C. From the supernatant fluid, 150 mL is transferred to a flat-bottomed microtiter plate (Bio-Rad, iMark, USA), and the absorbance is measured spectrophotometrically at 450 nm. The hemolysis percentage should be calculated by the following equation (Fig. 4.20):

$$\%\text{Hemolysis} = \frac{\text{A450 of test compound treated sample} - \text{A450 of buffer treated sample}}{\text{A450 of 1\%Triton X} - 100 \text{ treated samples} - \text{A450 of buffer treated sample}} \times 100$$

4.11.1.2 Method 2

A 10% solution of rabbit erythrocytes (from Hemostat Laboratories, Dixon, CA, USA) should be diluted in PBS (1:4) and added (100 µL/well) to the wells of a round-bottomed 96-well plate. The test compounds are dissolved in PBS and added to the wells. The contents of the plate should be mixed with a multichannel pipette and the plate is incubated at ambient temperature for 1 h. After incubation, the plate is at 1500 G for 5 min and 40 µL of the supernatant should be added to 160 µL of PBS in a flat-bottomed 96-well plate. The samples should be mixed properly and measured at 405 nm. The results of absorbance should be converted to percentage of hemolysis and compared with fully lysed rabbit red blood cells. The methodology is explained in Fig. 4.21. Also, the hemolytic activities of several plants are presented in Table 4.9.

4.12 TOTAL TANNIN CONTENT

Tannins are a particular class of natural products found in plants of different families. They are polyphenolics in nature. They may be divided grossly into hydrolysable, complex, and condensed tannins. Hydrolysable tannins are hydrolyzed on heating with dilute acid or enzymes, such as tannase, to produce simple molecules, such as gallic acid, ellagic acid, and pyrogallol. They may be classified into gallotannins and ellagitannins. On the other hand, the condensed tannins are not hydrolyzed on heating with acid or enzymes (Khanbabaee and Ree, 2001). They are based on a flavonoid basic skeleton. They are proanthocyanidins in nature. Tannins are known to carry out tanning, in which cross-linking of the collagens in the animal skin takes place. This property has been used in the leather industry. Specifically, this property is elicited in the goldbeater skin test of tannins (Price, 1923).

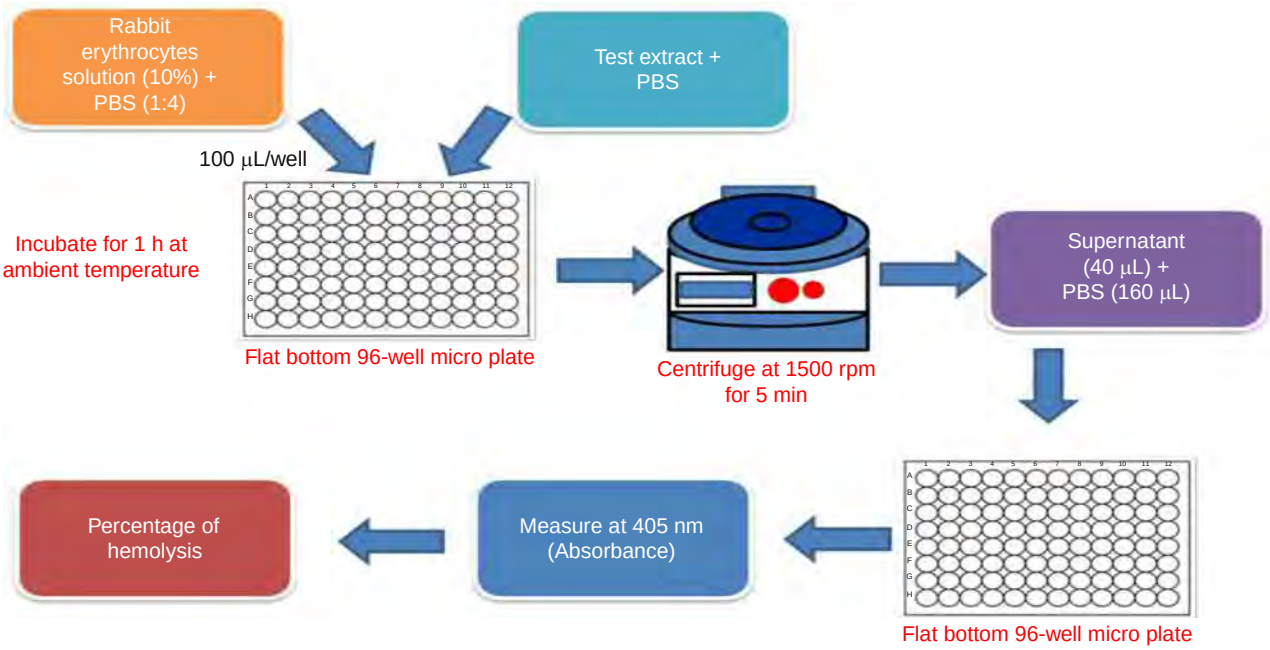

FIG. 4.21 Determination of hemolytic index, method 2.

TABLE 4.9 Hemolytic Activity of Several Plant Species

Plant Name	Hemolytic Activity	Reference
Anagallis arvensis L.	≥15 μg/mL	Soberóna et al. (2017)
Juniperus oxycedrus	2.5%–4.4%	Chaouchea et al. (2015)
Areca catechu	≤20%	Khan Mohd et al. (2011)
Pasteurella multocida	Titer of >1 in 32	Diallo and Frost (2000)
Apostichopus japonicus	Increases 1.18-fold	Liang et al. (2016)
Pandaros acanthifolium	HC50 15.0–30.2 μM	Regalado et al. (2011)
Platycodon grandiflorum A.DC	HI = 1600	Urbańska et al. (2009)
Smilax macrophylla	4.51%–5.32%	Zubair et al. (2017)

4.12.1 Hydrolysable Tannins

These are glycosidic compounds, comprising the C- or O-glycosides of gallic acid. They are formed by several molecules of ellagic acid or gallic acid, united by ester linkage to a central glucose residue. They were formerly known as pyrogallol tannins because, on dry distillation, the gallic acids and similar components are converted into pyrogallol. There may be different polyol residues present in the gallo- and ellagitannins. They are as follows: HHDP (hexahydroxydiphenoyl), meta-digalloyl, metadepsides, flavogallonyl, valoneoyl, anguisorboyl, dehydrohexahydroxydiphenoyl, gallagyl, elaeocarpusoyl, dehydrodigalloyl, metadigalloyl, chebuloyl, trilloyl, dehydrochebuloyl, and brevifolyl.

The hydrolysable tannins are classified into two classes.

4.12.1.1 Gallotannins

Tannins containing galloyl or diverse polyol-, catechin-, or triterpenoid units are considered to be gallotannins. This is the hydrostable and simplest form of a tannin, having a polyphenolic and a polyol residue, though different varieties of polyol residue are observed. Isolated gallotannins from medicinal plants having a polyol residue are derivatives of D-glucose. The polyol residue (hydroxy groups) is substituted with galloyl units. For partial substitution, the remaining hydroxy groups of galloyl residues are substituted with other residues or remain unchanged. For example, glycosidic residue having an anomeric carbon in the center, which is substituted with the α or β form to produce an ester or acetal, sometimes remains unchanged in an α,β mixture. Several plant species contain gallotannins 2,3,4,6-tetra-*O*-galloyl-D-glucopyranose (TGG) and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (β-PGG), which serve as important intermediates for the biosynthesis of hydrolysable plant polyphenols. Sometimes, polyol residues of gallotannins are associated with cinnamoyl or coumaroyl groups.

4.12.1.2 Ellagiotannins

This type of tannin contains at least two galloyl units, which are coupled to each other. Ellagitannins do not contain any catechin unit coupled by glycosidic linkage. Ellagitannins are the largest group of tannins isolated from natural products. Gallotannins serve as the precursor to produce ellagitannins through oxidative coupling of two or more galloyl units, yielding chiral HHDP (axial) unit. This chirality is formed due to the substitution of ortho groups to the biaryl axis and atropisomerism.

HHDP units of ellagitannins are linked through the 4,6- or 2,3-positions of their D-glucosyl unit to form an (*S*)-configured HHDP unit, while (*R*)-configured HHDP units are formed for the 3,6-positions linkage of the D-glucosyl unit. In natural products, HHDP units are linked with the 2,3- or 4,6- or 1,6-positions of D-glucopyranose, which have an (*S*)-configuration, and the 2,4- or 3,6- position of linkage D-glucopyranose, which favors the (R)-configuration.

The chemistry and different substitution products of HHDP are presented in Fig. 4.22.

For each of the C-glycosidic ellagitannins and, therefore, the ellagitannins with a D-gluconic acid unit, the coupling of the HHDP unit via the 2,3- and 4,6-positions of the D-glucosyl is extremely characteristic. The C-glycosidic bond between the aliphatic sugar and, therefore, the rough substituent, is usually formed at C-1 of the sugar. Typical examples of these groups of drugs are Vescalagin, with a C-glycosidic bond, and Lagerstannin C-15, with a D-gluconic acid unit.

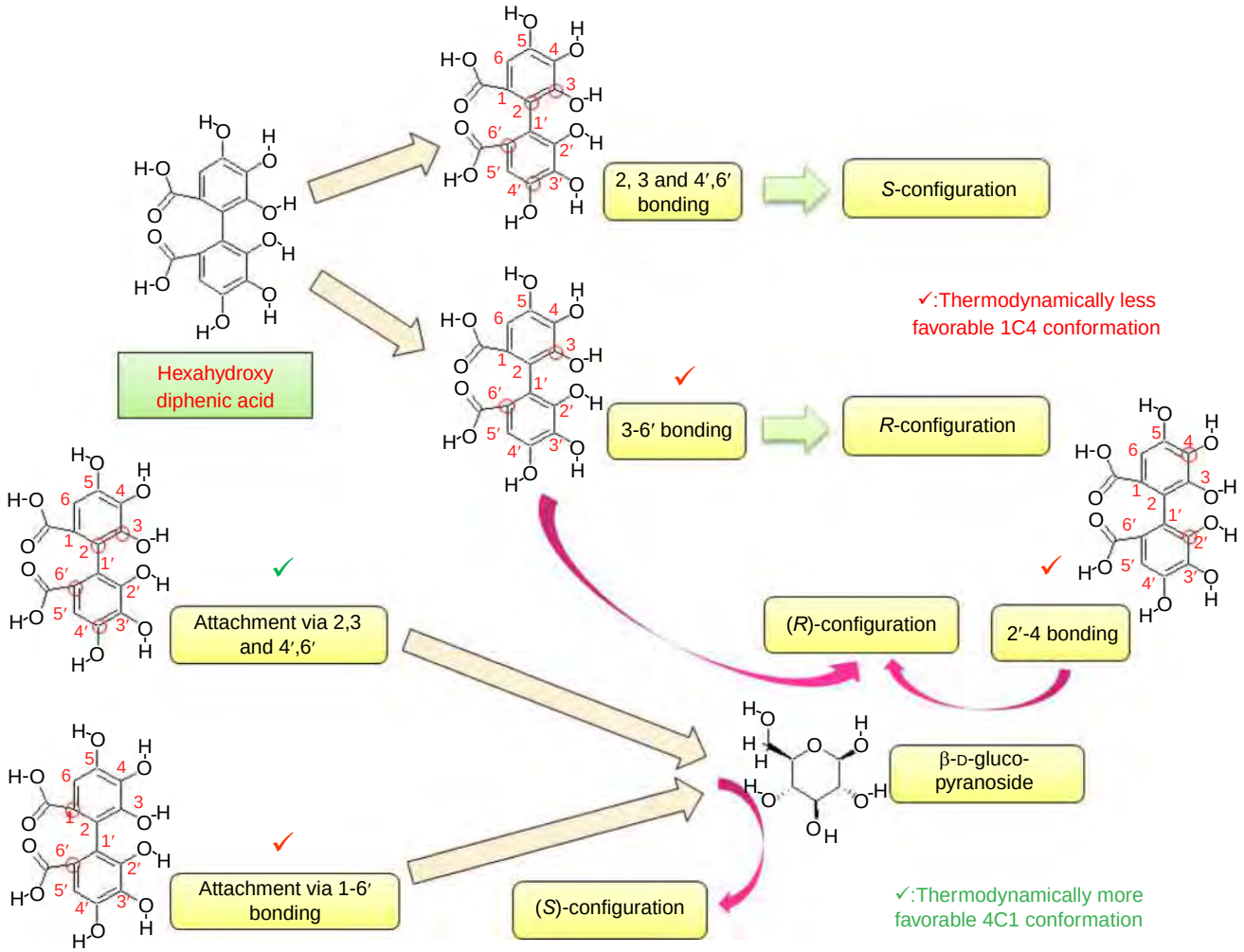

FIG. 4.22 Chemistry and substitution products of HHDP (hexahydroxydiphenoyl).

4.12.2 Complex Tannins

4.12.2.1 Condensed Tannins

Complex tannins consist of a gallotannin or ellagitannin unit along with a catechin unit. Acutissimin A is a complex type of tannin, which contains a flavogallonyl unit linked through a glucosidic linkage at C-1, and three ester bridges (hydrolysable) are linked to the polyol derivative of D-glucose.

These types of tannins are the oligomeric and polymeric proanthocyanidins that consist of catechin units (coupled flavan-3-ol). During biosynthesis of condensed tannins, the condensation of a single building block with polymerization of 2 or 50 blocks occurs. Condensed tannins are biosynthesized from catechins and anthocyanidins (Khanbabaee and Ree, 2001). Procyanidin B2, proanthocyanidin A1, and proanthocyanidin C1 are examples of typically condensed tannins. The chemistry and the different substitution products of condensed tannins are presented in Fig. 4.23.

4.12.3 Pseudotannins

Low-molecular-weight polyphenolics that do not respond to the goldbeater skin test are considered to be pseudotannins. Several examples include:

Gallic acid: Present in rhubarb and most materials containing gallitannin; Catechins—catechu, acacia cutch, Australian kinos, cocoa, and guarana; Chlorogenic acid—Mostly present in unroasted coffee, Nux vomica seed, etc.; Ipecacuanhic acid from Ipecacuanha (Mukherjee, 2002).

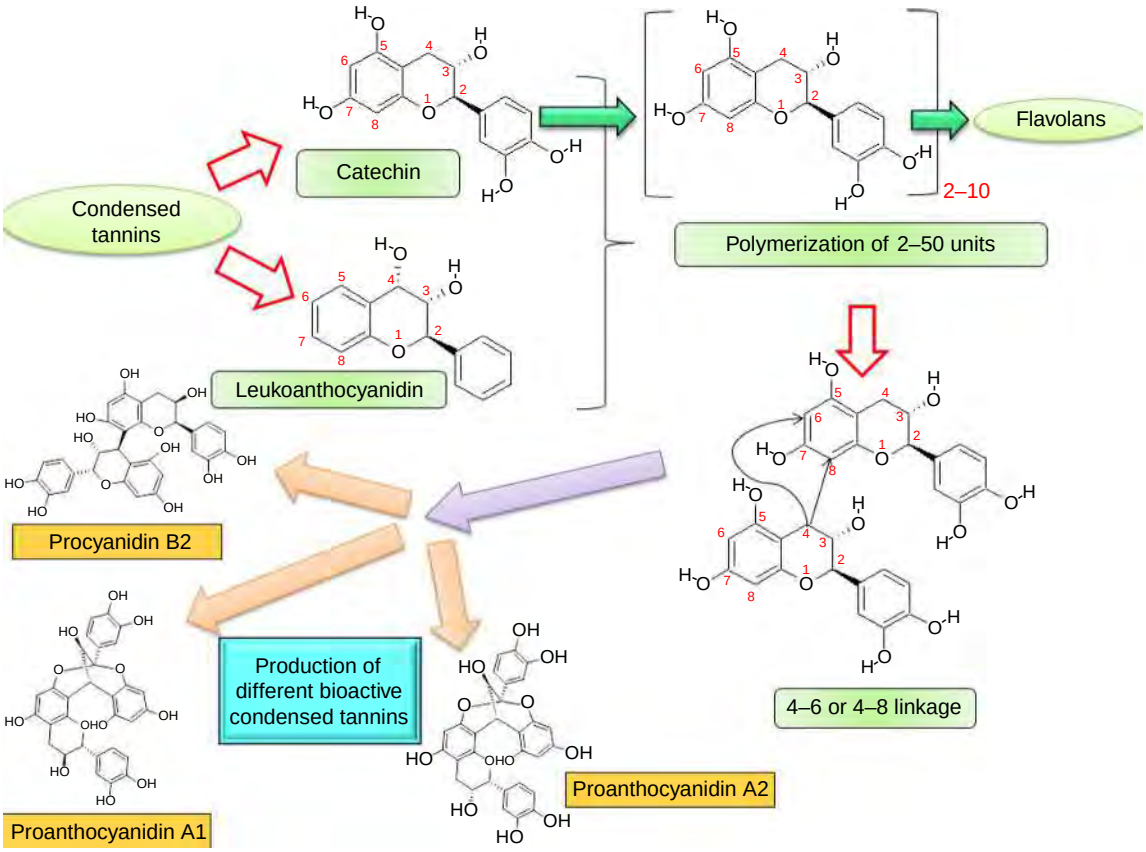

FIG. 4.23 Biosynthetic origin and chemical diversity of condensed tannins.

4.12.4 Determination of the Tannin Content (WHO, 1998)

Estimation of total tannin can be performed in several ways, including solvent-solvent extraction, alkaline ethanol precipitation, and the titrimetric, chromatographic, and spectroscopic methods. In many papers, a determination of tannin content has been performed for various plants, plant extracts, and even for marketed formulations. The basic principle behind this method is that tannins react with proteins, especially the collagens present in hide powder, which results in cross-linking of the collagens. This procedure is called tanning of the hide. In this process, the polyphenolics are first extracted by decoction with water. Then, the concentrated extract is reacted with the hide powder, filtered, and concentrated to get a dry residue. This residue is the dissolved hide powder and the tannins that reacted with the hide powder. Therefore, the amount of the tannins not bound to the hide powder is determined. The solubility of the hide powder is also determined. From there, amount of the tannin present in the dry residue is obtained. Thus, an estimate of the tannin content in the crude drug is measured. The tannin content can be determined by the method depicted in Fig. 4.24.

4.12.4.1 Titrimetric Method

The titrimetric method for the determination of the tannin content in a sample is shown in Fig. 4.25. In this titrimetric method, the $KMnO_4$ is first standardized with Oxalic acid (0.1 N) and then this standardized $KMnO_4$ solution is used as the titrant. The endpoint color is due to the formation of a complex between the phenolic acids and potassium permanganate. One example that may be given is the case of gallic acid. The reaction between gallic acid and potassium permanganate results in the formation of the complex $K_4[Mn(C_4H_4O_6)_2]\cdot 5H_2O$, which is a reddish brown complex. Similarly, the other tannins also form complexes with potassium permanganate. This ultimately gives rise to the formation of a golden-yellow color (Fig. 4.25).

4.12.4.2 Spectrophotometric Method

In an alkaline medium, the total phenolics reduce the mixture of the phosphomolybdate and the phosphotungstic acid that are present in the Folin-Ciocalteu phenol's reagent to molybdenum and tungstic oxides, proportional to the concentrations

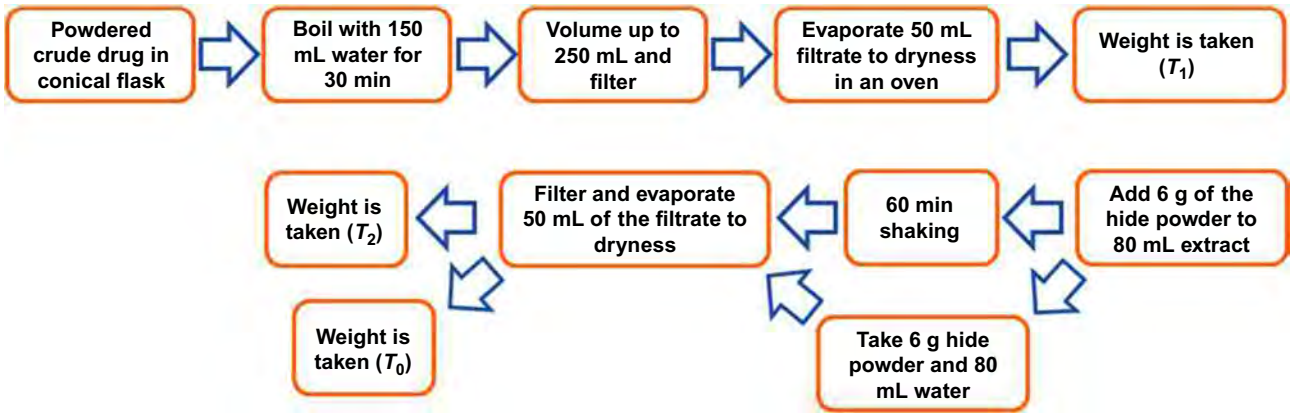

FIG. 4.24 Method for determination of tannin content.

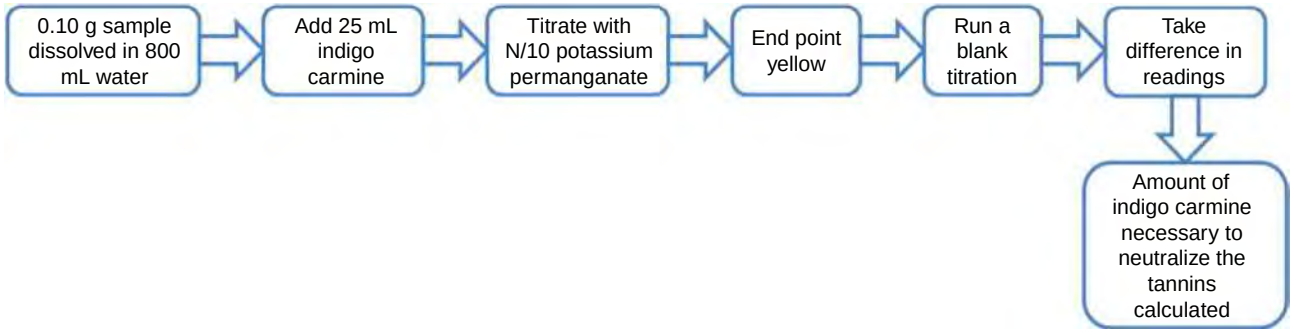

FIG. 4.25 Determination of tannin content by titrimetric method.

of the phenolic compounds that characterize the method. In the total phenol determination, the total amount of the tannin and nontannin phenolic content is determined. After that, the extract is heated with casein, which precipitates the tannins, and the nontannin portion remains in the solution. This is subtracted from the total phenolic to get the amount of the precipitated phenolic, that is, the tannins (Amorim et al., 2008). The method is explained in Fig. 4.26.

4.12.4.3 Alkaline Ethanol Precipitation Method

Gong et al. (2014) reported on tannin estimation by the alkaline ethanol precipitation method. A schematic diagram is given below. Here, the sample is extracted twice with ethanol, followed by addition of sodium hydroxide to separate the supernatants. A schematic diagram is shown in Fig. 4.27.

4.12.5 The Tannin Content of Several Medicinal Plants

The determination of total tannin content, as well as an estimation of individual compounds, is very important from many perspectives. Tannins, as well as various polyphenolics, are quite important from the perspectives of antioxidant, anticancer, antidiabetic, diuretic, and antihyperlipidemic activities. The traditional Ayurvedic formulation, Triphala, is a combination of three tannin-containing plant extracts.

4.13 TOTAL PHENOLIC CONTENTS

Plant extracts are prepared according to a standard protocol. Prepared plant material (10 g) is transferred to dark-colored flasks and mixed with 200 mL of solvents with different polarities (water, methanol, ethyl-acetate, acetone, petroleum ether) and stored at room temperature. After 24 h, infusions should be filtered through Whatman No. 1 filter paper and the residue to be reextracted with an equal volume of solvents. After 48 h, the process is repeated. Combined supernatants

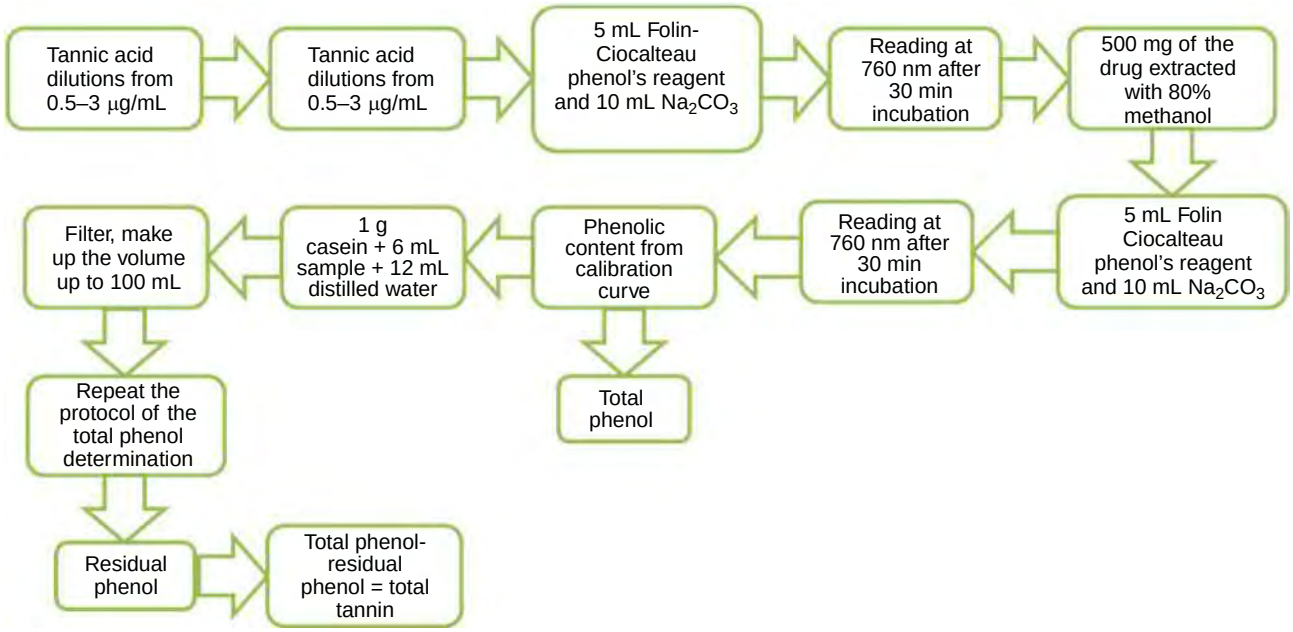

FIG. 4.26 Determination of tannins by the spectrophotometric method.

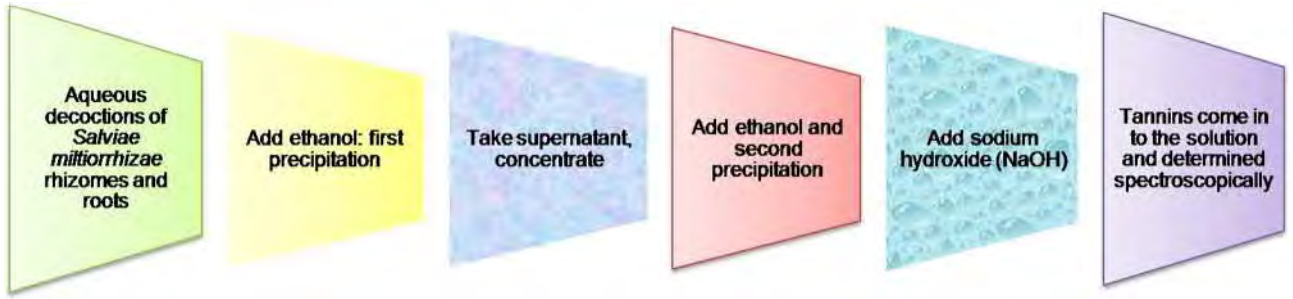

FIG. 4.27 Determination of total tannins by the alkaline ethanol method.

should be evaporated to dryness under vacuum at 40°C using a rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4°C.

The total phenolic contents of the extracts can be determined by the spectrophotometric method. Take a diluted plant extract (1 mL) or gallic acid standard phenolic compound and add into a 25-mL volumetric flask containing 9 mL of distilled water. Take 1 mL of Folin-Ciocalteu's phenol reagent and shake the mixture. Add 10 mL of 7% Na_2CO_3 solution after 5 min and mix well into the test sample. After that, dilute the solution with 25 mL distilled water and mix thoroughly. Keep the mixture in the dark for 90 min at 23°C. Take the absorbance reading at 750 nm. The total phenol content can be determined from an extrapolation of the calibration curve of the gallic acid solution. The total phenolic content is expressed as milligrams of gallic acid equivalents (GAE) per gram of dried sample.

A methanolic solution of the extract at a concentration of 1 mg/mL is used in the analysis. The reaction mixture is prepared by mixing 0.5 mL of methanolic solution of extract, 2.5 mL of 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 mL 7.5% $NaHCO_3$. A blank is concomitantly prepared, containing 0.5 mL methanol, 2.5 mL 10% Folin-Ciocalteu's reagent dissolved in water, and 2.5 mL of 7.5% $NaHCO_3$. The samples are thereafter incubated in a thermostat at 45°C for 45 min. The absorbance is determined using a spectrophotometer at $\lambda_{max} = 765$ nm. The samples are prepared in triplicate for each analysis and the mean value of absorbance are obtained. The same procedure is repeated for the standard solution of gallic acid and a calibration line is construed. Based on the measured absorbance, the concentration of phenolics are read (mg/mL) from the calibration line; then, the content of the phenolics in the extracts is expressed in terms of GAE (mg of GA/g of extract). The detailed methodology for a determination of phenolics is explained in Fig. 4.28.

TABLE 4.10 Tannin Content of Several Plants

Plant Species/Formulation	Tannins	Reference
Bambusa vulgaris	55.0 mg/100 g	Abidemi (2013)
Euphorbia hirta	125 mg/100 g	
Lawsonia inermis	96.7 ± 2.89 mg/100 g	
Mimosa pudica	180 mg/100 g	
Bidens pilosa	108 ± 3 mg/100 g	
Croton zambesicus	150 mg/100 g	
Persea americana	80.0 mg/100 g	
Myracrodruon urundeuva	13.58 ± 0.30% (w/w)	Amorim et al. (2008)
Anadenanthera colubrine	13.46 ± 0.13% (w/w)	
Ocimum campechianum	5.04 ± 0.03% (w/w)	
Spondias tuberosa	4.82 ± 0.04% (w/w)	
Ocimum gratissimum	3.51 ± 0.10% (w/w)	
Maytenus rigida	3.27 ± 0.10% (w/w)	
Hyptis suaveolens	2.71 ± 0.20% (w/w)	
Tabebuia impetiginosa	1.18 ± 0.09% (w/w)	
Plumbago scandens	0.81 ± 0.02% (w/w)	
Tephrosia purpurea	0.52 ± 0.01% (w/w)	
Cereus jamacaru	0.15 ± 0.01% (w/w)	
Tea Fresh leaf	21.4%–23.2% (w/w)	Barua et al. (2007)
Leaf fermented for 2.5 h	14.8%–17.5% (w/w)	
Terminalia chebula	99.55456 mg/g	Mishra et al. (2014)
Terminalia bellerica	9.95568 mg/g	
Terminalia arjuna	54.96288 mg/g	
Saraca indica	57.4869 mg/g	

4.14 TOTAL FLAVONOID CONTENTS

Flavonoids are the most widely distributed natural product in plants, with over 2000 different compounds reported that occur in both the free state and as glycosides. The major general structural categories are flavones, flavanones, flavanols, anthocyanidins, and isolflavones. In addition to glycosylated derivatives, methylated, acylated, prenylated, or sulfated derivatives also occur. A variety of flavonoids have been assigned different roles in nature as antimicrobial compounds, stress metabolites, or signaling molecules (Mukherjee, 2002).

There are several methods that have been developed for the determination of total flavonoid contents in the medicinal plants.

4.14.1 Method 1

The total flavonoid content is determined by the aluminum chloride method by using catechin as a standard. Place 1 mL of test sample and 4 mL of water into a volumetric flask (10 mL volume). Add 0.3 mL of 5% sodium nitrite and 0.3 mL of 10% aluminum chloride after 5 min. Incubate for 6 min at room temperature, then add 2 mL of 1 M sodium hydroxide into the reaction mixture. Make up the final volume to 10 mL with distilled water. Take the absorbance reading of the reaction

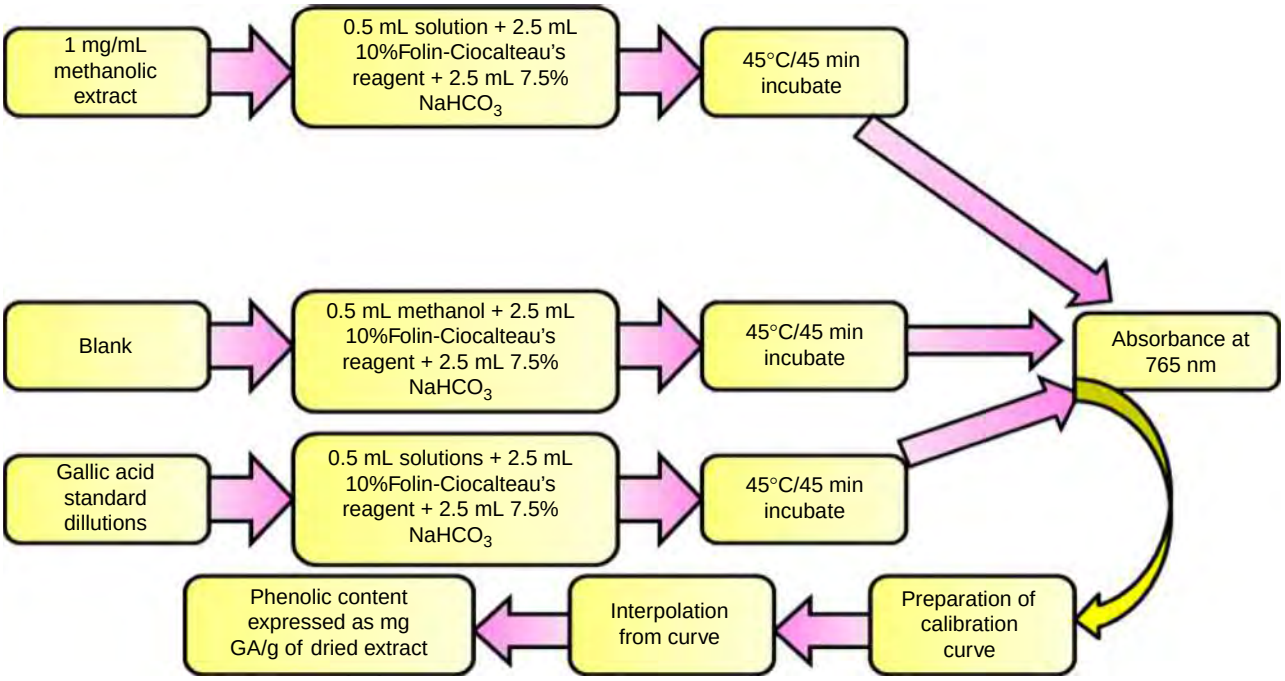

FIG. 4.28 Determination of total phenolic contents.

mixture at 510 nm against a blank spectrophotometrically. Take the absorbance of the reaction mixture at 510 nm by using a UV-visible spectrophotometer. Prepare the calibration curve by using quercetin solutions at concentrations of 2–8 µg/mL in methanol. The total flavonoid content is expressed as milligrams of quercetin equivalents per gram of dried sample (Mukherjee, 2002).

4.14.2 Method 2

Take 2–4 g of the dried crude plant material and extract with petroleum ether in a Soxhlet apparatus. Collect the marc and macerate with 50% methanol under mild heating on a water bath (40–50°C). Evaporate the methanol. The residue obtained is triturated with diethyl ether followed by ethyl acetate. The diethyl ether fraction and the ethyl acetate fraction are both evaporated to dryness to obtain residues (Mukherjee, 2002). Weigh the residue and calculate the total flavonoids content in the plant material by using the following formula:

$$\%\text{Total flavonoids} = 100 \times \text{weight of residue of ether fraction} + \text{weight of residue of ethyl acetate / weight of the sample taken}$$

4.14.3 Method 3

The content of flavonoids in the examined plant extracts is determined using a spectrophotometric method (Quettier-Deleu et al., 2000). The sample contained 1 mL of methanol solution of the extract in a concentration of 1 mg/mL and 1 mL of 2% $AlCl_3$ solution is dissolved in methanol. The samples should be incubated for an hour at room temperature. The absorbance is determined using a spectrophotometer at $\lambda_{max} = 415$ nm. The samples should be prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration line are construed. Based on the measured absorbance, the concentration of flavonoids are read (mg/mL) on the calibration line; then, the content of flavonoids in the extracts is expressed in terms of rutin equivalent (mg of RU/g of extract) (Stanković, 2011). A schematic diagram of the determination of total flavonoid content is shown in Fig. 4.29.

4.15 HEAVY METALS

Medicinal plant materials can be contaminated with arsenic and other heavy metals, which can be attributed to many causes, including environmental pollution and traces of pesticides. As these components are dangerous even in trace amounts, they

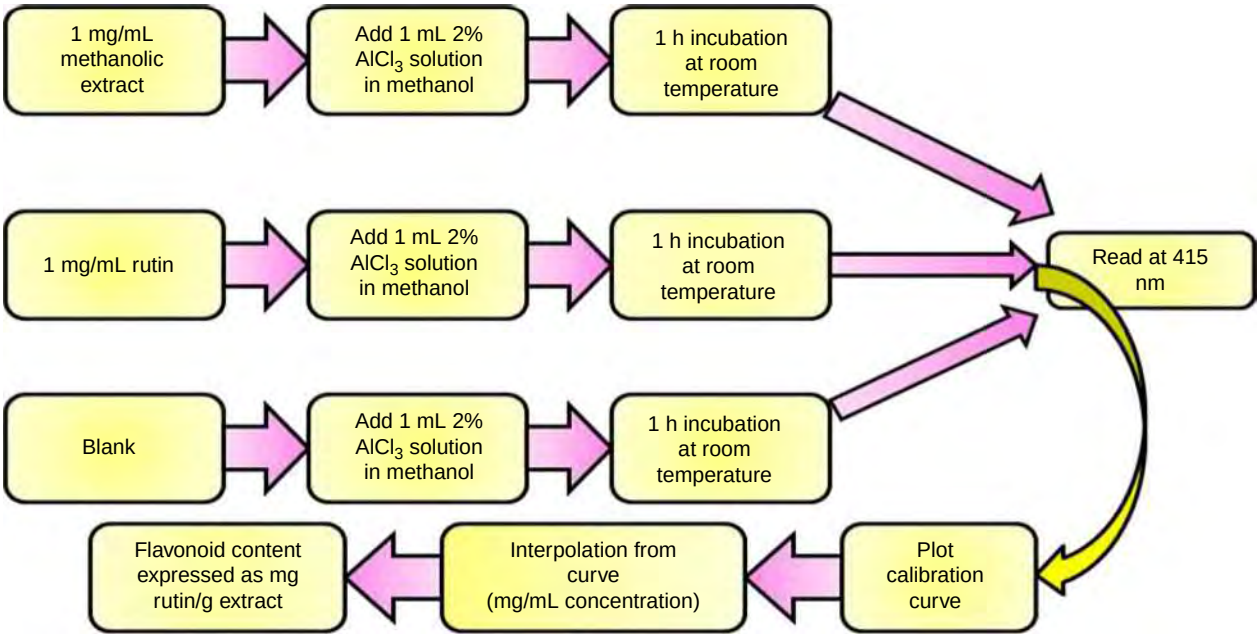

FIG. 4.29 Determination of total flavonoid content.

have to be removed from herbal drugs. Limit tests for these materials have been prescribed in almost all the Pharmacopeia throughout the world. As per WHO, the following procedures have been recommended for their respective limit tests.

4.15.1 Toxic Elements

There are different toxic elements present in the environment. They include the heavy metals, such as arsenic, cadmium, lead, and mercury. All of these are potentially hazardous elements for the human body. They can modulate the enzymatic activities of the CYP450 family of isoenzymes. Some of them are also carcinogenic, such as arsenic. Lead and mercury are fetopathic. For each heavy metal, a Provisional Tolerable Weekly Intake (PTWI) limit has been fixed. Several regulations have already been established worldwide for medicinal plants and related marketed herbal products, such as the U.S. Pharmacopeia (USP), Italian Pharmacopeia (FUI), and European Pharmacopeia (Ph. Eur.). Moreover, there are legal frameworks at national and/or regional levels that are designed to regulate the quality of plant-based products (Sarma et al., 2011). Before 1988, only 14 WHO member states had regulations relating to herbal medicine products, but by 2003 this had increased to 53 member states (37%). Of those without laws/regulations, 49% declared that such regulations were in the process of being developed (WHO, 1998).

The limits for heavy metal content, as specified by WHO, are as follows: mercury 5 (μg/kg b.w.), arsenic 15 (μg/kg b.w.), lead 25 (μg/kg b.w.), and cadmium 7 (μg/kg b.w.). Several countries, including Canada, China, Malaysia, Singapore, and Thailand, have developed their own national guidelines to ensure satisfactory levels of heavy metals in medicinal plants and plant-based products (Table 4.12). WHO (1998) recommends maximum permissible levels in raw materials for Cd and Pb that amount to 0.3 and 10 mg/kg, respectively. Even though certain essential elements can be toxic at high levels; the WHO limits for these metals have not yet been established (Street, 2012; Kosalec et al., 2009).

Though most of the heavy metal contaminants are detrimental to human health, some of the metals are essential for human health, such as copper and zinc. As they do not accumulate gradually in the tissues, for them, another separate parameter called the PTDI has been designed. This signifies the maximum TDI of different metals: copper (Cu) is 0.05 mg/kg b.w. to 0.5 mg/kg b.w. and zinc is (Zn) 0.3 mg/kg b.w. to 1 mg/kg (Kosalec et al., 2009).

The heavy metal content is also subject to environmental and geographic variation. Also, heavy metal contamination varies between plant species. As per the European Pharmacopeia, there are some limits for some medicinal plants known to accumulate heavy metals (Kosalec et al., 2009). The maximum permissible limits of cadmium and lead for Linseed (*Linum usitatissimum* L.) were found to be 0.5 and 90 mg/kg, respectively. The same were found to be 4 and 5 mg/kg for *Fucus vesiculosus* L. Also, the maximum permissible limit for mercury was found to be 0.1 mg/kg in the latter case.

Controlled growth (under GACP) and processing environments (under good manufacturing practice) need to ensure that heavy metal contamination of medicinal plant material is kept to a minimum. For the medicinal plant industry, cultivated plant material is preferred as it is easier to control the supply chain, plus contamination is nominal (Street, 2012). A group of Bulgarian and Turkish authors studied the content of arsenic, cadmium, and lead in commonly used herbal teas and their aquatic infusions. Lead was found to be virtually water insoluble, although its concentration in herbal materials was higher than that of arsenic and cadmium. The extraction efficiency of arsenic (12%–61%) and cadmium (9%–74%) varied by plant species. Fractionation showed that most of the arsenic and lead in herbal infusions was bound to biomacromolecules. The authors believe that these macromolecules are large polyphenolic compounds ubiquitous in the plant kingdom. They have also assumed that arsenic and lead from herbal infusions are virtually not bioavailable because they resorb poorly, and are therefore less toxic (Kosalec et al., 2009). Table 4.11 displays the toxic effects of toxic heavy metals.

Particles in urban areas represent one of the most significant atmospheric pollution problems, and are responsible for decreased visibility and other effects on public health, particularly when their aerodynamic diameters are smaller than 10 µm, because these small particles can penetrate deep into the human respiratory tract. There have been many studies measuring the rainwater concentrations of toxic metals, such as Ag, As, Cd, Cr, Cu, Hg, Ni, and Pb, and their deposition into surface waters and on soil. Natural sources of aerosols include terrestrial dust, marine aerosols, volcanic emissions, and forest fires. Anthropogenic particles, on the other hand, are created by industrial processes, fossil fuel combustion, automobile mufflers, worn engine parts, and corrosion of metallic parts (Mukherjee, 2002). The pathophysiological phenomena caused by heavy metals are presented in Table 4.11.

Numerous abiotic factors influence the availability of metal to plants, including pH, temperature, redox potential, cation exchange capacity, and organic matter. Furthermore, the interactions of soil-plant roots-microbes play vital roles in regulating heavy metal movement from the soil to edible plant parts. The accumulation of metals by both roots and leaves increases with increasing available metal concentration in the external medium. Factors such as reduced biomass, root length, and shoot length are common indicators of heavy metal toxicity. Changes at the cell, tissue, and organ level are either a result of a direct interaction between the metal and structural components at the sites or a consequence of changes in signal transduction and/or metabolism. Plant responses to heavy metals should be investigated for the articular soil-plant environment. The term "hyperaccumulator" describes plant species that have the ability to grow on metalliferous soils and to accumulate extraordinarily high levels of heavy metals (in comparison to the majority of species) without displaying phytotoxic effects. However, hyperaccumulators are habitually confined to metal-enriched soils, such as those soils found on serpentine outcrops and other metalliferous rocks (Street, 2012). The regulation of limits of heavy metals in different types of herbal materials varies greatly in different countries (Table 4.12). Daily permissible limits for lead, arsenic, chromium, mercury have been stipulated by different pharmacopeia and regulatory bodies (see Table 4.13).

4.15.2 Effects of Heavy Metals on Secondary Metabolites

Mineral elements are involved in the structure of some secondary metabolites, yet can also have undesirable effects on their regulation. Nonetheless, a few studies have addressed the effects of heavy metals on the ultramorphological characteristics

TABLE 4.11 Common Toxic Effects of Heavy Metal Contaminants in the Human Body (Kosalec et al., 2009)

Element	Common Industrial Use	Principal Toxic Effects
Arsenic	Pesticides, herbicides	Lung cancer, skin diseases
Cadmium	Batteries, plastics, pigments, plating	Kidney damage, lung cancer, bone disorder
Chromium	Plating, alloys, dyes, tanning	Respiratory effects, allergic dermatitis
Lead	Batteries, wire and cable, alloys	Neurological effects, hematopoietic system damage, reproductive effects
Manganese	Pesticides, ceramics, batteries, steel	Central nervous system effects
Mercury	Chloroalkali industry, pesticides, thermometer, batteries	Neurological effects, kidney damage
Nickel	Coins, jewelry, alloys, plating, batteries	Dermatitis
Thallium	Electronics, batteries	Neurological, heart, lung, kidney, and liver effects

TABLE 4.12 Regulations on the Limits of Heavy Metals in Different Types of Herbal Materials

Name of the Authority	Type of Material	Arsenic (As)	Lead (Pb)	Cadmium (Cd)	Chromium (Cr)	Mercury (Hg)	Copper (Cu)
Canada	HD	5	10	0.3	2	0.2	
	HP (mg/day)	0.01	0.02	0.006	0.02	0.02	
China	HD	2	10	1		0.5	
Malaysia	HP	5	10			0.5	
Republic of Korea	HD						
Singapore	HP	5	20			0.5	150
Thailand	HD, HP	4	10	0.3			
WHO (World Health Organization)	HD		10	0.3			
USP (United States Pharmacopeia)	HE						
(FUI) Italian Pharmacopeia	HD		3	0.5		0.3	
Ph. Eur. Draft Monograph Herbal Drugs (54)	HD		5	0.5		0.1	
Regulation (EC)	FS		3	1 (3 for seaweed products)		0.1	

HD, crude herbal drugs; HP, finished herbal products; HE, herbal extracts; FS, food supplements.

TABLE 4.13 Daily Permissible Limits of Heavy Metals in Different Pharmacopeias

Pharmacopeia	Lead	Arsenic	Cadmium	Mercury
European Pharmacopeia	Not more than 5 ppm	Not more than 2 ppm	Not more than 0.3 ppm	Not more than 0.1
United States Pharmacopeia	Total not more than 20 ppm			
Indian Pharmacopeia	Total not more than 20 ppm			
World Health Organization	Not more than 10 ppm	Not more than 3 ppm	Not more than 0.3 ppm	
Govt. of India for ASU Herbal Products	10 ppm	03 ppm	0.3 ppm	1 ppm

and the therapeutically active constituents in medicinal plant parts. Plants exposed to heavy metal stress show varying degrees of secondary metabolite response. Chromium (Cr) stress induced the production of eugenol, a major component of the essential oil of *Ocimum tenuiflorum* (Lamiaceae) (15%, 25%, 17%, and 4% more eugenol from 10, 20, 50, and 100 µM Cr exposed plants, respectively). Similarly, the therapeutically active compounds Phyllanthin and Hypophyllanthin were enhanced at certain levels of Cd stress in *Phyllanthus amarus* Schum and Thonn (Phyllanthaceae). On the contrary, heavy metal pollution of soil and air at a distance of 400 m from the source of pollution suppressed the growth of *Mentha piperita* L. (Lamiaceae) and *Mentha arvensis* var. piperascens Malinv. with the yield of essential oil up to 14% compared

with the control. This, however, did not negatively affect the essential oil content and its quality. It is debatable whether screening medicinal plants by means of in vitro assays is the most effective approach to validation; though, in Africa, the active compound(s) of so few medicinal plants have been identified that simply screening medicinal plant extracts using biological assays is the norm. Nevertheless, reporting on the biological activity of crude plant extracts without the isolation and identification of an active compound raises concern, as the activity may be due to the presence of toxic substances (Mukherjee, 2002). A recent study investigated Cd accumulation and its effect on COX-1 and COX-2 antiinflammatory activity in *Eucomis autumnalis* (Hyacinthaceae) and *Eucomis humilis*. When treated with Cd, 2 mg/L, *E. humilis* bulbous extracts exhibited lower inhibitory activity than the control for both COX-1 and COX-2 while *E. autumnalis* bulbous extracts had greater COX-1 activity compared with the control with suppressed COX-2 activity. The study cautioned researchers to be cognizant of the consequence of environmental contaminants when reporting on the biological activity of crude plant extracts. It is clear that heavy metal-induced stimulation of medicinal plants is strongly influenced by several factors, including plant growth stage, concentration and duration of treatment, and composition of growth medium. As a result, optimizing nutrient supply is a key factor in the quality of medicinal plants. It has been suggested that certain medicinal plants be grown in polluted soils for higher secondary metabolite yield. This will, however, depend on the plant part used as consumer safety needs to be first and foremost.

Heavy metal contamination in natural medicines and marketed natural products is of utmost importance from the point of view of a pharmacist. Numerous studies have been conducted worldwide to determine heavy metal levels in medicinal plants and plant-based products. Both developed and developing countries have shown high levels of potentially toxic heavy metals in products available to the public. Such products are not only from local sources but are often imported. A study examining heavy metal content in traditional Asian herbal remedies purchased in the United States, Vietnam, and China revealed that the majority of products had detectable levels of heavy metals, with nearly 74% containing amounts greater than current recommended public health guidelines. An effective solution to the importation of traditional medicines containing heavy metals presents a great challenge. These products are expected to be imported in small quantities by numerous different routes, including via the postal service and with intercontinental travelers. Formal labeling and packaging may be deceptive as it gives the public a false sense of product safety. In Africa, formalization and registration of herbal products are not the norm and preparations often lack appropriate labeling, such as contents, contraindications, place and date of manufacture, and expiry date. Medicinal plant collection is often from the wild and locations are habitually undisclosed. Medicinal plants have been shown to be both a rich source of essential metal ions and a potentially dangerous source of nonessential metals. Poisonings from traditional medicine products containing heavy metals is well documented. The toxic effects of heavy metals are due to their hindrance of the regular body biochemistry in normal metabolic processes. Arsenic, chromium, and magnesium are the heavy metals most frequently implicated in morbidity and death in South Africa. An investigation of the Johannesburg forensic record over a period of 5 years identified 206 cases in which a traditional remedy was either declared to be the grounds for death or was found to be present in a case of poisoning with an unidentified substance. Heavy metals were responsible for 10% of these poisonings. A study on heavy metal concentrations in plants, plant-based remedies, and urine from patients treated with traditional remedies concluded that out of the 12 concoctions investigated, copper (Cu) levels were extremely high in 4 of the concoctions. A large number of patients (34%) showed elevated zinc (Zn) concentrations. In one of the patients, the Zn concentration was 10 times the upper limit of the reference range. After a week of vomiting with hepatomegaly, and dehydration, the patient died of hepatic failure. A further report revealed that a seven-month-old infant was hospitalized after the intake of a traditional medicine, which resulted in a severe case of multiple-metal poisoning. It is known that numerous traditional medicines give rise to severe renal pathology, the mechanism of which is uncertain but which could be associated with heavy metal toxicity. It has been suggested that potassium dichromate ($K_2Cr_2O_7$) toxicity should be suspected in cases of unexplained renal failure. Several cases of dichromate poisoning has been reported after the use of purgative solutions obtained from traditional healers. One patient who ingested dichromate died from massive gastro-intestinal hemorrhage. Six patients took dichromate solutions as rectal enemas, two were left with impaired renal function, and one patient required a permanent colostomy caused by extensive peri-anal necrosis. Similar reports have indicated that Cr(VI)-containing traditional remedies have been the cause of poisonings and morbidity in young children. In Nigeria, a study reported high levels of heavy metal in blood from unknown sources and African traditional medicines cannot be ruled out. Unfortunately, African traditional medicine products do not customarily contain details, such as place of production/manufacture; thus, there is no accountability for adverse reactions. Strict regulations with regard to classification and labeling may prevent further poisonings. Moreover, certain substances should not be available to the general public for self-administration, such as $K_2Cr_2O_7$. Regrettably, adverse reactions and poisoning profiles of traditional medicines are not well documented and substances are often inadequately categorized. For example, potassium permanganate, which could be classified as a household chemical or antiseptic, is also used in South African traditional medicine; however, this substance may be incorrectly profiled with regard to causative agents in acute and chronic poisonings (Street, 2012).

4.15.3 Atomic Absorption Spectrometry (AAS)

AAS is used to determine the content of heavy metals present in a sample, including environmental samples, industrial wastes, and biological samples, through measuring the absorption and emission of ultraviolet or visible light by the toxic elements. This analytical method is used for the determination of toxic elements qualitatively and quantitatively. In this method, the energy absorbed by the sample in terms of photons is measured. An AAS detector measures the emitted light by the sample in comparison with the absorbed light. Then, a signal processor integrates the changes and records the spectral lines. The concentration calculation is achieved based on the principle of the Beer-Lambert law. The quantification of heavy metals is done using a calibration curve, which is prepared by different known concentrations of individual elements.

4.15.3.1 Sample Preparation

Organic and inorganic samples can be analyzed by AAS. Soil, sediment, clay, and metal are considered as inorganic samples, while organic samples include biologicals, pharmaceuticals, and petrochemicals. Organic samples can be prepared by solubilizing the sample using dry or wet methods in a closed/open system. Decomposition methods are employed by dry ashing (burning at 450–550°C). In this method, the samples can easily dissolve in a small quantity of acid and can detect a small quantity of elements. In an open system, wet digestion is carried out by acid or a mixture of acids followed by heating. This method can also be achieved in a microwave system. This method reduces the chance of contamination and also has greater solubilizing ability. For high organic content, microwaves are used to solubilize the sample (Pouzar et al., 2001; Wasilewsk et al., 2002). For inorganic samples, a fusion method can be applied to prepare the sample with high-temperature fluxes (300–1000°C). The solubility of product (ash) is done by an acid or a mixture of acids (Oliveira, 2003).

4.15.4 Instrumentation

Two atomizers, including a flame burner and graphite furnace, are used in an atomic absorption spectrometry instrument. The flame burner uses acetylene and air fuels and the graphite furnace is used for the analysis of trace metal. The basic configuration of the instrument is the same, while different manufacturers change the configuration in terms of analytical demand and advancement of technologies. The source of radiation continuously emits light from the visible to the infrared range. Emitted light is differentiated by the modulator. The atomizer helps to remove the analytes and generates atomic vapor in the ground state, which further absorbs the emitted light.

Selection of photons is done by the monochromator and transferred to the detector. The transformation of photon energy into signals and subsequent amplification is achieved by the detector. Then, the intensity of signal is used in data acquisition and processing.

4.15.5 Techniques Based on Atomic Absorption Spectrometry

Several common fondants, including lithium metaborate, sodium carbonate, and potassium nitrate, are used to solubilize the samples with the application of a high-temperature reaction. Calibration curves, the method of standard additions, and the internal standard method are used to detect heavy metals. The calibration curve method is considered to be the most widely used method. A flowchart of atomic absorption chemical analysis is shown in Fig. 4.30.

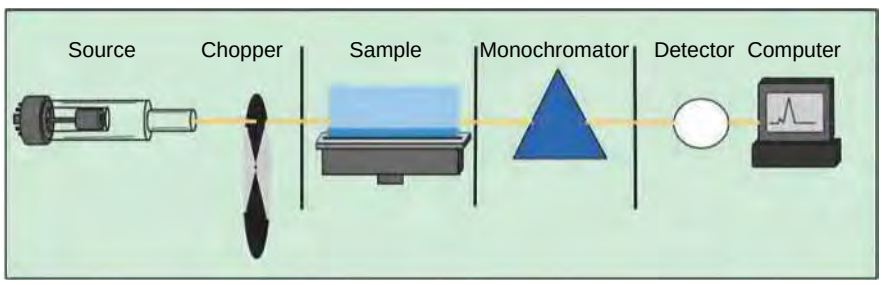

FIG. 4.30 Main components of an atomic absorption spectrometer.

4.15.5.1 Calibration Curve Method

Different concentrations of standard solution (minimum of three different concentrations) are prepared. The absorbances are recorded and a calibration curve is plotted from the values obtained. After that, the absorbance of the test sample is measured within the concentration range to determine the concentration of the heavy metal.

4.15.5.2 Standard Addition Method

In this method, standard and equal volumes of different test solutions (two/more) are added. The absorbance for each solution is measured and plotted against the concentration of a standard heavy metal. The concentration of the added standard element is plotted on the abscissa and the measured absorbance on the ordinate. The calibration curve is extended by joining the plotted points. The concentration of the element is determined from the calibration curve. In this method, the calibration curve should pass through the origin.

4.15.5.3 Internal Standard Method

Several solutions are prepared with constant amounts of the standard element. The absorbance of the standard element and the internal standard element is measured. The result is expressed as a ratio of the absorbance of each of the samples and the internal standard element. A calibration curve is prepared by plotting the concentrations of the standard element (abscissa) versus the ratios of absorbance (ordinate). Then, measure the absorbance of the prepared test solutions by adding the internal standard element (same amount). Prepare a calibration curve by obtaining the ratio of the absorbance. The determination of element concentration is achieved by the calibration curve.

4.15.6 Limit Test for Arsenic

The content of arsenic present in a medicinal plant is quantified by comparing the color depth with a standard stain. This can be performed by the process outlined below.

4.15.6.1 Preparation of the Sample by Acid Digestion

Coarsely ground plant material (35–70 g) is placed in a Kjeldahl flask (capacity 800–1000 mL). Water (10–25 mL) is added along with nitric acid (50 mL) (~1000 g/L), followed by 20 mL of sulfuric acid (~1760 g/L), drop by drop, until all the organic matter is destroyed. Continuous heating is required to get a clear solution having copious vapors of sulfur trioxide. After cooling, water (75 mL) and ammonium oxalate (25 mL) (25 g/L) are added. To develop sulfur trioxide vapors, heating is employed. After cooling, place it in a volumetric flask (250 mL), and make up the volume with water.

4.15.6.2 Apparatus and Methodology

The apparatus consists of a bottle of about 120 mL with a rubber stopper. Ordinary glass tubes (20 cm × 5 mm) are inserted through the rubber stopper. The second glass tube is held in contact with the first glass tube, so that the openings of the two tubes meet together and form a tube. An amount of 50–60 mg of lead acetate cotton is inserted in the tube and also a disc of mercuric chloride paper is placed between the flat surfaces to perform the experiment. The methodology of this experiment is described in Fig. 4.31.

4.15.6.3 Preparation of Standard Stain

The standard stain is prepared by adding hydrochloric acid (10 mL) and diluted arsenic (1 mL). The dilution of arsenic is carried out by adding arsenic solution (1 mL) to water (50 cc). Stannated acid (~250 g/L) (As) TS and 1 mL of dilute arsenic (As) TS to 50 cc of water. The final solution yields a stain on mercuric bromide paper, referred to as the standard stain (Mukherjee, 2002).

4.15.6.4 Limit Test for Cadmium and Lead

The limit test to measure cadmium and lead requires a consistent and sensitive method, which allows comparison with the reference standard. The procedure given by WHO is outlined below.

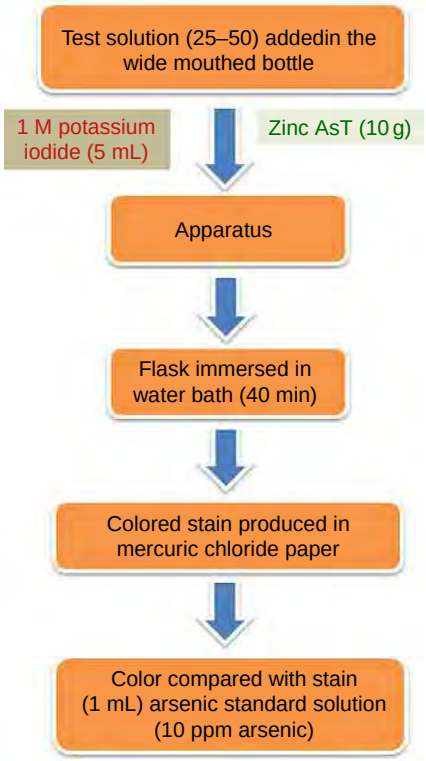

FIG. 4.31 Methodology for the limit test of arsenic.

4.15.6.4.1 Apparatus

A digestion vessel, along with a vitreous silica crucible (DIN 12904) (capacity 75 mL) with a cover of vitreous silica, are used for preparation of the apparatus.

- The digestion mixture is prepared by two parts of nitric acid (~1000 g/L) TS and one part of perchloric acid (~1170 g/L).
- *Olea europaea* leaves are used as reference materials.

The digestion vessels and other equipment are cleaned carefully with nitric acid followed by washing with water (several times) and drying (120°C).

4.15.6.5 Preparation of the Sample

The wet digestion method is carried out in an open system using dried plant extract (200–250 mg) of air-dried plant material. The digestion mixture (1 mL) is added and the crucible is then covered. The complete system is then placed in an oven with a controlled temperature. The system is heated at 100°C for 3 h, after which the heating temperature is increased to 120°C for 2 h. The temperature is then slowly raised to 240°C for 4 h. The resulting dry inorganic residues are then dissolved in nitric acid (2.5 mL) and analyzed in comparison with a blank to determine the heavy metals.

4.15.6.5.1 Method

The inverse voltammetric method or atomic absorption spectrophotometry are applied to determine the contents of lead and cadmium in a sample. The maximum content of heavy metals in a dried plant material is based on the ADI value (lead, 10 mg/kg; cadmium, 0.3 mg/kg). Lead is also determined by the method described in Fig. 4.32.

4.15.7 Limit Test for Mercury

4.15.7.1 Materials and Methods

Use analytical grade nitric acid (HNO_3) and 70% perchloric acid ($HCLO_4$) supplied from Fischer Scientific as reagents for wet digestion of samples. Prepare solutions using deionized water. Thoroughly wash all the glassware and rinse before use.

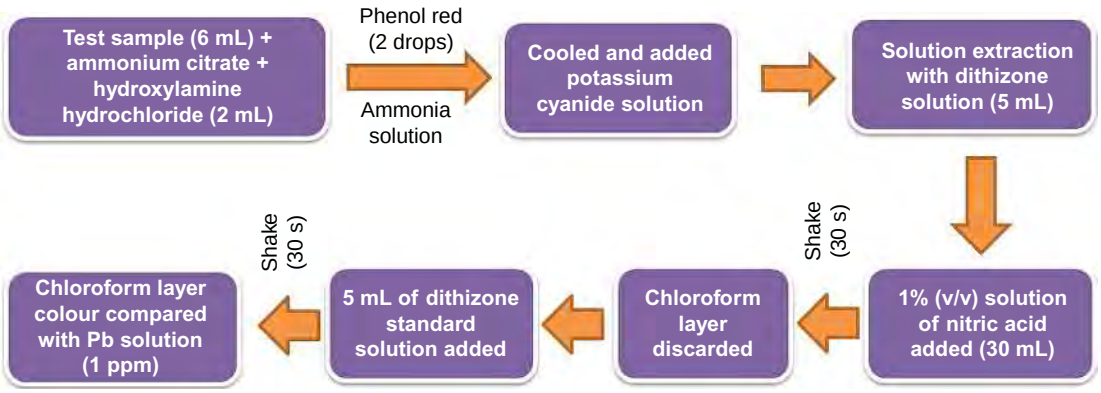

FIG. 4.32 Methodology for determination of lead.

4.15.7.2 Apparatus

Determination of heavy metals in all the medicinal plants should be performed by using atomic absorption spectroscopy. Standard operating parameters are set as required. The hollow cathode lamps for Cu, Cr, Cd, Co, Ni, Pb, Zn, and Fe are used as radiation sources and air acetylene is used as fuel. All of the samples and the standard are run in duplicate.

4.15.7.3 Standard Preparation

Prepare the standard solutions for all the heavy metals under study in three to five different concentrations to obtain a calibration curve by diluting a stock standard solution of concentration 1000 ppm.

4.15.7.4 Analysis Method

First, digest the samples under study using the wet digestion method. Place approximately 0.2 g of the sample in a 100-mL volumetric flask, add about 4 mL of HNO3, and allow to stand for a few hours Heat carefully over a water bath until the red fumes coming from the flask cease completely. Allow the flask to cool at room temperature, then add about 4 mL of perchloric acid and heat again over the water bath to evaporate until a small portion remains. Filter through Whatman filter paper no. 42 and make up the volume using distilled water up to 100 mL. As per the National Sanitation Foundation draft proposal, the maximum permissible limit for mercury in medicinal plants is 0.02 mg/day (Akram et al., 2015).

4.16 PESTICIDES

Pesticides are used on plants and herbs for protection from insects and pests, which can damage the plants. Although they help in getting rid of pests, the accumulation or presence of pesticides are harmful on plants used for food and medicines. Thus, pesticides can be considered a curse in the disguise of a blessing. Pesticides are mixtures of different substances that help in preventing and controlling any unwanted pests or plants that interfere with or destroy the production, processing, storage, transport, or marketing of vegetable and herbal drugs. Pesticides are biocides used to reduce or repel insects, weeds, rodents, fungi, or other organisms that threaten public health. Pesticides are toxic by design, but have numerous beneficial effects, including crop protection, preservation of food and materials, and prevention of vector-borne diseases. The mode of action of a pesticide is by targeting systems or enzymes in the pests, which may be very similar to systems or enzymes in human beings (National Resource Council, 1993).

In 1940, synthetic pesticides were used for the first time. Over time, consumption has increased substantially. Initially, most pesticides were used in agriculture, but in 1999 about 74% of households in the United States were reported to use at least one pesticide. Developing countries use only 25% of the pesticides produced worldwide, but they experience 99% of the deaths. This is because the use of pesticides tends to be more intense and unsafe, and regulatory, health, and education systems are weaker in developing countries (Jeyaratnam, 1990).

4.16.1 Pest Control and Pesticide

The term pest control means to destroy or ward off those plant or animal organisms that in any way, directly or indirectly, threaten human health or nutrition. This can be done with biological, physical, or chemical agents. Detrimental effects on

the biological equilibrium should be avoided as far as possible by enforcing an integrated method of pest control in which biological pest control is coupled with physical and chemical methods. Medicinal plant materials are liable to contain pesticide residues, which accumulate from agricultural practices, such as spraying, treatment of soils during cultivation, and administration of fumigants during storage. It is therefore recommended that every country producing medicinal plant materials (naturally grown or cultivated) should have at least one control laboratory capable of performing a determination of pesticides in accordance with the procedure outlined below. Biological pest control uses the natural enemies of the pests to destroy them or promotes the colonization of the cultivation area by these enemies. Biological methods are also used for the protection of stored products. The simplest and certainly the oldest example is to keep cats in granaries and mills. "Artificial" biological methods are sometimes used; one example is the use of a preparation of *Bacillus thuringiensis*, which selectively attacks about 100 species of caterpillars but not useful insects. The physical methods include mechanical weeding and the application of grease bands to fruit trees. Biological and physical methods are, however, far exceeded by chemical pest control. Only with their aid success was obtained in controlling pests so effectively that crop yields have increased considerably. Pest control is used for the protection of growing plants, plant products in warehouses, materials, timber, wood, and also in general hygiene. The protection of medicinal plants includes all branches of agriculture, such as the cultivation of arable crops, fruit, wine, medicinal plants, hops, vegetable and ornamental plants, as well as grasslands, greenhouse crops and, not least, forest. Plant protection also includes the treatment of seeds and soil. The objective of warehouse protection is to deter and destroy pests, such as insects, mites, and rodents, in harvested products (Mukherjee, 2002).

4.16.2 Chemical Pest Control

Pesticides can be classified according to their chemical composition, function, and mode of action in organisms. Chemically, the compounds can be divided into three groups; biological (vegetable, bacterial, or fungal), inorganic, and organic pesticides.

The organochlorine, organophosphorus, carbamate, and triazine compounds mainly constitute the last group, which is the largest and has pronounced physiological activity. Chemical pesticides have, of course, been the cause of certain unpleasant side effects, such as the occasional disturbance of the biological equilibrium (particularly after incorrect use of the chemical agent). The occurrence of resistance to these pesticides in the case of pests that rapidly pass from one generation to the next and the persistence of some of the pesticides or their metabolites in plant and other harvested material is a growing cause for concern.

Chemical pest control agents are used in the form of suitable preparations (formulations). Depending on the properties of the active substances and the products they are to protect, they contain suitable carriers (e.g., Kaolin, talc, silicic acid), adjuvants, such as wetting agents and emulsifiers, organic solvents, or propellant gases.

4.16.3 Different Pesticides and Chemical Composition

Pesticide residues having different chemical classes and composition include chlorinated hydrocarbons (Aldrin, HCH, HCB, Chlordane, etc.), chlorinated phenoxyalkanoic (2,4-D; 2,4,5-T), organophosphorus pesticides (carbophenothion, coumaphos, demeton, dichlorvos, etc.), carbonate insecticides (carbaryl), dithiocarbamate fungicides (Ferbam, Nabam, etc.), inorganic pesticides (aluminum phosphate, calcium arsenate, lead arsenate), and others (bromopropylate, chloropicrin, ethylene dibromide, etc.). Beside these, pesticides of plant origin are used, including tobacco leaf and nicotine, pyrethrum flower, pyrethrum extract and pyrethroids, and rotenoids (Mukherjee, 2002).

In the case of chlorinated hydrocarbons and a few organophosphorus materials, pesticides have very short residual actions. So, the plant material is being tested to determine the presence of these substances. Different groups of insecticides and pest control agents, including those from plant sources, are shown in Fig. 4.33.

4.16.4 Various Forms of Preparations of Pesticides Used

- *Spray or wettable powders*

The active substance, together with the carrier material and the wetting and dispersion agents, are finely ground to a particle size of 1–20 and suspended in water before use.

- *Powders*

The active substance is finely ground to a particle size of 10–50 in low concentration with the carrier material and any necessary addition of adhesives, and used without any further processing.

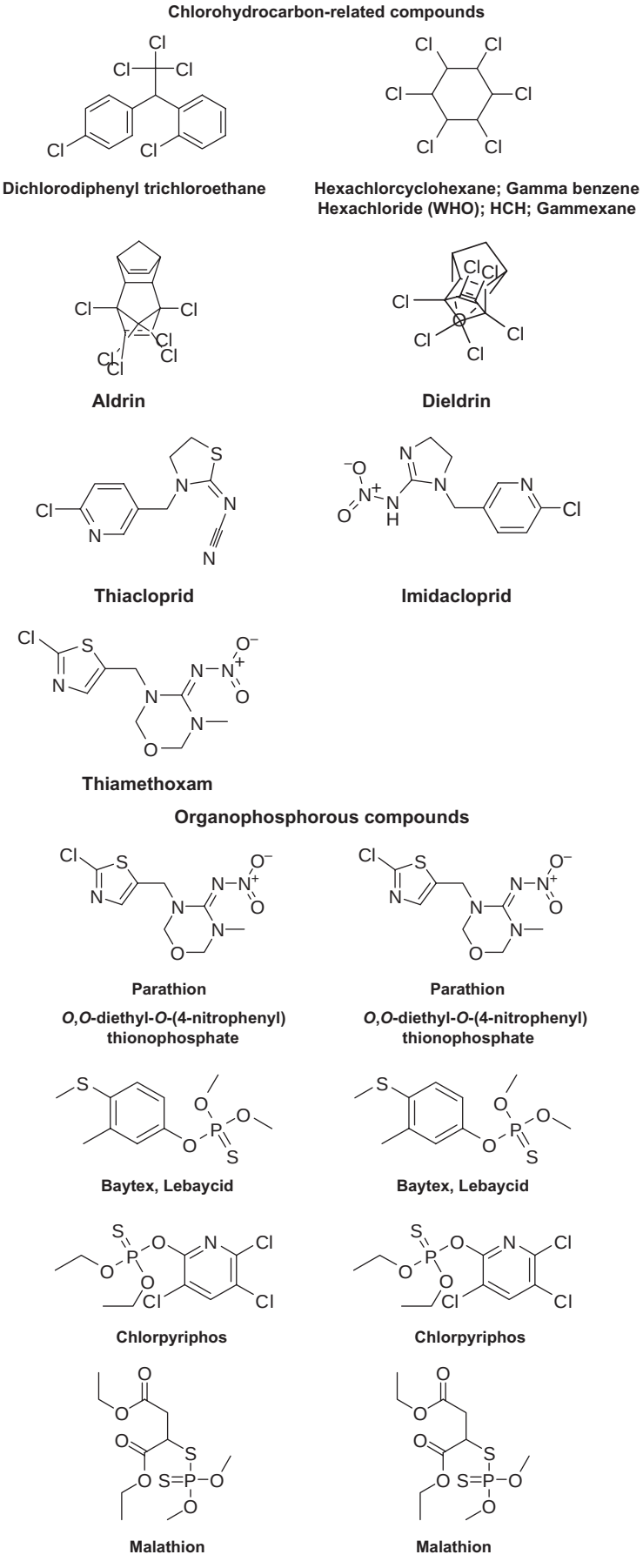

FIG. 4.33 Different groups of pesticides used for the cultivation of herbal drugs.

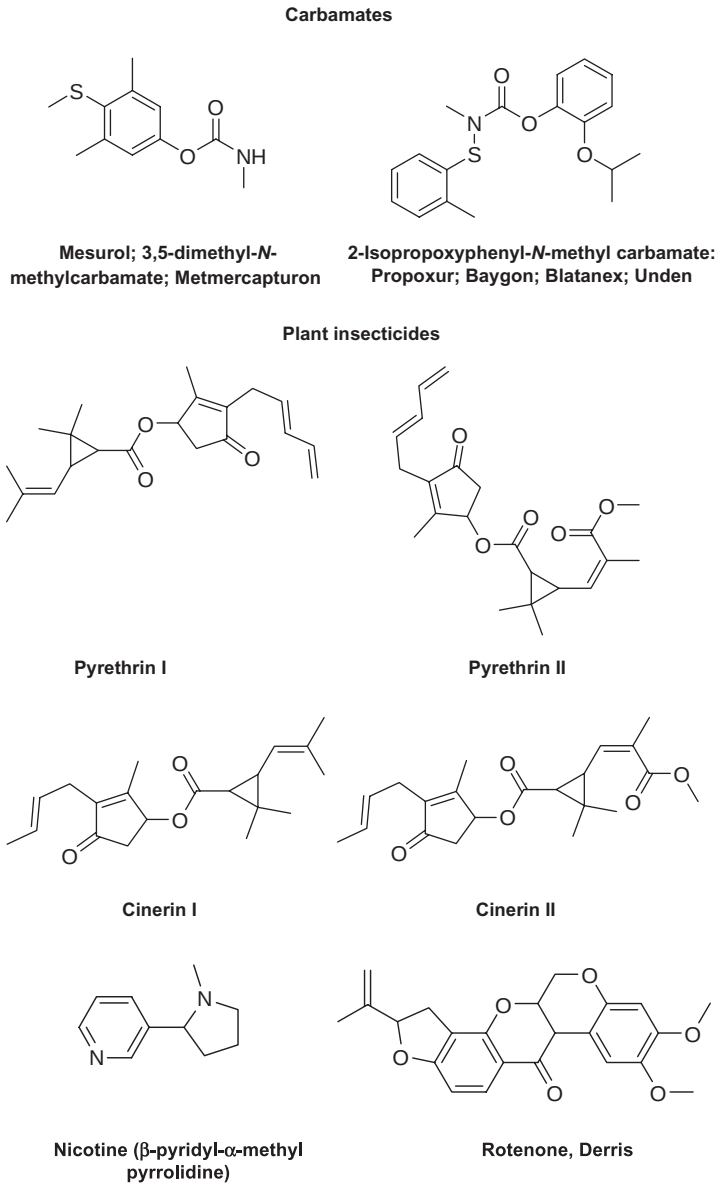

FIG. 4.33, CONT'D

- *Granulates*

Here the active substance is adsorbed on to a granulated carrier material (granule size 300 mm and higher) and thus disseminated.

- *Self-emulsifying concentrates*

The active substance, to which emulsifiers have been added, is dissolved in as high a concentration as possible in an organic solvent, usually mineral oil. Immediately before use, the concentrate is poured into the prescribed quantity of water, where it emulsifies spontaneously.

- *Solutions in mineral oil fractions*

These do not contain any emulsifiers and are applied to the plants to be protected, either directly or as aerosols.

- *Fumigants*

The active substance is contained in a salt mixture acting as a trap from which it vaporizes when heated. This type of preparation is used in closed rooms (storeroom, greenhouses, etc.). Examples of such fumigants are ethylene oxide, ethylene chlorohydrin, and methyl bromide.

4.16.4.1 Classification of Pesticides

4.16.4.1.1 Fungicides

They are used to prevent or control the plant diseases caused by phytopathogenic fungi and are mostly used as seed or soil fungicidal disinfectants. They can be of different types based on their use as follows.

(a) Disinfectants for seeds

These fungicides give prior protection to seeds against fungal disease-causing pathogens, which are admixed with the seed in the form of spores, hyphae, and scleratia. The substances used for this purpose include dithiocarbamates, organophosphorus compounds, pyridine derivatives, and carboxins.

(b) Disinfectants for soil

There are some fungicides that can disinfect the soil and thereby kill the phytopathogenic bacteria that cause fungal infestation through the soil. However, this is more difficult and expensive.

(c) Leaf Fungicides

These are a class of fungicides used for the protection of leaves and fruits. Compounds of this type include Bordeaux mixture (liquor), copper oxy chlorides, colloidal sulfur, barium sulfide, thiurams and dithiocarbamates, and nitro compounds, such as dichloro nitroaniline. Some of these compounds possess both fungicidal and acaricidal activity.

4.16.4.1.2 Herbicides

Herbicides are weed killers, used for destroying unwanted plants or to prevent their growth. There are several types that differ in their mode of action as follows:

(a) *Total herbicides*: prevent or destroy the growth of all plants
(b) *Selective herbicides*: prevent or destroy the growth of undesirable plants but do not affect cultivated plants.
(c) *Water weed killers*: these agents act against aquatic plants in flowing or still water.
(d) *Harvesting aids*: help in mechanical harvesting; for example, harvesting aids cause the defoliation of cotton plants and hence make possible the mechanical harvesting of cotton.

Practically, only the selective herbicides are used in the cultivation of medicinal plants. The compounds most commonly used are carbamates, urea derivatives, growth promoters, quaternary ammonium compounds, and di- and triazines.

4.16.4.1.3 Insecticides

The insect controlling agents are of different categories, as follows

- Insecticides
- Insect repellants
- Insect attractants
- Insect sterilizing agents

Of all these items, insecticides are the most important category. Others have not been tested sufficiently and hence they can be used only for experimental purposes. Insecticides can only be used when the materials to be protected are already infested with insects at various stages of herbal drug development. They cannot be used previously as protective insecticides. Various varieties of insecticides are available due to the varieties of insects and the resistances they have developed through different mechanisms. There is a search for insecticides with a low level of toxicity and that are selective, that is, active only against harmful insects. The way in which the insecticides are used is governed by whether the target insects are biting insects, such as beetles or caterpillars, or sucking insects, such as aphids and bugs. Insects can absorb the active substance in insecticides by respiration, in their food, or by contact. Therefore, the insecticides consist of three types:

(i) Respiratory toxins
(ii) Food toxins
(iii) Skin toxins

Beside these, they can be classified by the mode of action as follows:

(i) General cell toxins (protein precipitants)
(ii) Enzyme toxins (phosphoric acid ester and N-alkyl carbamates)
(iii) Nerve toxins (e.g., chlorinated hydrocarbons)

The insecticides used for the protection of medicinal plants can be of two types based on the site of their action:

(i) Those that do not penetrate into the plant.
(ii) Those that are absorbed rapidly and almost quantitatively—these can be differentiated into different compounds, such as those that are rapidly metabolized, and others which remain unaltered in the plant sap for some time and act against sucking insects. These are known as systemic insecticides (Mukherjee, 2002). A list of the major groups of insecticides is given in Table 4.14.

The inorganic insecticides, for example, arsenic compounds, such as lead and calcium arsenate, and fluorine compounds, such as sodium fluoride, sodium fluorosilicate, and cryolite, have a large number of toxic effects and they have largely been replaced by organic ones. Because of their natural origin, the organic insecticides from various *Tanacetum* species, such as *Pyrethrum* and rotenone from *Derris* roots, are used particularly in the hygiene sector. Because of their high price, they are rarely used in cultivation of medicinal plants. Thus, the insecticides can be of different groups as described in Table 4.15. As there are so many synthetic insecticides (and in many cases it is not known which pesticides have been used by medicinal plant growers), it is expedient when testing for pesticide residues to look for insecticide groups.

TABLE 4.14 List of the Major Groups of Insecticides

Name of the Insecticides	Origin
Natural insecticides	
Pyrethrum	A crude extract of plant containing Pyrethrin I and II and Cinerin I and II
Rotenone	Root extract of *Derris indica*
Nicotine (β-pyridyl-α-methyl pyrrolidine)	Leaf of *Nicotiana tabacum*
Chlorohydrocarbons and related compounds	
Dichlorodiphenyl trichloroethane (DDT)	Synthetic source
Hexachloro cyclohexane	Synthetic source
Gamma-benzene hexachlodium	Synthetic source
Organophosphorus compounds	
O,O-Diethyl-*O*-(4-nitrophenyl) thionophosphate, e.g., parathion; E 605, etc.	Synthetic source
Methyl parathion	Synthetic source
Fenitrophion	Synthetic source
Baytex; Lebaycid	Synthetic source
O,O-Diethyl-*O*-[2-(ethylthio) ethyl] Phosphorothioate, such as Demeton, Systox, etc.	Synthetic source
Carbamates	
3,5-Dimethyl-4-methylthiphenyl-*N*-methyl carbamates, such as Metmercapturon, Mesurol, etc.	Synthetic source
2-Isopropoxyphenyl-*N*-methylcarbamate, such as Propoxure, Baygon, Blattanex, Unden, etc.	Synthetic source

TABLE 4.15 Limits for Pesticides Prescribed by the Ayurvedic Pharmacopeia (Part II, Volume 2)

Substance	Limit
Alachlor	0.02
Aldrin and Dieldrin (sum of)	0.05
Azinphos-methyl	1.0
Bromopropylate	3.0
Chlordane (sum of *cis*-, *trans*-, and Oxychlordane)	0.05
Chlorfenvinphos	0.5
Chlorpyrifos	0.2
Chlorpyrifos-methyl	0.1
Cypermethrin (and isomers)	1.0
DDT (sum of p,p′-DDT, o,p′-DDT, p,p′-DDE, and p,p′-TDE)	1.0
Deltamethrin	0.5
Diazinon	0.5
Dichlorvos	1.0
Dithiocarbamates (as CS2)	2.0
Endosulfan (sum of isomers and endosulfan sulfate)	3.0
Endrin	0.05
Ethion	2.0
Fenitrothion	0.5
Fenvalerate	1.5
Fonofos	0.05
Heptachlor (sum of heptachlor and heptachlorepoxide) 0.05	0.05
Hexachlorobenzene 0.1	0.1
Hexachlorocyclohexane isomers (other than γ)	0.3
Lindane (γ-hexachlorocyclohexane)	0.6
Malathion	1.0
Methidathion	0.2
Parathion	0.5
Parathion-methyl	0.2
Permethrin	1.0
Phosalone	0.1
Piperonylbutoxide	3.0
Pirimiphos-methyl	4.0
Pyrethrins (sum of)	3.0
Quintozene (sum of quintozene, pentachloroaniline and methyl pentachlorophenyl sulfide)	1.0

4.16.4.1.4 Acaricides

They are substances that are used to control the infestation caused by mites, especially spider mites. These mites belong to the arthropods and multiply very rapidly. They cause rapid damage to the foliage of cultivated plants. These insects rapidly acquire resistance to chemicals because of their rapid multiplication and, therefore, new acaricides have to be developed.

These substances contain both fungicides and insecticides. Several classes of compounds, such as phosphoric and thiophosphoric acid esters, carbamidic esters, and chlorinated aromatics with and without sulfur in the molecule, exhibit this property and are, therefore, found in the appropriate group when tested for pesticide residues.

4.16.4.1.5 Nematocides

They are used for the control of phytopathogenic thread worms, that is, nematodes living free in the soil and also occurring in the plants. The agents responsible for the control of nematodes are halogenated hydrocarbons, carbamidic and thiocarbamidic acid derivatives, and thio-phosphoric acid esters.

4.16.4.1.6 Rodenticides

They are agents used to prevent the infestation of cultivated crops by rodents. Nowadays, their use is very much restricted for various bio-diversity reasons.

4.16.4.1.7 Bactericides

These are agents used mostly for the control of bacterial infestation. With regard to the microbial purity of medicinal substances (which do not necessarily have to be sterile), harvested medicinal plants have to be free from pathogenic bacteria and are permitted to contain at most 10^4 apathogenic bacteria/g and 100 fungal mold or yeast cells/g. Plant drugs that have been processed further should not contain more than 10^4 bacteria/g and here every effort should be made to ensure that the final product contains the prescribed limit of apathogenic bacteria. As the fumigation procedures for reducing the bacterial count must be more rigorous than those for the destruction of vermin, the latter is only to be regarded as an added phenomenon of antimicrobial treatment. Other agents, such as molluscicides, bird deterrents, and rodent deterrents, are also sometimes used for the protection of cultivated medicinal plants. The range of chemical pest control agents is not only very large but is also continually changing. Many of the substances listed here are no longer permitted in developed countries or have been replaced by new compounds. DDT, for example, is no longer permitted in Western industrialized countries. However, it is still used in other countries, especially for the control of the malaria-transmitting *Anopheles* mosquito.

4.16.5 Adverse Effects of Different Pesticides

The organochlorine pesticides, for example, 1,1,1-trichloro-2,2-di-(4-chlorophenyl)-ethane (DDT) are an extensive group of compounds that have been widely used as insecticides. Owing mainly to their persistence in human beings, their use has been restricted or even banned in many countries. The toxicity of individual compounds, and hence the potential hazard to animals and human beings, varies widely. Aldrin, dieldrin, and endrin are considered to be the compounds that commonly cause poisoning. In the main, these insecticides produce symptoms, such as overstimulation of the central nervous system, which may be worsened by solvent effects. The organophosphorus pesticides, for example, parathion, malathion, and diazinon, are potent cholinesterase inhibitors and can be very toxic. CNS symptoms include restlessness as well as depression of the respiratory or cardiovascular system. Repeated exposure may have a cumulative effect, although these pesticides are, in contrast to organochlorines, rapidly metabolized and excreted and are not appreciably stored in body tissues. Among some of the other groups cited, the carbamates (*N*-substituted esters of carbamic acid) are cholinesterase inhibitory. They differ from the organophosphorus pesticides because their inhibitory effect is generally less intense and more rapidly reversed. Furthermore, they do not seem to enter the CNS as readily, so severe central effects are uncommon.

LD_{50} is the amount of a toxicant necessary to kill 50% of the test population within a specified period. It is used to estimate oral and dermal pesticide toxicity, in terms of weight of the chemical per unit body weight (mg/kg). For example, pesticides, such as (2,4-dichlorophenoxy) acetic acid (2,4-D), dicamba, parathion, and carbofuran, are included in the highly toxic class (LD_{50} 0–50 mg/kg). However, LD_{50} alone is not sufficient to evaluate toxicity because it gives information only about the acute toxicity of a compound in relation to a certain animal species and does not describe possible alteration that may occur due to prolonged exposure, that is, chronic toxicity. Chronic toxicity refers to the capacity of a substance to cause poisonous effects over the long term at a low level of exposure. Data from animal or human exposure tests have indicated a number of examples of chronic toxicity, including carcinogenicity caused by various compounds, such as (2,4 dichlorophenoxy) acetic acid (2,4-D), triazines, endrin, linuron, rotenone, captan, and maleic hydrazide, teratonicity by

cyanazine, carbaryl, rotenone, benomyl, and maleic hydrazide, neurotoxicity by ethyl p-nitrophenyl thiobenzene phosphate (EPN), and reproductive effects by dibromochloropropane (DBCP), dimethoate, rotenone, and benomyl.

The abusive and disordered usage of pesticides has caused a series of problems of an ecological and public health nature. Some of these are the appearance of new pests, extermination of pollinator insects, and bio-accumulation and existence of residues in the atmosphere, lithosphere, and hydrosphere. The fate and behavior of pesticides in a soil-plant atmosphere system, which is mediated by water dynamics, are determined by inherent properties of these climatic conditions, soil characteristics, and plant morphology. In such a system, pesticides will undergo a fairly limited number of reactions through physical, physio-chemical, chemical, or biochemical processes, such as adsorption, desorption, ion-exchange, free radical reactions, oxidation, reduction, hydrolysis, alkylation, dealkylation, decarboxylation, and isomerization (Mukherjee, 2002).

4.16.6 Maximum Limit of Pesticide Residues for Herbal Drugs

To promote uniformity in the use of pesticides and thereby for preparation of an international list of pesticides suitable or unsuitable for utilization on cultivated medicinal plants, the WHO has made a series of recommendations on the basis of national consultations. They have also established a maximum residue limit (MRL) for the biocides in medicinal plant cultivation as well as development of appropriate methodologies for their analysis.

The MRL is calculated after safety tests in human beings, which indicate toxicologically accepted levels according to the most reliable assay available. Owing to the complexity and specific requirements of herbal preparations, the MRL determination cannot be based solely on that of foodstuffs. The toxicological evaluation of pesticide residue in herbal drugs must be considered based on the intake of the material by a patient. Considering the huge differences in the patterns of consumption within the population, as well as the sensitivity of different categories of populations, such as babies, children, pregnant women, and old and very ill people, toward these biocide compounds, a test for the limit of pesticide residues is essential. In establishing tolerable contaminant limits, many problems may occur when dealing with phytopharmaceuticals. Thus, the toxicological evaluation of pesticide residues present in a medicinal plant is based on intake by patients. The highest nontoxic dose of pesticides obtained by experiment with animals cannot be directly converted to a dose for humans because, besides considering bio-accumulation, bio-reactivity, and synergic interactions, the toxicological assessment of a specific pesticide is essential in different ways. The highest dose (mg pesticides/kg bodyweight/day) that produces no observable toxic effects in the most sensitive species is called the nonobservable effect level (NOEL). This is derived from chronic toxicity tests and used to set the ADI for humans.

$$\text{ADI} = \text{NOEL} \times \left[\text{safety factor}\left(1/100 \text{ to } 1/2000\right)\right]$$

The ADI includes a variable safety factor, 1/100, applied in uncomplicated cases in which all the required toxicological data are available. According to WHO, generally the amount of pesticide residue intake from medicinal plants should be restricted to be within 1% of the total intake from all sources (food and drinking water). Several plant materials contain higher concentrations of pesticide residue, but the quantity decreases after extraction due to the insolubility in water/ethanol. So, a determination of the actual concentration of pesticide residue consumption is essential to prepare the final doses. For example, it would not be admissible if 40%–50% of the ADI for pesticide residues primarily derived from food and drinking water were to be accounted by the additional consumption of vegetable drugs (Mukherjee, 2002). Some pharmacopeias have fixed the limits for pesticides and some have not yet done so. The European Pharmacopeia (1997) has fixed the limits for a series of pesticides that fall in the range of mg of pesticides/kg of plant material. However, if the limit of a specific pesticide does not appear in the Pharmacopeia, it can be calculated by using the following formula:

$$\text{MRL} = \text{ADI} \times W / \text{MDI} \times \left[100 \times \left(\text{safety factor}\right)\right]$$

where MRL is maximum residue limits (mg/kg); ADI is acceptable daily intake (mg compounds/kg body weight); W is body weight (kg); and MDI is mean daily intake of drug.

When an herbal crude drug is used to prepare extracts, tinctures, or other phytopharmaceutical formulations in which manipulation may influence the pesticide concentration of the final product, the MRL is calculated as:

$$\text{MRL} = \text{ADI} \times W \times E / \text{MDI} \times \left[100 \times \left(\text{safety factor}\right)\right]$$

where E is the extraction coefficient of the pesticide, which depends on the method of preparation and needs to be experimentally determined.

Some limits of the pesticides higher than those fixed by the European Pharmacopeia can be tolerated in exceptional cases, for example, when the plant requires special management for cultivation. Determination tests for pesticide residues can be partially or totally eliminated when the complete treatment history of a lot is known and conducted with controlled and precise procedures (Mukherjee, 2002).

The nature of the pesticide present in a plant material is unknown, so a determination of the content of total chlorine is sufficient and the calculation is performed on the basis of the acceptable residue level (ARL) of most pesticides containing toxic chlorine. As per FAO and WHO, calculations of ARL (in mg of pesticide per kg of plant material) are performed on the basis of the maximum daily intake of the pesticide for humans (ADI). The following formula may be used:

$$\mathrm{ARL} = \mathrm{ADI} \times 60 / \mathrm{MDI} \times 100$$

where ADI stands for the maximum daily intake of pesticide (mg/kg of body weight), *E* stands for the extraction factor, and MDI is mean daily intake of medicinal plants or products.

4.16.7 Limits for Pesticides

In order to standardize the use of pesticides, the WHO and various countries have made a series of recommendations on the permissible limits for pesticides and on a list of pesticides acceptable for use on herbal medicinal plants. Unless otherwise indicated in the monograph, the drug to be examined at least complies with the limits indicated in Table 4.15, The limits applying to pesticides that are not listed in the table and whose presence is suspected for any reason comply with the limits set by European Community directives 76/895 and 90/642, including their annexes and successive updates. Limits for pesticides that are not listed in Table 4.15 nor in EC directives are calculated using the following expression:

$$\frac{\mathrm{ADI} \times \mathrm{M}}{\mathrm{MDD} \times 100}$$

where ADI is the acceptable daily intake, as published by FAO-WHO, in milligrams per kilogram of body mass, *M* is body mass in kilograms (60 kg), and MDD is daily dose of the drug (kg).

If the drug is intended for the preparation of extracts, tinctures, or other pharmaceutical forms whose preparation method modifies the content of pesticides in the finished product, the limits are calculated using the following expression:

$$\frac{\mathrm{ADI} \times M \times E}{\mathrm{MDD} \times 100}$$

where *E* is extraction factor of the method of preparation, determined experimentally.

Higher limits can also be authorized, in exceptional cases, especially when a plant requires a particular cultivation method or has a metabolism or a structure that gives rise to a higher-than-normal content of pesticide. The competent authority may grant total or partial exemption from the test when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after the harvest) of the treatment of the batch is known and can be checked precisely (Table 4.15).

4.16.8 Pests and Pest Control

Microbes, insects, noninsect pests, and weeds are examples of pests that grow undesirably near medicinal plants and herbs.

4.16.8.1 Microbes

Fungus, bacteria, and viruses are included in this category. Generally, fungus grows on the soil, which is frequently damp. The plants affected by fungus and other pathogens develop to have shorter shoots. As soon symptoms develop on the plants, a dark root-like structure grows in the soil. It has been reported that many viruses lead to necrosis of leaves and other plant parts. The growth of fungus and other similar microbial pathogens can be avoided with the help of fumigation of the soil with chemicals, removal of the infected part by cutting, and genetic modification of the plant so that it can resist fungi and other microbes (Mukherjee, 2002).

4.16.8.2 Insects

A common insect that harms herbs and medicinal plants is the ant. Ants are found in different varieties and types. Ants make nests in the soil and feed on the honeydew secreted in plants and, eventually, they spoil the plant. Ants feed on plants in such a way as to make the plants weak and when the shoot is of length 10–12 in., even a gust of wind can cause the plant to break. Ants can feed and injure buds, so that there is less chance of the buds developing. These insects can render the cells of a leaf empty by removing the contents of the leaf cells. For this type of pest, digging in the soil and killing the eggs, larvae, and pupae can help. As for adult insects, the use of insecticide may help (Mukherjee, 2002).

4.16.8.3 Noninsect Pests

Generally, noninsect pests refer to rats, monkeys, spiders, mites, and others. These pests cause yellow spots on leaves. Noninsect pests can be controlled by the construction of effective and distinct warehouses, traps, and rodenticides (Mukherjee, 2002).

4.16.8.4 Weeds

Weeds are plants that grow and feed on the nutrition of healthy cultivated plants. They compete for water and nutrients with the desired plant and stunt its growth. It is important to control weeds as the absence of weeds near a healthy plant enhances the growth and yield of the healthy cultivated plant. A skilled person should have proper tools for weed removal and these tools also vary with the time of the year and soil conditions. Using lower amounts of herbicide can control weeds. Herbicides are of two types in general: one, which acts against the germination of weed seeds, and the other, which acts against growing plants. There are some herbicides that have the capacity to act on both pre- and postsurfacing. The herbicides that act in a preemergent way act on the soil and hinder the germination of weeds. This type of herbicide leaches in the soil with the help of water obtained by irrigation and rain, and hence act on germinating seeds. Herbicides are degraded by the harmful rays of the sun if not leached into the soil with water. Even though herbicide may be present on the surface, it is useless unless it reaches within the soil and weeds emerging during this time cannot be controlled. Herbicides applied after the growth of weeds in an area are of the postemergent type. Their application is generally used to provide instant relief from weed growth (Ayurvedic Pharmacopeia, part II, Volume 2). Pesticide analysis generally consists of a common extraction step and clean-up followed by detection. Two separate groups of references have formed using two criteria: first, the number of pesticides included in the method, and second, the matrices analyzed by the method. The methods that include a large number of pesticides from different families are considered to be multiresidue methods. These are commonly applied to different matrices, including grapes. Methods that focus on the matrix grape and that do not include a large number of pesticides are considered as single-residue methods or specific methods for grapes. These methods often include some of the by-products of grapes as additional matrices (Grimalt and Dehouck, 2016) (see Table 4.16). Also, see Table 4.17 for miscellaneous types of pesticides.

4.16.9 Different Classes of Pesticide Based on Chemical Composition

- Chlorinated hydrocarbons and related pesticides
- Chlorinated phenoxyalkanoic acid herbicides

TABLE 4.16 General Methods of Pest Control (Ayurvedic Pharmacopeia of India Part II, Volume 2)

Controlling Techniques	Steps Involved
Cultural	Changing the time of sowing and harvesting maintenance of storage, special cultivation methods, proper cleaning, using trap crops and resistant varieties
Physical	Mechanical control utilization of physical factors (temperature, less oxygen concentration, humidity, passing of O_2)
Biological	Using predators, parasites, pathogens, sterilization, genetic manipulation, pheromones
Chemical	Use of pesticides, herbicides, antifeedants

TABLE 4.17 Pesticides That Help to Control a Variety of Organisms (Ayurvedic Pharmacopeia of India Part II, Volume 2)

Class	Protection From
Acaricide	Controls ticks and mites
Algicides	Algae and other aquatic vegetation
Antiseptics	Microorganisms
Arboricides	Defoliates and destroys trees and shrubs
Bactericides	Bacterial infections
Molluscicides	Controls mollusks, including gastropods
Nematicides	Nematodes

- Organophosphorus pesticide
- Carbonate insecticide
- Dithiocarbamate fungicides
- Inorganic pesticide
- Pesticide of plant origin
- Miscellaneous

4.16.10 Method for Determination of Pesticides

4.16.10.1 Method 1

In this method, powdered test samples (10 g) are treated with acetone (100 mL) and kept for 20 min. After adding 1 mL carbophenothion in toluene solution (1.8 µg/mL), homogenization is carried out in a high-speed blender for a time period of 3 min. The total system is subjected to filtration and the filter cakes obtained are then washed thoroughly with two quantities of acetone (25 mL each). The washing material, along with the filtrate, is heated at a temperature below 400°C by a rotary evaporator until complete solvent evaporation is achieved. Toluene is added to the obtained residue until the acetone is completely removed. Toluene is used to dissolve the residue and filtered by a membrane filter (45 µm). Filtration is done with the addition of toluene. Dilution is carried out with the same solvent (solution A). To determine the pesticide residue in the test samples, chromatography methods are also applied. In the chromatographic method, a column made of stainless steel (0.30 m long and 7.8 mm in internal diameter) packed with styrene-divinylbenzene copolymer is used. Toluene is used as the mobile phase with a flow rate of 1 mL/min. To purify the test sample, a suitable volume of solution A is injected and chromatography is performed. Fractions are collected and considered as solution B. Organophosphorus insecticides are eluted between 8.8 and 10.9 mL, whereas organochlorine and pyrethroid insecticides are eluted between 8.5 and 10.3 mL. To determine the concentration of organochlorine and pyrethroid insecticides, concentrated solution B is diluted with a suitable volume of toluene (200 µL to 1 mL) and transferred into the column. Toluene is used as the mobile phase. The collected fraction is considered as solution C (Ayurvedic Pharmacopeia of India, Vol. 2).

4.16.10.2 Method 2

A sensor film should be prepared by using 16 mg of HRP (944 units), 10 mg of N,N_0-bis acrylamide, and 150 mg of acrylamide, which should be dissolved in 800 µL of a 0.1 mol/L phosphate buffer solution of pH 6. The mixture should be bubbled with nitrogen for 5 min, and 4 µL of a 10% (w/v) $(NH_4)\ 2S_2O_8$ solution and 1 µL of the commercial TEMED solution should be added. Batch absorption measurements may be obtained by placing 2 mL phosphate buffer solution in a quartz cuvette, adding 0.5 mL of the HRP solution, and registering absorbance measurements at 400 and 424 nm. When the signal becomes stable, 100 µL of the corresponding H_2O_2 solution should be added. For pesticide determination, 200 µL of the corresponding pesticide and 1800 µL of the phosphate buffer solution should be placed in the cuvette, 0.5 mL of the HRP solution added, and the absorbance registered at 400 and 424 nm. When the signal becomes stable, 100 µL of a 6.7×10^{-5} mol/L H_2O_2 solution should be added.

The sensor film should be placed in a flow cell, described elsewhere, and settled in the cuvette compartment of the spectrophotometer. A 5×10^{-5} mol/L tyrosine solution in phosphate buffer solution of pH 6 should flow at 1 mL/min thorough the flow cell with a peristaltic pump. The variation in absorbance at 400 and 424 nm should be registered and, when the signal becomes stable, 1 mL of a 2.9×10^{-5} mol/L H_2O_2 solution should be injected with a manual sample injection valve. For determination of pesticide, 1 mL of the corresponding mixture of pesticide-H_2O_2 should be injected.

4.16.10.3 Method 3

Pesticide should be dissolved in acetone or methanol to obtain a stock standard solution with a concentration of 5000 mg/L. A fresh standard solution containing pesticides with concentration of 100 mg/L should be prepared in acetone every week and stored at 4°C. A determination of the target analytes should be performed using a gas chromatograph. Helium (purity 99.9999%) should be employed as the carrier gas at a flow rate of 1 mL/min. During the whole analysis, the injector should be operated in the splitless mode with an injector temperature of 280°C. The oven temperature initially should be set at 50°C (2 min hold), followed by a temperature ramp of 10°C/min to 200°C, and to be held for 5 min, then it should be increased to 280°C at a rate of 5°C/min (10 min hold), for a total run time of 55 min. The mass detector should be operated in electron impact (EI) mode at 70 eV. The inlet, MSD transfer line, MSD source, and quadrupole temperatures should be 280, 280, 230, and 150°C, respectively. The sample extracts, standards, and blanks should be injected (1 µL) into the GC. The MSD system should be routinely programmed in selective ion monitoring (SIM) according to the conditions selected for any pesticide, using two ions. Confirmation of the pesticides should be established by the retention time and the presence of the target ions. The target ion abundances should be determined by injection of individual pesticide standards under the same chromatographic conditions, but utilizing full-scan conditions with the mass/charge scan ranging from 40 to 550 m/z.

4.16.10.4 Method 4

Standard liquids of pesticide with appropriate concentrations of 0.8 mg/mL (4×10^{-3} mol/L) and 0.35 mg/mL (4×10^{-4} mol/L) should be obtained by titrating with carbon tetrachloride. A fluorescence spectrum of the standard liquid should be obtained using RF5301. Then, edible oil (rapeseed oil or peanut oil) should be diluted by carbon tetrachloride with a volume ratio of 1:20 and the fluorescence spectrums should be recorded. The two kinds of edible oil (3 mL) are placed in a cuvette and the pesticide standard solution is mixed with the edible oil by successive addition from 0.1 to 1.0 mL. After sufficient stirring, the edible oil and drug solution should be mixed well and their fluorescence spectra should be obtained with a certain excitation wavelength. A UV spectrometer should be used for scanning.

4.16.10.5 Method 5

QuEChERS stands for quick, easy, cheap, effective, rugged, and safe. This technique involves microscale extraction using acetonitrile and purifying the extract using dispersive solid-phase extraction (d-SPE). Since the development and publication of the method, QuEChERS has been gaining significant popularity. It is the method of choice for food analysis because it combines several steps and extends the range of pesticides recovered over older, more tedious extraction techniques.

The method has undergone various modifications and enhancements over the years since its first introduction. These have been designed to improve recovery for specific types of pesticides or types of food. The traditional methods of determining pesticides in food are usually multistage procedures, requiring large samples and one or more extract cleanup steps. Therefore, they are time-consuming, labor-intensive, complicated, expensive, and produce a considerable amount of waste. Moreover, the traditional methods often give poor quantitation and involve a single analyte or analytes from a single class of compounds. On the other hand, QuEChERS methodology reduces sample size and quantities of laboratory glassware. Clearly, QuEChERS requires fewer steps (no blending, filtration, large volume quantitative transfers, evaporation/condensation steps, or solvent exchanges required): this is very significant, as every additional analytical step complicates the procedure and is also a potential source of systematic and random errors.

The development of a new methodology requires a number of problems to be addressed, for example, the choice of extraction solvent. For determining pesticide residues in food matrices, the usual solvents have been acetone and acetonitrile, as both ensure large analyte recoveries. Although acetone is readily miscible with water, the separation of water from this solvent is impossible without the use of nonpolar solvents. On the other hand, ethyl acetate is only partially miscible with water, which renders superfluous the addition of nonpolar solvents to separate it from water, but the most highly polar pesticides do not separate in it. Acetonitrile extracts of food (fruit and vegetables) contain fewer interfering substances than the corresponding ethyl acetate and acetone extracts, and acetonitrile can be separated fairly easily from water (salting out); therefore, it is the preferred extraction solvent in the QuEChERS methodology.

4.17 AFLATOXINS AND OTHER MICROBIAL CONTENTS

The safety of herbal medicines is of great importance from a regulatory perspective. Plant-derived crude drugs are subject to contamination by microbial species, which includes bacterial and fungal spores as well as viable forms. Fungal species are also responsible for releasing mycotoxins, of which the most important one from the toxicological point of view are the aflatoxins. Other mycotoxins include the ochratoxins and penicillic acid. Microbial contamination in medicinal plant materials triggers degradation of the therapeutic constituents in the crude drug during storage. Also, they may be responsible for intoxication after ingestion by the patient as a finished herbal formulation. The bacterial species responsible for food poisoning in most cases are *Bacillus cereus* and *Clostridium perfringens*. The fungal species responsible for production of aflatoxins include mainly molds from the *Aspergillus* species. Predominantly, *Aspergillus flavus* and *Aspergillus parasiticus* are responsible for this type of contamination. Other than these species, the other bacterial species that infect plant samples include *Aeromonas hydrophila*, *Shigella* spp., *Enterobacter agglomerans*, *E. cloacae*, *Vibrio fluvialis*, *Pasteurella multocida*, *S. epidermidis*, *Acinetobacter lwoffii*, *Klebsiella* spp., *B. subtilis*, and *Pseudomonas aeruginosa*. On the other hand, the other fungal species infecting medicinal plant products include *Aspergillus*, *Penicillium*, *Mucor*, *Candida*, and *Trichosporium* species. Field Fungi infecting the plant include *Alternaria*, *Helminthosporium*, *Fusarium*, and *Cladosporium*, which infect the plant in the field. Molds infecting the plant during storage are *Aspergillus* and *Penicillium* (Aziz et al., 1998).

4.17.1 Factors Affecting Microbial Content

The microbial load in medicinal plants is affected by different processing parameters. First, medicinal plants are collected from natural sources, and so there remains ample chance for microbial contamination from environmental sources. Again, after collection of a plant sample, it is subject to drying, extraction, and ultimately formulation in a particular solid, liquid, or semisolid dosage form. How these various factors affect the microbial load is discussed below.

Drying parameters: The drying of a crude drug may be carried out by sun drying, shed drying, freeze drying, spray drying, infrared drying, and microwave drying. During sun drying or shed drying, uniform drying of the crude drug is desirable to prevent mold formation. Spray drying, though, subjects the crude drug to a higher temperature, but it lasts for a shorter span of time. So, it is not effective in removing microbial species from crude drugs. Drying at temperatures higher than 60°C is effective in removing most of the moisture content from a crude drug. This is effective in preventing microbial and, especially, Aspergillus growth in the sample.

Extraction parameters: Extraction of crude drugs may be done using the hot or cold method. The cold method includes the cold maceration method. This method is carried out at ambient temperature and it is not able to remove much of the microbial content. On the other hand, hot extraction procedures, such as decoction, infusion, and Soxhlet extraction, are to some extent able to remove the microbial load. One point to be noted is that the infusion process, though hot extraction in nature, provides a perfect niche for the growth of thermophilic bacteria, such as the *Bacillus species*. Leaving aside this instance, in all other hot extraction procedures, the microbial load is reduced by a few log scales.

Chemical constituent of the crude drug: If the crude drug itself contains some antimicrobial principle, it will prevent microbial growth to some extent. Crude drugs bearing volatile oils are found to possess antimicrobial properties. In brief, the factors affecting the microbial quality of medicinal plants are as follows:

Intrinsic factors: (i) Nature of the plant and natural barriers; (ii) structure of the plant; (iii) plant compositions; and (iv) intracellular microbial composition.

Extrinsic factors: (i) Climate; (ii) humidity; (iii) location; (iv) harvest method; (v) postharvesting; (vi) physical state; (vii) technological treatment; (viii) packaging and storage conditions; and (ix) exogenous microbial contaminations.

Possible pathways for the microbial contamination of medicinal plants are depicted in Fig. 4.34. The bacterial count depends on different factors, such as the distance of the plant part from the soil and the plant surface area to plant weight ratio. The total aerobic mesophilic count is an important determinant of the microbial load. Among the mesophilic bacteria are *Bacillus cereus* and *Clostridium perfringens*.

Official compendia, such as the ISO, EHIA, and European Pharmacopeia, have laid down guidelines regarding the permissible microbial limits and sampling plans. According to these testing procedures, a particular weight of the plant sample should be free from a particular strain of bacteria. The different bacterial strains and their importance in the microbial load determination are listed below (Kneifel et al., 2002):

- Total aerobic mesophilic count is important in determining the general hygiene and the quality parameters.
- Enterobacteria are important in determining the general hygiene and the quality parameters and fecal contamination.
- Coliforms and enterococci are also indicators of fecal contamination.
- Enterobacteria, coliform, enterococci, yeasts, and molds are ubiquitously present microbes.

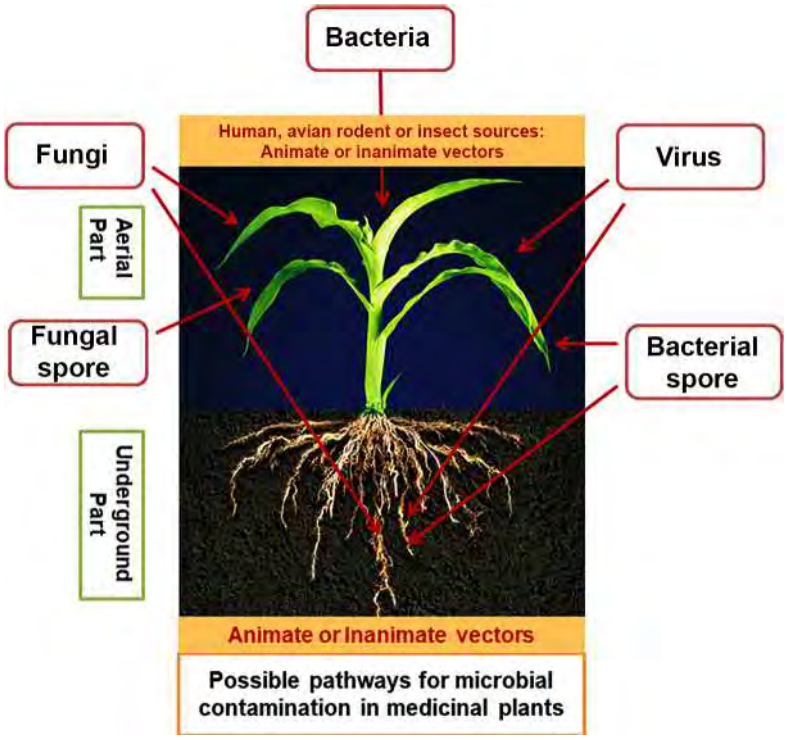

FIG. 4.34 Possible pathways for microbial contamination of medicinal plants.

4.17.2 Microbial Quality Standards of WHO

The most widely accepted and used technique is that recommended by WHO for the total count of microorganisms in plant materials. The specification of WHO for total aerobic microorganisms is not more than 10^7 CFU/g for plant materials used as teas and infusions at most 10^5 CFU/g for internal use. The specification of WHO for yeasts and molds is at most 10^4 CFU/g for plant materials used as teas and infusions and at most 10^3 UFC/g for internal use. High counts of fungi are a risk because of the possibility of producing mycotoxin, such as aflatoxin, which is a carcinogenic toxin. The WHO also recommends a test to detect the possible presence of aflatoxins, which are highly dangerous contaminants in any material of plant origin. The United States Pharmacopeia draws the following specifications for products for oral use: 10^4 aerobic bacteria/g or mL, 10^2 fungi/g, and the absence of *Salmonella* spp., *E. coli*, and *S. aureus*. However, the Brazilian Pharmacopeia also indicates the detection of other indicators of increased risk for oral administration, such as *P. aeruginosa*, *B. cereus*, *Enterobacter* spp., *Candida albicans*, *A. flavus*, and *A. parasiticus* (de Araújo and Bauab, 2012).

4.17.3 Principles for the Determination of Microbial Load

There are several official protocols for the determination of the microbial load in a sample. In general, the sample is suspended in peptone water, pH 7.0. This suspension is then plated in sterile agar of bacterial or fungal media. As per the WHO guidelines, as bacterial media, soybean casein digest media is used. As fungal media, Abouraud dextrose media is used containing 10% tartaric acid. This is followed by tests for specific pathogens (de Araújo and Bauab, 2012). According to the official compendia, there are some guidelines for sample preparation, preparation of the inoculum, and growth promotion by media. Specific media are there for the bacteria and fungi. For each specific strain of the bacteria, there are specific media and specific tolerance limits in the Pharmacopeia. In the following section, these will be discussed accordingly.

4.17.4 Procedures for the Determination of Microbial Load

4.17.4.1 Total Aerobic Viable Count

For determination of this parameter, an inoculum with particular strains of the target bacteria have to be prepared. This will be followed by inoculation, growth promotion by media, and then appropriate enumeration in the presence of media.

TABLE 4.18 Examples of Microbial Load With Incubation Temperature and Incubation Time

Name of the Microbial Species	Strain	Media	Incubation Temperature (°C)	Incubation Time (h)
Staphylococcus aureus	ATCC 6538	Casein-soybean digest medium	30–35	18–24
Pseudomonas aeruginosa	ATCC 9027	Casein-soybean digest medium	30–35	18–24
Bacillus subtilis	ATCC 6633	Casein-soybean digest medium	30–35	18–24
Candida albicans	ATCC 10231	Sabouraud Dextrose broth	20–25	48
Aspergillus brasiliensis	ATCC 16404	Sabouraud Dextrose agar with antibiotic	20–25	5–7

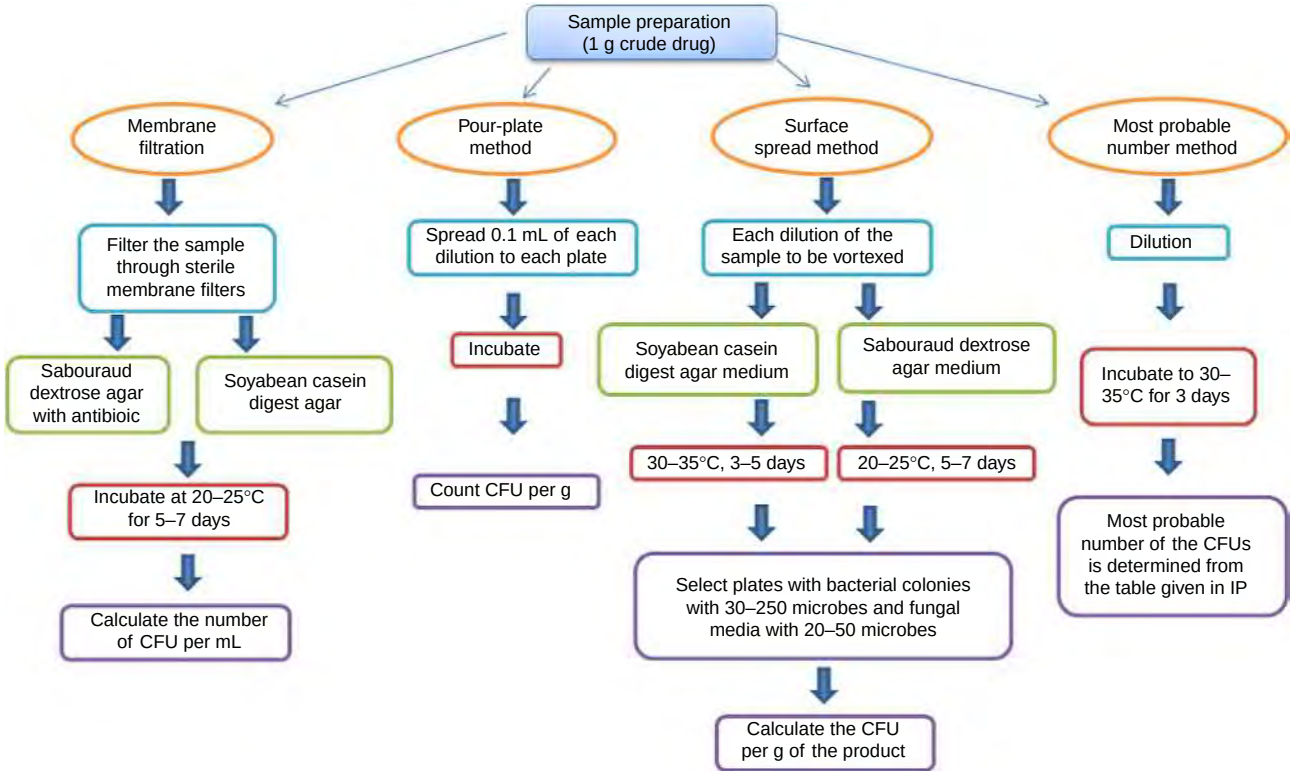

FIG. 4.35 General protocol for microbial load determination.

First, the inoculum should be prepared. For this, a particular strain of the bacteria or fungus is to be selected. This is then suspended in peptone water. Next, the strain is grown in a growth medium by incubating at a particular temperature for a specified time. The media and incubation used for the different strains are given in Table 4.18. Also, a brief overview of the processes is depicted in Fig. 4.35.

4.17.4.2 Growth Promotion by Media

For verification of growth promotion by the media prepared, a blank control is needed to be prepared first. It will contain only the diluent, that is, peptone water as inoculum. After that, the inoculation should be executed. Both liquid broth and agar plates of casein-soybean digest medium are inoculated with 100 CFU each of the bacterial strains. Similarly, the Sabouraud Dextrose medium is also inoculated as well as streaked on agar plates. After incubation, the negative control should not show any growth. If there is any, the reason should be investigated properly. After that, investigate the growth observable in the inoculated broth is investigated. Growth obtained on a solid medium must not differ from the calculated standardized inoculum by more than two factors (Mukherjee, 2002).

4.17.4.3 *Appropriateness of the Enumeration Method in the Presence of Product*

First, the sample needs to be prepared. For water soluble and water insoluble nonfatty and fatty products, separate preparation procedures should be adopted. They are described in what follows.

(i) *Water-soluble products*: They are simply dissolved in peptone water and filtered. The pH is adjusted to 7.0.
(ii) *Water-insoluble nonfatty products*: These products are insoluble, if not nonwettable in water. So, this should be suspended in peptone water, pH 7.0. For that, 0.1% Tween 80 may be used for wetting the material. This will be used as the inoculum.
(iii) *Fatty materials*: As they are totally insoluble and nonwettable by water, they need to be prepared as an emulsion form. The material should be first dissolved in isopropyl myristate. Then, the matter should be emulsified in peptone water with the help of Tween 80. The volume will be made up by Tween 80 (Fig. 4.36).

After inoculation and incubation of the nutrient media with the prepared sample, as described above, the antimicrobial efficacy of the material needs to be neutralized with certain diluents. The concentration of the diluents varies with the microbicidal activity of the specific chemical constituents in the product. The type of antimicrobial agent and the type of inhibitor that should be used are presented in Table 4.19.

After the sample has been subjected to the above procedures, the next step is the enumeration of the approximate microbial load in the sample. This may be carried out by (i) membrane filtration; (ii) plate pour method; (iii) surface spread method; and (iv) most probable number (MPN) method.

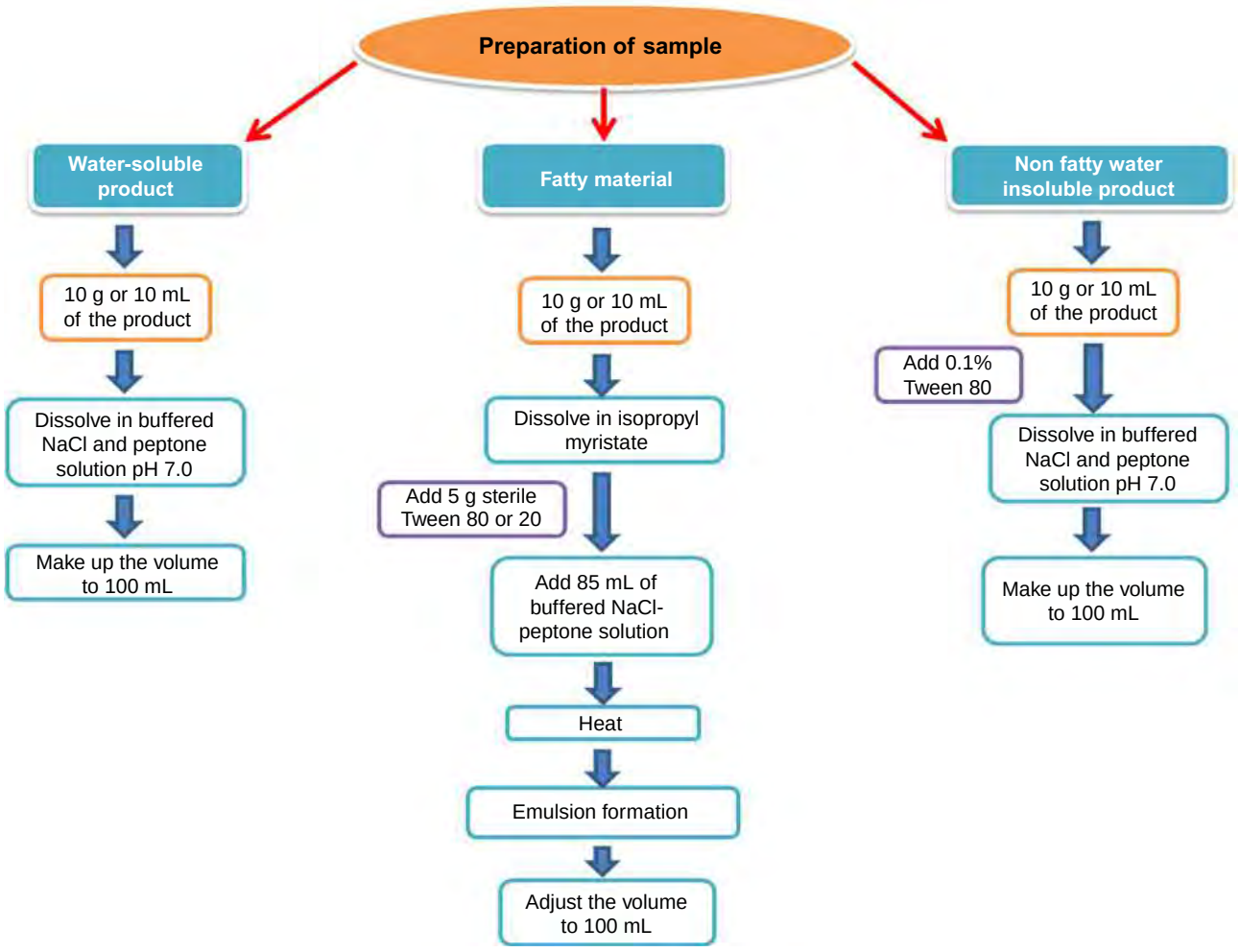

FIG. 4.36 Sample preparation for different types of crude drug samples.

TABLE 4.19 Antimicrobial Agents and Inactivators

Antimicrobial Substance	Inactivator	Concentration
Phenolics, parahydroxybenzoate	Tween 80	30 g/L
Iodine and quaternary ammonium compounds	Lecithin, SLS	3 g/L, 4 g/L
Alcohol, aldehyde, sorbates	Dilution	—
Mercurial halogens	Sodium thiosulfate	5 g/L

4.17.4.4 Membrane Filtration Method

In this method, the liquid broth containing the incubated inoculum and inactivating agent is filtered through a 0.45-μ cellulose nitrate filter. It is then washed with a sodium chloride solution of pH 7.0. After that, it is transferred to the surface of casein-soybean digest medium for bacterial growth and to the surface of Sabouraud Dextrose agar for fungal growth. The number of colonies will be counted to approximately determine the number of microbes per g or per mL of the product.

4.17.4.5 Pour Plate Method

In this method, 1 mL of the liquid broth is poured on a nutrient agar plate. After that, it is incubated for a particular period of time. Then, the number of CFU per mL or g is calculated based on the colonies formed. For bacteria, the casein-soybean digest medium is used and for the fungi, the Sabouraud Dextrose medium is used. The surface spread method is a similar method, in which the inoculum is spread with a surface spreader.

4.17.4.6 Most Probable Number (MPN) Method

This method is used when no other determination method is available. In this method (Fig. 4.37), three dilutions of the sample solution are made. They should be of 10-fold dilution. From each dilution, three aliquots of 1 g or 1 mL are taken and inoculated in three test tubes. After incubation, the growths are observed in the nine test tubes. The number of tubes showing growth is an indirect estimate of the number of microbial species per unit amount of the product. If detection is not easy, the culture on agar plates of the corresponding media should be inoculated.

The total aerobic count (TAC) is considered to be equal to the number of CFU found on casein-soybean digest agar. If colonies of fungi are detected on this medium, they are counted as part of the TAC. The total fungal count (TFC) is considered to be equal to the number of CFU found using Sabouraud Dextrose agar with antibiotic.

The acceptance criteria for microbiological quality should be interpreted as follows:

10^1 CFU: Maximum acceptable count 20.
10^2 CFU: Maximum acceptable count 200.
10^3 CFU: Maximum acceptable count 2000, and so forth.

4.17.5 Examples of the Importance of Microbial Load According to the Indian Pharmacopeia

A list of several nutrient media useful for the determination of the microbial load are given below:

1. Buffered sodium chloride-peptone solution pH 7 (sample preparation)
2. Casein-soybean digest broth
3. Casein-soybean digest agar
4. Sabouraud Dextrose broth
5. Sabouraud Dextrose agar
6. Enterobacteria enrichment broth-Mossel
7. Violet red bile glucose agar
8. MacConkey broth
9. MacConkey agar
10. Rappaport Vassiliadis Salmonella Enrichment broth

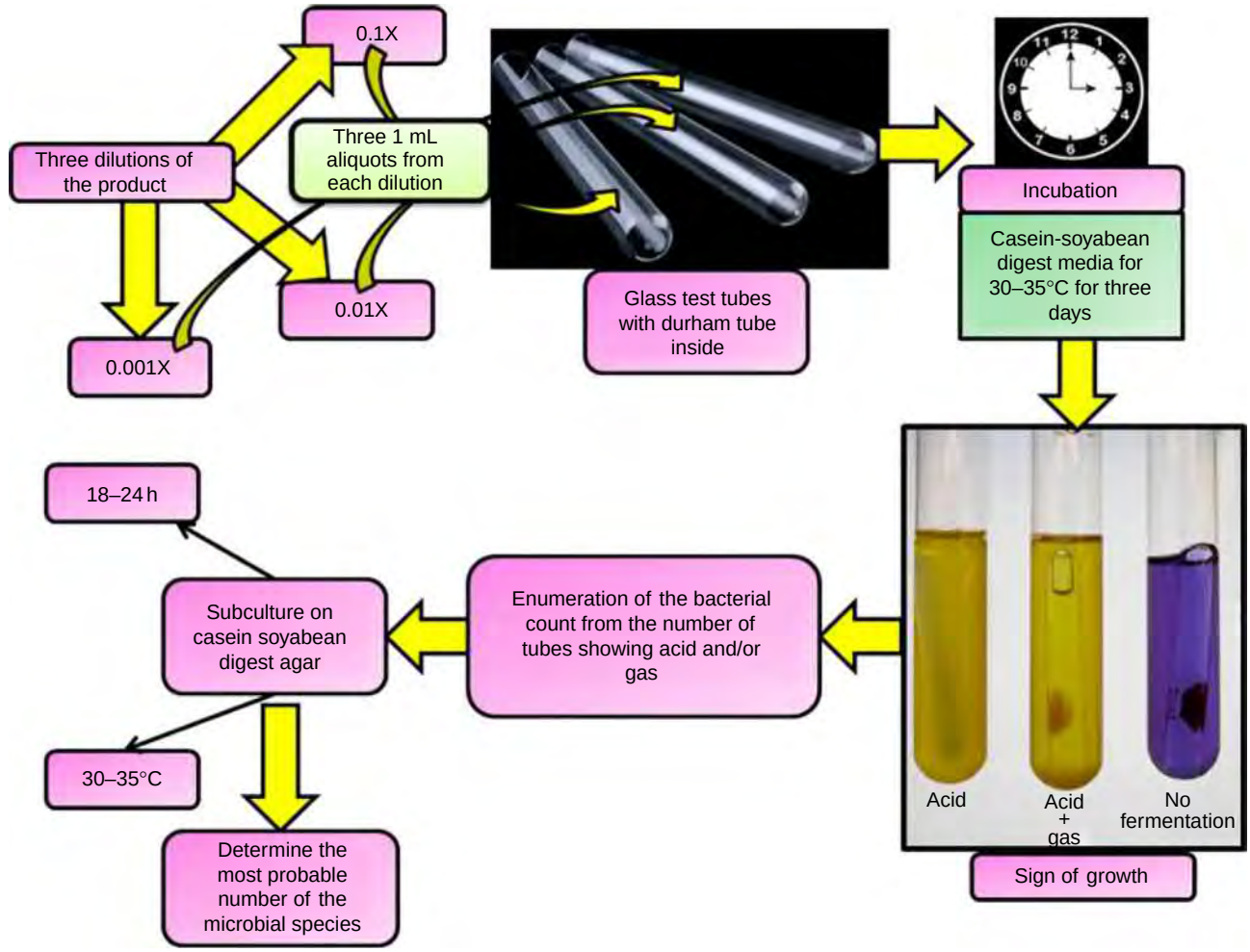

FIG. 4.37 Most probable number method.

11. Wilson and Blair's BBS agar
12. Complete medium
13. GN broth
14. Xylose-lysine-deoxycholate agar
15. Cetrimide agar
16. Mannitol salt agar medium
17. Reinforced medium for clostridia
18. Columbia agar.

4.17.6 Detection of the Presence of Specific Microorganisms

4.17.6.1 Escherichia coli

This is one species under the family Enterobacteriaceae. The sample is first inoculated in the casein-soybean digest medium. After incubation, it is subcultured in MacConkey's broth. After that, it is subcultured in MacConkey's nutrient agar media. After incubation for 18–72 h, pink, the formation of nonmucoid colonies indicates the presence of *E. coli*. The details of the method are presented in Fig. 4.38.

4.17.6.2 Salmonella *Species*

This is used for the confirmation of the presence of *Salmonella* sp. in the sample. Here also, the sample solution is first cultured in a casein-soybean digest medium. After incubation, it is subcultured in a Rappaport Vassiliadis *Salmonella*

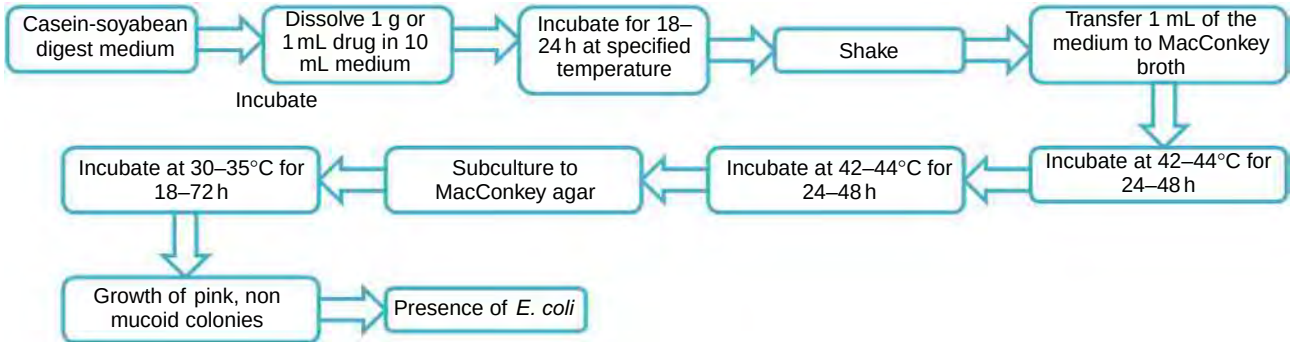

FIG. 4.38 Detection of the presence of *E. coli*.

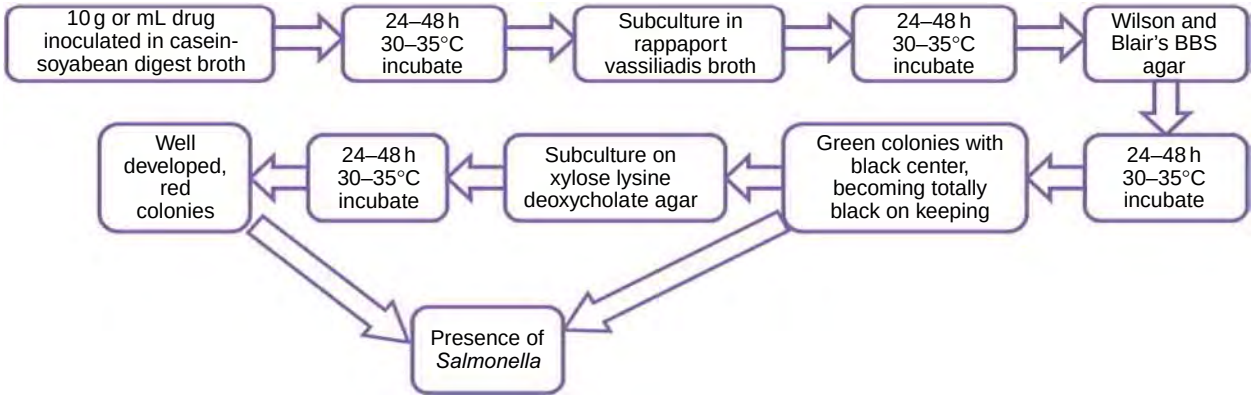

FIG. 4.39 Detection for the presence of *Salmonella*.

enrichment broth. After incubation again, subculture is done on a Wilson and Blair's BBS agar. Formation of green colonies indicates a possibility of the presence of *Salmonella*. If red colonies form on subculturing on xylose lysine Deoxycholate agar, it is indicative of the possibility of the presence of *Salmonella*. Method details have been presented in Fig. 4.39.

4.17.6.3 Pseudomonas *Species*

A quantity of 10 mL of the drug is inoculated in a casein-soybean digest medium first. After incubation, it is subcultured on a cetrimide agar plate. After incubation, the appearance of greenish colonies indicates the probability of the presence of *P. aeruginosa*. In the absence of any such colonies, the drug passes the test. If colonies are found, the drug needs to be further investigated in additional identification tests. The details of the method are presented in Fig. 4.40.

4.17.6.4 Candida albicans

This is a dimorphic fungus. The sample is first inoculated in a Sabouraud Dextrose broth and then subcultured on Sabouraud Dextrose agar. Growth of cream-colored colonies indicates the possible presence of *C. albicans*. Such presence will be confirmed by identification tests later. The absence of these colonies indicates the absence of *C. albicans*. The details of the method are presented in Fig. 4.41.

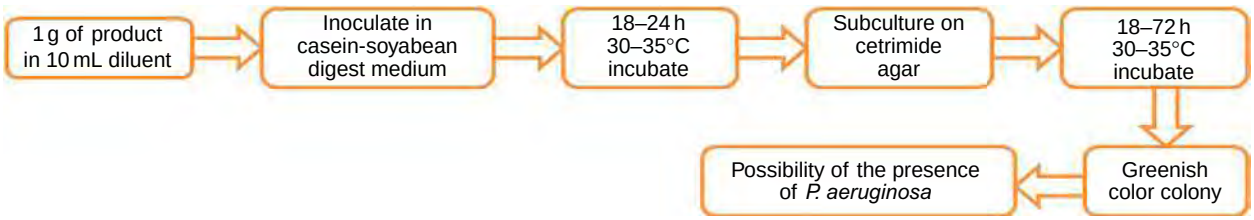

FIG. 4.40 Detection for the presence of *P. aeruginosa*.

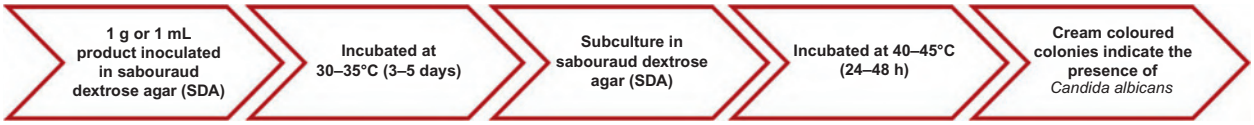

FIG. 4.41 Detection for the presence of *Candida albicans*.

4.17.7 Determination of Aflatoxins

Aflatoxins are a class of compounds with the molecular mass of $C_{17}H_{12}O_6$. They are mainly caused by contamination by *A. flavus* and *A. parasiticus*. They are very much injurious to the liver. They may cause hepatic carcinoma. So, their percentage needs to be controlled carefully. The WHO has given guidelines regarding how to test for aflatoxins in natural products. Mainly B_1, B_2, G_1, and G_2 are monitored in herbal products. The following is a schematic diagram representing the determination of aflatoxins in herbal material (Mukherjee, 2002). Method details have been presented in Fig. 4.42.

The crude drug is extracted with a methanol: water mixture by continuously shaking. After that, zinc acetate and aluminum chloride is added to this solution. It is then stirred and diatomaceous earth is added to it. After that, a 10% sodium chloride solution is added to it. Also, pet ether is added to it. After separation of the layers in a separating funnel, the aqueous layer is separated and it is subsequently washed with dichloromethane (CH_2Cl_2) twice. After that, the combined chloroform layers are concentrated under vacuum and 0.2 mL chloroform: CAN is added to it. After that, it is vortexed until homogeneous. Then, TLC of the extract is run. In parallel, standard aflatoxins are run. By using densitometric analyses, a quantitative estimate of the aflatoxins is obtained (Mukherjee, 2002).

Aflatoxins were extracted with a mixture of methanol and water, followed by purification by solid-phase clean-up using a polymeric sorbent for the determination of the toxins. The eluted extract was injected into the chromatographic system using a reversed-phase C18 short column with an isocratic mobile phase composed of methanol-water (30:70). A single-quadruple mass spectrometry using an electrospray ionization source operating in the positive ion mode was used to detect

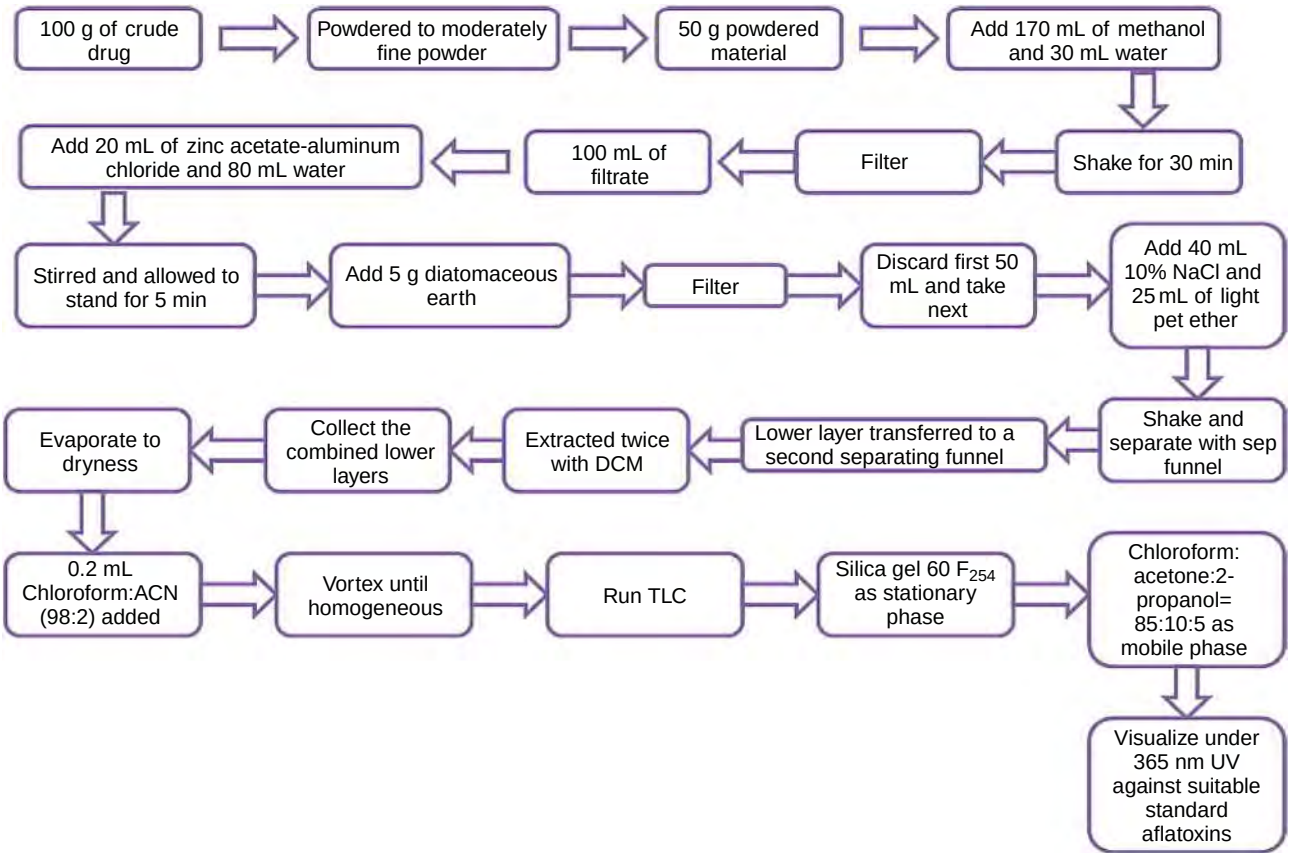

FIG. 4.42 Determination of the presence of aflatoxins.

aflatoxins due to derivatization, presenting several disadvantages. Recoveries of the full analytical procedure were 110% for aflatoxin B1, 89% for aflatoxin B2, 81% for aflatoxin G1, and 77% for aflatoxin G2. The detection limit (S/N = 3) was 10 ng and the quantification limit (S/N = 10) was 25 ng, calculated as the amount in the medicinal herb (Ventura et al., 2004). The total aerobic viable counts, as well as the individual bacterial and fungal counts, are important parameters for the quality and safety of herbal materials. Aflatoxins are toxic metabolites produced by *Aspergillus* sp., which are very much injurious to the health of consumers. This parameter may be monitored by TLC or LC/MS technology. The microbial load may be determined by membrane filtration or the plate count method. Alternatively, it may also be determined by the MPN method.

4.18 ESSENTIAL OIL IN CRUDE DRUGS

Essential oils are volatile liquids obtained from plants belonging to certain families. They are highly volatile in nature and are responsible for the pleasant smells of different aromatic plants. The yield of the essential oils in medicinal plants is generally very low. These oils are used as antimicrobial agents, and aromatic and flavoring agents in pharmaceutical preparations. The quality control of essential oils is very important as they are susceptible to adulteration (Rao, 2006). The principal plant families for essential oils are: Annonaceae, Cupressaceae, Lauraceae, Pinaceae, Asteraceae, Graminae, Orchidaceae, Rutaceae, Burseraceae, Geraniaceae, Myrtaceae, Verbenaceae, Compositae, Labiatae, Piperaceae, and Zingiberaceae. Essential oils are derived from different plant parts, such as:

A. *Leaves*: Basil, Bay leaf, Cinnamon, Eucalyptus, Lemon Grass, Melaleuca, Oregano, Patchouli, Peppermint, Pine, Rosemary, Spearmint, Tea Tree, Wintergreen, Thyme, etc.
B. *Flowers*: Chamomile, Clary Sage, Clove, Geranium, Hyssop, Jasmine, Lavender, Manuka, Marjoram, Orange, Rose, Ylang-Ylang.
C. *Peel*: Bergamot, Grape fruit, Lemon, Lime, Orange, Tangerine.
D. *Seeds*: Almond, Anise, Celery, Cumin, Nutmeg Oil.
E. *Woods*: Camphor, Cedar, Rosewood, Sandalwood.
F. *Berries*: Allspice, Juniper.
G. *Bark*: Cassia, Cinnamon.
H. *Resin*: Frankincense, Myrrh.
I. *Rhizome*: Ginger.
J. *Root*: Valerian.

Essential oils are well known for a wide range of pharmacological activities as well as therapies. One special use of volatile oils is in aromatherapy. Other pharmacological uses include antimicrobials, expectorants and diuretics, spasmolytics and sedatives, cholagogues, antiinflammatories, and cicatrizing (Rao, 2006).

Essential oils comprise different types of chemical constituents, such as mono-, sesqui, and diterpenes, alcohols, and aldehydes. However, for preliminary determination of the quality of a medicinal plant containing a volatile oil, hydrodistillation or solvent-free microwave extraction (SFME) is employed for volatile oil content determination in a particular plant. Afterward, total Eugenol content, total cineole content, total aldehyde content, and other parameters are measured.

4.18.1 Principles and Methods for Determination of Essential Oil Content

4.18.1.1 Distillation

The distillation method for the determination of the volatile oil content of medicinal plants is quite popular. The hydrodistillation method comprises the following phenomena: (1) hydrodiffusion, (2) hydrolysis, and (3) decomposition by heat.

4.18.1.2 Hydrodiffusion

For the essential oil in the vacuoles to be distilled with water, the oils must be freed from the vacuoles first. In the case of distillation with water only, the cell membranes get swollen and the oil dissolved in the water leaves the cells by diffusion and then gets vaporized with water. In the case of steam distillation, the soaked plant material swells and the oil coming out of the cells is then vaporized with steam. The water and oil ultimately get condensed and form separate layers in the graduated tube. After this, the components may be decomposed due to prolonged heating and get hydrolyzed to simpler compounds. Hydrodistillation can be of three main types: (1) water distillation, (2) water and steam distillation, and (3) direct steam distillation (Mukherjee, 2002).

4.18.1.3 Water Distillation

In this process, the crude drug to be extracted is immersed in water, the ratio of the drug and water (or water/glycerol mix) being 1:8. After that, the heating of the flask may be carried out by direct fire, steam jacket, closed steam jacket, closed or open steam coil, microwave, etc. The main characteristic of this process is that there is direct contact between the boiling water and the plant material.

There are some drawbacks of water distillation, such as (1) ester-type compounds in the oil are susceptible to hydrolysis and the hydrocarbons and aldehydes are susceptible to polymerization. These may be degraded during extraction process. (2) Oxygenated components, such as phenols, tend to dissolve in the still water, so their complete removal by distillation is not possible. (3) As water distillation tends to be a small operation (operated by one or two persons), it takes a long time to accumulate much oil, so good-quality oil is often mixed with bad-quality oil. (4) The distillation process is treated as an art by local distillers, who rarely try to optimize both oil yield and quality. (5) Water distillation is a slower process than either water and steam distillation or direct steam distillation (Mukherjee, 2002).

4.18.1.4 Water and Steam Distillation

In water and steam distillation, the steam can be generated either in a satellite boiler or within the still, although separated from the plant material. Like water distillation, water and steam distillation is widely used in rural areas. Moreover, it does not require a great deal more capital expenditure than water distillation. Also, the equipment used is generally similar to that used in water distillation, but the plant material is supported above the boiling water on a perforated grid. In fact, it is common that persons performing water distillation eventually progress to water and steam distillation (Mukherjee, 2002).

4.18.1.5 Direct Steam Distillation

In this case, the steam is generated in a separate boiler and passed through the chopped crude drug, which is kept on a perforated platform. This enables the crude drug to be extracted under high pressure with the steam generated, and also facilitates the removal of the crude drug after the extraction is complete. The distillate, which ultimately leads to the separation of the water and the oil into separate layers, is collected in a Florentine flask, a glass jar, or more recently in a receptacle made of stainless steel with one outlet near the base and another near the top (Mukherjee, 2002).

4.18.1.6 Cohobation

Cohobation is a procedure that can only be used during water distillation or water and steam distillation. It uses the practice of returning the distillated water to the still after the oil has been separated from it so that it can be reboiled. The principal behind it is to minimize the loss of oxygenated components, particularly phenols, which dissolve to some extent in the distillate water. For most oils, this level of oil loss through solution in water is less than 0.2%, whereas for phenol-rich oils, the amount of oil dissolved in the distillate water is 0.2%–0.7%. As this material is being constantly revaporized, condensed and revaporized again, any dissolved oxygenated constituents will promote hydrolysis and degradation of themselves or other oil constituents. Similarly, if an oxygenated component is constantly brought in contact with a direct heat source or side of a still that is considerably hotter than 100°C, then the chances of degradation are enhanced.

Cohobation is another circulatory distillation method for the extraction of volatile oils. Here, the water part of the distillate is redirected to the flask. It prevents loss of the oxygenated phenolics from the distillate, which dissolve to some extent in the distillate water. For most oils, this level of oil loss through solution in water is less than 0.2%, whereas for phenol-rich oils the amount of oil dissolved in the distillate water is 0.2%–0.7%. The disadvantage is that prolonged heating may trigger hydrolysis of these compounds. As a result, the practice of cohobation is not recommended unless the temperature to which oxygenated constituents in the distillate are exposed is no higher than 100°C. Isolation of the volatile oil after the hydrodistillation process may be performed in varied ways. The apparatus used for laboratory-scale hydrodistillation is a circulatory distillation apparatus, which is most commonly known as a Clevenger apparatus (Mukherjee, 2002).

4.18.1.7 Hydrodistillation Through Clevenger Apparatus

A Clevenger apparatus is used for a determination of the percentage of volatile oils present in an oil-bearing material. Place a weighed quantity of material in a sample flask and add water. Generally, the ratio of the material to water is 1:8. Connect the flask with the Clevenger apparatus and open the water tap for running a flow of water in the condenser. Start heating on a heating mantle and control the heat so that the oil with the water vapor comes into the graduated distillate receiving tube and the excess water goes back into the flask. Continue heating for 8 h, cool the assembly and remove the water from

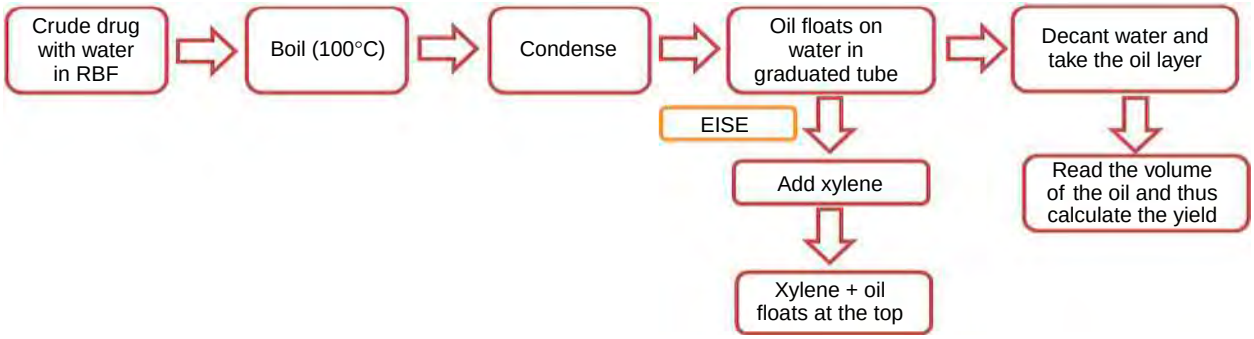

FIG. 4.43 Essential oil determination using the Clevenger apparatus.

the distillate receiving tube, and take the oil into a rotary evaporator for removing traces of water and cool the flask in a desiccator (Kumar and Tripathi, 2011). The details of the method are presented in Fig. 4.43. After cooling, weigh it accurately and calculate as follows:

$$\%\text{of oil} = \text{weight of oil} \times 100 / \text{weight of material taken}$$

The separated oil generally has a specific gravity lower than that of water and floats at the top of the water layer. In case the oil is heavier than water, it submerges below the water layer. In that case, xylene is added to the system. As a result of this, the oil, dissolved in xylene floats at the top of the water layer. By subtracting the volume of the xylene added to the system, the amount of volatile oil extracted may be calculated. The state of the crude drug being extracted is an important controller for the yield of the volatile oil. One example is that the lemon peel to be extracted should be crushed under water (Mukherjee, 2002). A further improvement in the hydrodistillation technique involves the introduction of extraction-solvent extraction in the process. To improve the yield, the water part is extracted with a water-immiscible organic solvent, such as dichloromethane. These types of extract are suitable for GC/MS analysis. The extraction of volatile oils in a Soxhlet apparatus, using liquid carbon dioxide produced a solvent-free sample suitable for HRGC analysis.

4.18.1.8 Microdistillation Method

A further advancement in the determination of the volatile oil content with yet smaller samples may be achieved via microdistillation techniques. The preparation of micro amounts of the volatile oil may be done by a modified Marcusson device. An amount of 0.2–3 g of the powdered material is heated with 50 mL water and then the oil is collected in a 100 µL tube, in Pentane. Such experiments are important in doing the chemotaxonomic investigations. Another modification of the microdistillation technique is performing it in a 20-mL crimp-cap glass vial with a Teflon-lined rubber septum containing 10 mL water and 200–250 mg of the material to be investigated. This vial, which is placed in a heating block, is connected with a cooled receiver vial by a 0.32-mm-I.D. fused silica capillary. By temperature-programmed heating of the vial by a heating block, the volatile oils are vaporized and collected in a receiver vial (Mukherjee, 2002).

4.18.2 Simultaneous Extraction Coupled to TLC

A simple device for the rapid extraction of volatiles from natural plant drugs and the direct transfer of these substances to the starting point of a thin-layer chromatographic plate has been described by in publications by Stahl. A small amount of the sample (100 mg) is introduced into a glass cartridge with a conical tip together with 100 mg silica gel containing 20% of water, and heated rapidly in a heating block for a short time at a preset temperature. The tip of the glass tube projects 1 mm from the furnace and it points to the starting point of the thin-layer plate, which is positioned 1 mm in front of the tip. Before introducing the glass tube, it is sealed with a silicone rubber membrane. This simple technique has proven to be useful for many years in numerous investigations, especially in quality control, identification of plant drugs, and rapid screening of chemical races. In addition to the aforementioned microhydrodistillaion with the TAS procedure (T = thermomicro and transfer; A = application; S = substance), several further applications, for example, in structure elucidation of isolated natural compounds, such as zinc dust distillation, sulfur and selenium dehydrogenation, and catalytic dehydrogenation with palladium, have been described in the microgram range (Mukherjee, 2002).

4.18.3 Direct Sampling From the Secretory Structures

The investigation of the essential oils by direct sampling from secretory glands is of fundamental importance in studying the true essential oil composition of aromatic plants, because the usual applied techniques, such as hydrodistillation and extraction, are known to produce several artifacts in some cases. Therefore, only direct sampling from secretory cavities and glandular trichomes and properly performed successive analysis may furnish reliable results. One of the first investigations with a kind of direct sampling has been performed by Hefendehl (1966), who isolated the glandular hairs from the surfaces of *M. piperita* and *Mentha aquatica* leaves by means of a thin film of polyvinyl alcohol, which was removed after drying and extracted with diethyl ether. The composition of this product was in good agreement with the essential oils obtained by hydrodistillation. In contrast to these results, Malingré et al. (1969) observed some qualitative differences in the course of their study on *M. aquatica* leaves after isolation of the essential oil from individual glandular hairs by means of a micromanipulator and a stereomicroscope. In the same year, Amelunxen et al. (1969) published results on *M. piperita*, who separately isolated glandular hairs and glandular trichomes with glass capillaries. They found identical qualitative composition of the oil, but differing concentrations of the individual components. In addition to these methods, volatile oil extraction may be done using the cold expression, enfleurage, deflurage, Sfumatrice, or Pelatrice methods. Modern methods for the determination of the volatile oil content include supercritical fluid extraction, microwave distillation, accelerated solvent extraction, and headspace trapping techniques (Mukherjee, 2002).

4.18.4 Supercritical Fluid Extraction

Critical pressure is the temperature above which, a gas cannot be converted to liquid even by application of pressure. At the critical temperature, the pressure required to convert the gas into liquid is known as the critical pressure. In the case of supercritical fluid extraction, the crude drug is extracted at a temperature and pressure above the critical temperature of the gas. The most widely used gas for supercritical fluid extraction is CO_2. Other organic liquids and gases also have been tried, such as N_2O, acetone, ether, chloroform and supercritical water. The advantage of using CO_2 as the extracting fluid is the low critical temperature and the critical pressure of it (31.1°C and 73.8 bar).The extraction efficiency depends on the thermodynamic phenomena, extraction kinetics, temperature, and pressure. Several experiments have been done using supercritical fluid extraction on different crude drugs, such as chili, ginger, and black pepper. Also, the solubility of the individual compounds, such as limonene and citral in carbon dioxide, were also tested. The use of supercritical fluids, especially carbon dioxide, in the extraction of plant volatile components has increased during the last two decades due to the expected advantages of the supercritical extraction process. Supercritical fluid extraction (SFE) is a rapid, selective, and convenient method for sample preparation prior to the analysis of compounds in the volatile product of plant matrices. Also, SFE is a simple, inexpensive, fast, effective, and virtually solvent-free sample pretreatment technique. The factors affecting extraction by supercritical fluid extraction include pressure and temperature, modifiers, extraction time, flow rate, particle size and packing density, water, and drying (Pourmortazavi and Hajimirsadeghi, 2007). There are several advantages of SFE: (1) dissolving power of the SCF is controlled by pressure and/or temperature; (2) SCF is easily recoverable from the extract due to its volatility; (3) nontoxic solvents leave no harmful residue; (4) high-boiling components are extracted at relatively low temperatures; (5) separations not possible by more traditional processes can sometimes be effected; and (6) thermally labile compounds can be extracted with minimal damage as low temperatures can be employed in the extraction. There are some disadvantages of SFE as follows: (1) elevated pressure required; (2) compression of solvent requires elaborate recycling measures to reduce energy costs; and (3) high capital investment for equipment (Mukherjee, 2002).

4.18.5 Microwave-Assisted Extraction

The extraction of crude drugs using hydrodistillation in a Clevenger type apparatus is also possible in a microwave-aided manner. The heating of the crude drug and the water is carried out using the application of microwaves on a round-bottom flask containing the crude drug and water. The solvent-free microwave extraction apparatus is an original combination of microwave heating and dry distillation at atmospheric pressure. SFME was conceived for laboratory-scale applications in the extraction of essential oils from different kinds of aromatic plants. Based on a relatively simple principle, this method involves placing plant material in a microwave reactor, without any added solvent or water. The internal heating of the in situ water within the plant material distends the plant cells and leads to rupture of the glands and oleiferous receptacles. This process thus frees essential oil, which is evaporated by the in situ water of the plant material. A cooling system outside the microwave oven condenses the distillate continuously. The excess of water is refluxed to the extraction vessel in order

to restore the in situ water to the plant material. Three aromatic herbs: basil (*Ocimum basilicum* L.), garden mint (*Mentha crispa* L.), and Thyme (*Thymus vulgaris* L.) belonging to the Labiatae family, were studied in an experiment and the yield of the essential oil was compared with that obtained in the conventional hydrodistillation method (Mukherjee, 2002).

One of the advantages of the SFME method is rapidity. The extraction temperature is equal to the boiling point of water at atmospheric pressure (100°C) for both the SFME and HD extraction methods. To reach the extraction temperature (100°C) and thus obtain the first essential oil droplet, it is necessary to heat for only 5 min with SFME compared with 90 min for HD. An extraction time of 30 min with SFME provides yields comparable to those obtained after 4.5 h by means of HD, which is the reference method in essential oil extraction. The ultimate yields of essential oils obtained by solvent free microwave extraction (SFME) from the three aromatic herbs were 0.029% for basil, 0.095% for crispate mint, and 0.160% for thyme. The ultimate yields obtained by HD were 0.028% for basil, 0.095% for crispate mint and 0.161% for thyme (Mukherjee, 2002).

On comparison of the chemical profiles of the two types of volatile oils, it was found that substantially higher amounts of oxygenated compounds and lower amounts of monoterpenes hydrocarbons are present in the essential oils of the aromatic plants extracted by SFME in comparison with HD. Monoterpenes hydrocarbons are less valuable than oxygenated compounds in terms of their contribution to the fragrance of the essential oil. Conversely, the oxygenated compounds are highly odoriferous and, hence, the most valuable. The greater proportion of oxygenated compounds in the SFME essential oils is probably due to the diminution of thermal and hydrolytic effects compared with hydrodistillation, which uses a large quantity of water and is time and energy consuming. Water is a polar solvent, which accelerates many reactions, especially reactions via carbocation as intermediates. The greatest difference between the chromatograms for the two methods can be noted for basil. Linalool and Eugenol were the main components in the essential oil extracted from basil but the relative amounts differed for the two extraction methods. Eugenol is the most abundant component of the SFME extract (43%) and linalool the second most abundant (25%), whereas the HD extract is dominated first by linalool (39%) and then by eugenol (11%). The essential oil of garden mint isolated both by SFME and HD is characterized by an important content in the oxygenated compound carvone, respectively, 65% and 52%. Limonene, a monoterpene which is the second most abundant compound, is present at 9.7% and 20%, respectively, for SFME and HD. The essential oil of thyme isolated either by SFME or HD contains the same three dominant components: thymol (51% and 41%), γ-terpinene (17% and 23%), and p-cymene (7.5% and 11%). There are slightly fewer compounds present in the chromatograms of essential oils extracted by SFME compared with those obtained by HD. Essential oil from basil extracted by HD and the essential oil from thyme extracted by SFME are the richest in terms of the number of organic compounds. Relatively few new compounds were found as a result of SFME extraction but these were present in very small amounts. The loss of some compounds in SFME compared with HD is probably not because these compounds are not extracted but rather that the reduction in extraction time and the amount of water in the SFME method reduces the degradation of compounds by hydrolysis, trans-esterification, or oxidation, and hence there are fewer degradation products noted in the analysis. The reduced cost of extraction is clearly advantageous for the proposed SFME method in terms of energy and time. The energy required to perform the two extraction methods are 4.5 kWh for HD and 0.25 kWh for SFME. At the same time, the calculated quantity of carbon dioxide rejected in the atmosphere is dramatically more in the case of HD (3600 g CO_2 per gram of essential oil) than for SFME (200 g CO_2 per gram of essential oil). Hydrodistillation required an extraction time of 270 min for heating 6 kg of water and 500 g of plant material to the extraction temperature, followed by evaporation of water and essential oil. The SFME method required heating for 30 min only of the plant matter and evaporation of the in situ water and essential oil of the plant material (Lucchesi et al., 2004b).

4.18.6 Illustrative Example of the Determination of the Essential Oils in Crude Drugs

Table 4.20 gives the official specifications for crude drugs that contain volatile oils (Mukherjee, 2002). Also, Table 4.21 gives the extraction procedures, along with the yields of some medicinal plants.

4.18.7 Preliminary Quality Control Parameters for Essential Oils

4.18.7.1 Total Eugenol Content

This is calculated as the unabsorbed oil after treatment of the crude oil with potassium hydroxide solution. First, use a graduated 150-mL flask, with a neck, in which 10 mL liquid occupies at least 15 cm of the length of the flask. This is then rinsed with Sulfuric acid and then with water. 10 mL oil and 80 mL of the KOH solution is taken in it. This is kept for 1 h with occasional shaking. After that, quite meticulously, the volume of the liquid is made up to the graduated region. It is kept overnight and then the amount of unabsorbed liquid is calculated. The details of the method are presented in Fig. 4.44. This is indicative of the amount of eugenol content (Mukherjee, 2002).

TABLE 4.20 Official Specifications for Essential Oil Content in Different Plants

Crude Drugs Used	Weight of Sample in Grams	Distilled With	Distillation Time in Hours	% (v/w) Oil in Sample
Caraway	20	Water	4	3.5
Cardamom	20	Water	5	4.0
Cinnamon	40	Water	5	1.0
Clove	4	Water/glycerol	4	15.0
Coriander	40	Water	3	0.3
Fennel	25	Water/glycerol	4	4.0
Nutmeg	15	Water	3	10.0

TABLE 4.21 List of Medicinal Plants With the Method of Extraction and the Percentage Yields

Plant Extracted	Method for Isolation of Volatile Oil	% Yield	Reference
Cymbopogon martini	Hydrodistillation using Clevenger apparatus	0.5%	Jain and Sharma (2017)
Achillea sintenisii, aerial parts	Hydrodistillation using Clevenger apparatus	0.6%	Schimleck et al. (2003)
Hypericum perforatum, aerial parts	Solvent free microwave extraction	0.365%	Abdelhadi et al. (2015)
Juniperus excels leaves	Hydrodistillation using Clevenger type apparatus	0.5%	Moein and Moein (2010)
Ocimum gratissimum L. fresh aerial parts	Hydrodistillation by Clevenger apparatus, and thereafter removal of the essential oil by hexane	0.2%–1% yield	Mohr et al. (2017)
Mentha piperita (peppermint)	Hydrodistillation using Clevenger apparatus	60 min was found as the optimum extraction time (1.33% yield)	Oliveira et al. (2012)
Melaleuca cajuputi, leaves	Steam distillation by cohobation in a modified Dean and Stark apparatus	2.13%	Schimleck et al. (2003)
Laurus nobilis, leaves	Hydrodistillation using Clevenger apparatus	Fresh plant—0.56%	Sellami et al. (2011)
		Air dried—1.13%	
		Microwave dried—0.56%	
		Oven dried 45°C—0.62%	
		Oven dried 65°C—0.32%	
		Infrared dried 45°C—0.78%	
		Infrared dried 65°C—0.49%	

TABLE 4.21 List of Medicinal Plants With the Method of Extraction and the Percentage Yields—cont'd

Plant Extracted	Method for Isolation of Volatile Oil	% Yield		Reference
Lippias idoides, fresh leaves	Hydrodistillation using Clevenger apparatus	1.06%		Veras et al. (2013)
Cuminum cyminum L., seeds	Roasted and microwave heated seed samples were extracted by hydrodistillation	*Roasted*		Behera et al. (2012)
		Fresh	5.6 (±0.05)% yield	
		100°C, 5 min	4.4 (±0.10)% yield	
		100°C, 10 min	3.8 (±0.05)% yield	
		125°C, 5 min	3.6 (±0.03)% yield	
		125°C, 10 min	3.0 (±0.05)% yield	
		150°C, 5 min	2.6 (±0.08)% yield	
		150°C, 10 min	1.8 (±0.03)% yield	
		Microwave heated		
		Fresh	5.6 (±0.05)	
		175 W, 10 min	4.4 (±0.03)	
		175 W, 20 min	4.0 (±0.05)	
		385 W, 10 min	4.0 (±0.08)	
		385 W, 20 min	3.6 (±0.05)	
		595 W, 10 min	3.6 (±0.03)	
		595 W, 20 min	2.8 (±0.10)	
		730 W, 10 min	2.8 (±0.08)	
		730 W, 20 min	2.4 (±0.10)	
		800 W, 10 min	2.4 (±0.08)	
		800 W, 20 min	2.0 (±0.10)	
Ocimum basilicum *Mentha crispa* *Thymus vulgaris*	Comparison of conventional hydrodistillation with microwave-assisted extraction was done	Hydrodistillation *O. basilicum*—0.028% *M. crispa*—0.095% *T. vulgaris*—0.161% *MW extraction* *O. basilicum*—0.029% *M. crispa*—0.095% *T. vulgaris*—0.160%		Lucchesi (2004a)

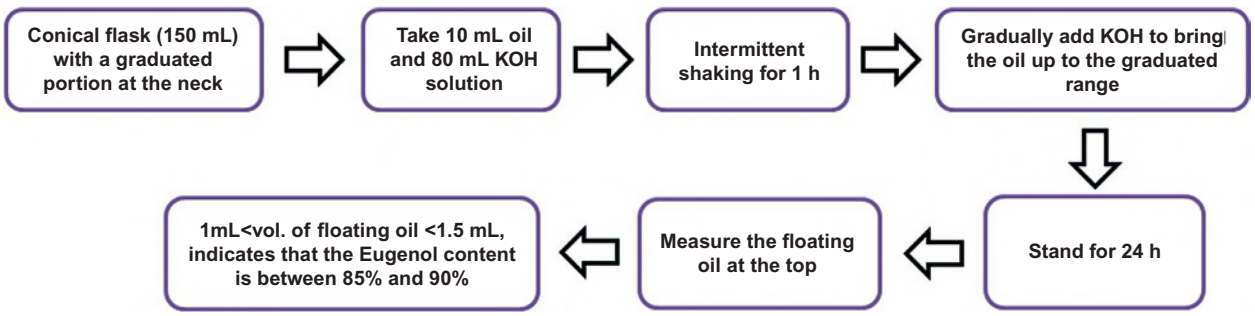

FIG. 4.44 Determination of total eugenol content.

4.18.7.2 Determination of Total Cineole Content

This is performed by precipitating the cineole by addition of o-cresol, freeze thawing the mixture by a freezing point determination apparatus and then by a water bath. During crystallization, the rise in the temperature as well as the maximum temperature is noted. This freeze-thaw cycle is continued until the difference in two successive maximum temperatures is less than 0.2°C. Then, crystallization of the cineole is carried out by addition of a crystal of cineole and o-cresol. The details of the method are presented in Fig. 4.45. The cineole content is determined according to a table given in the BP (Mukherjee, 2002).

4.18.7.3 Determination of Total Aldehyde Content in Volatile Oil

It is performed by first converting the aldehydes present in the volatile oil into oximes by treatment with hydroxylamine. Then, the oxime is titrated with the help of 0.5 M ethanolic potassium hydroxide. A quantity of 0.5 mL 0.5 M ethanolic potassium hydroxide is added to it and then the aldehyde content is determined as specified in the individual monograph. The details of the method are presented in Fig. 4.46.

4.18.7.4 Determination of the Carvone Content of Volatile Oil

The carvone content in a volatile oil is determined by a method similar to the aldehyde content determination method. Here, the volatile oil is treated with a hydroxylamine solution and then the red-colored solution is titrated with 90% ethanolic KOH solution, with heating on a water bath. Small increments of potassium hydroxide solution are added to neutralize the solution. After 40 min, the final yellow color indicates completion of the reaction. The carvone content is determined from the endpoint. If necessary, the process may be repeated by the addition of 0.5 mL of 0.5 M KOH and then 1 mL 0.5 M KOH (90%) in ethanol is equivalent to 0.1514 g carvone (Mukherjee, 2002). The details of the method are presented in Fig. 4.47.

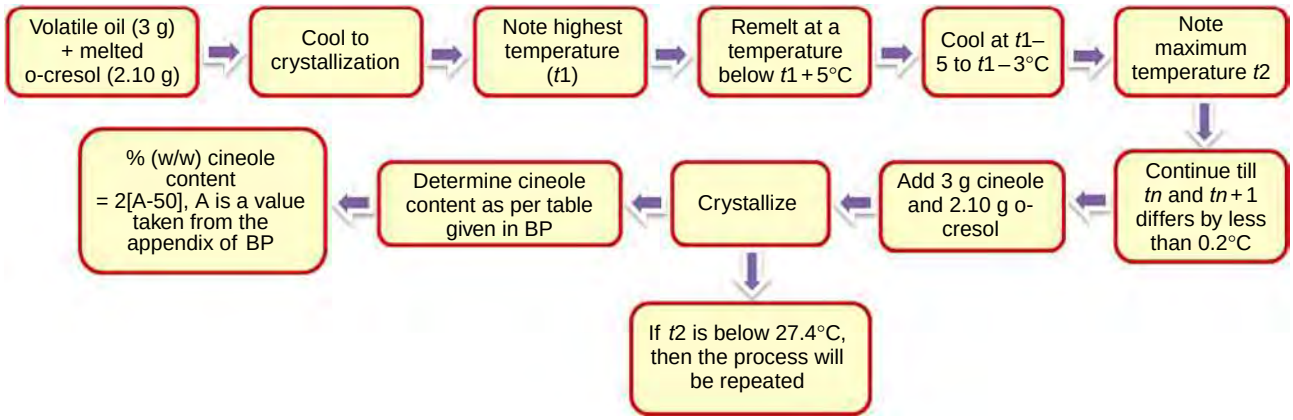

FIG. 4.45 Determination of the cineole content in volatile oil.

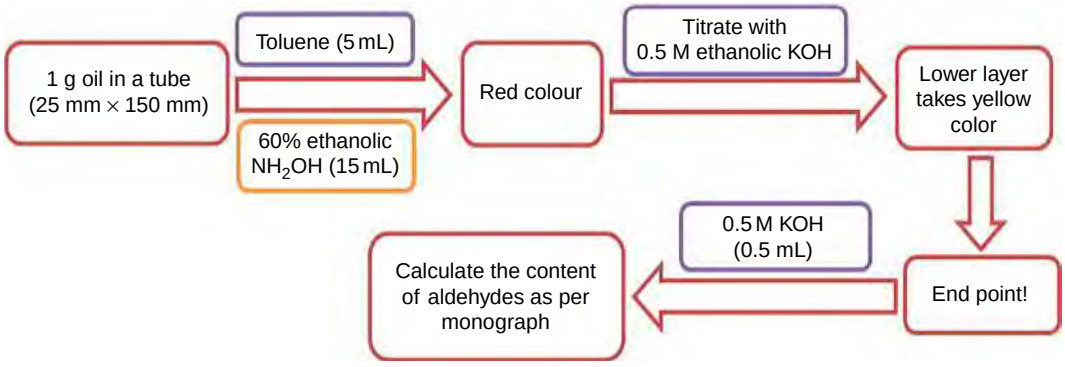

FIG. 4.46 Determination of aldehyde content in the volatile oil.

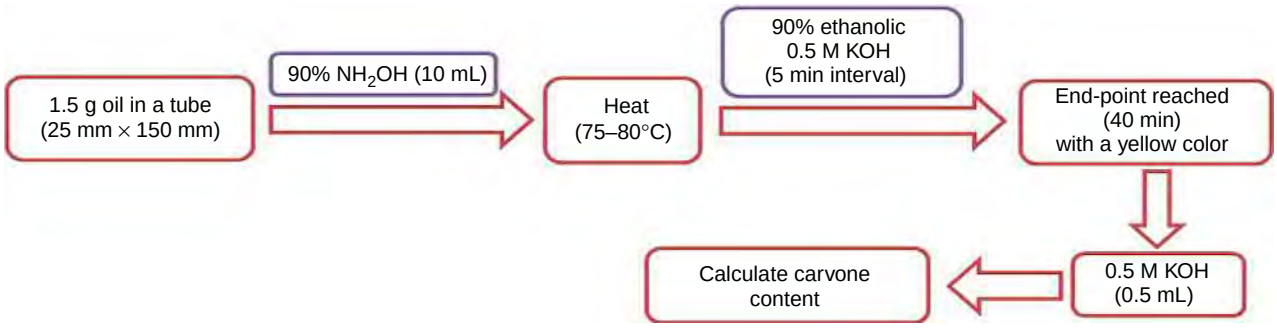

FIG. 4.47 Determination of the carvone content in volatile oil.

After reviewing several research papers, books and official compendia, It is concluded that the essential oil content determination in a crude drug is very important in the case of plants containing volatile oil. The essential oil content is determined by hydrodistillation using a Clevenger apparatus, steam distillation, solvent-free microwave extraction, micro-distillation, the head space analysis method, the solid phase microextraction method, and others. The most commonly used methods are the hydrodistillation method using a Clevenger apparatus and the solvent-free microwave extraction method. The drying method and the extraction method affect the volatile oil content of the crude drug by a significant amount. It is recommended to dry the crude drug under mild conditions, such as ambient air drying, vacuum drying, or infrared drying at low temperature (45°C), which helps in retaining the volatile oil to a great extent. This requires less time also for the extraction process. There are several important parameters that the Pharmacopeias and Formularies recommend for measurement, such as flash point, optical rotation, refractive index, and acid value, which should be estimated according to the official guidelines. There are several other parameters to be estimated for a volatile oil, such as the eugenol content, cineole content, and aldehyde content. These measurements may be performed using titrimetric methods or gas chromatography coupled to mass spectroscopy (Mukherjee, 2002).

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Chapter 5

Morphological and Microscopical Evaluations

Chapter Outline

Quality Control and Evaluation of Herbal Drugs. https://doi.org/10.1016/B978-0-12-813374-3.00005-3

5.1 ORGANOLEPTIC EVALUATION

Medicinal plant materials are categorized according to sensory microscopic and macroscopic characteristics depending on the variations in the sources of crude drugs and their chemical nature; they are standardized by using different techniques, including the different methods for estimation of the chief active constituent. For this purpose, an authentic specimen of the material under study and samples of pharmacopoeial quality should be available to serve as a reference standard (Shah, 2009).

Pharmacognosy is the study of botanicals having medicinal properties. This study includes the physical, chemical, biochemical, and biological properties of herbs for the purpose of searching for new drugs of natural origin. This is a simple and reliable tool to screen commercial varieties, substitutes, and adulterants for complete information of the crude drugs. Authentication of herbal materials includes morphological, microscopic, and chemical identification. This study has some limitations, which are especially important for closely related species and plant parts that share similar morphological or chemical structures (Anonymous, 1998).

Adulteration in the plant samples is a serious issue that involves the deliberate or inadvertent mixture of a plant species by other inferior species, plants of other genera, or even toxic materials. Some important medicinal plants used in several ailments are also adulterated with different plant species. For example, *Hoodia gordonii* is often adulterated with different other inferior species of *Hoodia*. Ginseng is adulterated with other different species, such as the *Platycodon grandiflorus* (Jacq.), *Panax notoginseng*, and is cross-contaminated with different *Panax* species. *Illicium verum* Hook. F, commonly known as the Chinese star Anise fruit, has been adulterated with Japanese star anise fruit (*Illicium anisatum* L.), *I. anisatum*, and others. Such an admixture has been proven to result in serious neurologic disturbances when this plant was used for the treatment of infantile colic (Pferschy-Wenzig and Bauer, 2015).

The identification of a plant species may be carried out in various ways, including organoleptic (macroscopic evaluation), microscopic evaluation, analysis of powder characteristics, fluorescence evaluation, near-infrared evaluation, and evaluation of standardization parameters, such as the ash value, extractive value, moisture content, and total solids.

The authentication of plant material includes many parameters, such as macroscopic observation identification/authentication, including traits such as (1) woody/suffruticose (semiwoody)/herbaceous; (2) leaf shape, size, and morphology (e.g., leaf margins—entire, undulate, dentate, serrate, lobed, pinnatifid); (3) inflorescence characteristics, such as the type of inflorescence (e.g., spike, raceme, panicle, cyme, corymb, helicoid cyme, head); (4) floral morphology (e.g., epigynous/perigynous/hypogynous; stamen number and shape; number of carpels per ovary; number of seeds per carpel); and (5) root characteristics, including surface texture, type (corm, bulb, rhizome), and tissue layering (banding patterns). Macroscopic techniques may be used to discriminate between the desired plant species and plant parts and morphologically similar, yet distinguishable, species that could occur as potential adulterants. They also include chemical analysis and DNA fingerprinting (Smillie and Khan, 2010).

5.1.1 Attributes of Organoleptic Evaluation

There are several analytical aspects for organoleptic evaluation of medicinal plants as described below:

(i) Defined morphological and/or anatomical characteristics of the whole plant or individual plant parts (e.g., leaf, flower, fruit, seed, root and rhizome, bark).

(ii) Characteristic color, fracture, smell, or taste.

- *Identification* is achieved by comparing the morphological characteristics with authenticated or in-house plant reference material or an authoritative technical reference description or test that can assure the identity of the botanical ingredient.
- *Herbs and plant parts* that have been cut or ground to the point at which morphological characteristics are no longer apparent to the unaided eye are best analyzed by microscopic and/or chemical means.

Major attributes to be considered for organoleptic evaluation of medicinal plants are as follows:

(i) It is possible that processing variables may promote some difference in the taste or color of raw herbal materials, thus confounding proper and positive identification by macroscopic or organoleptic techniques. So, not only does the accuracy of the result depend on trained experts, but the robustness of the results is also a reflection of the presence and knowledge of diagnostic characters that enable one plant ingredient to be separated from another.

(ii) It is probable that confidence in the taxonomic or botanical characters, including macroscopic, anatomical, and organoleptic characteristics alone, may not confirm authenticity and possibly may not detect adulterants unless the tests are sufficiently accurate to discriminate the species from the standard material.

(iii) The harvest of plants for use as dietary ingredients often does not coincide with the flowering season of the plant. All of the distinguishing morphological characteristics of the plant are not present at this time. While this is not always a significant obstacle to identification, a manufacturer should use good judgment in determining whether this technique can effectively identify plant material at such times without an identity test that has been proven to distinguish the desired species from known and potential adulterants where the botanical is collected.

(iv) In the case of plants harvested from wild populations, it is possible that material from different locations and different collectors may be mixed prior to identification by the representative specimen. The integrity of such methodology is suspect in such situations unless the training of the collectors and the shipping of the material is sufficient to assure proper identity of all the material and that sampling protocols are designed to detect the adulteration of heterogeneous lots of material.

(v) However, judgment based on the sensory characteristics, such as odor and taste, may vary from person to person and time to time based on the nature of the individual. So, the description of these features are very difficult; often characteristics, such as odor and taste, can only be described as "characteristic" with reference made to the analyst's memory (Abdel-Farid et al., 2007).

The characteristics related to macroscopic or organoleptic evaluation are mostly subjective and substitution by an adulterant, which closely resembles the genuine material, occurs. It is often necessary to substantiate the findings of this evaluation by microscopic and/or physicochemical analysis. The essential basis of any authentication procedure is a comparison between the test sample and another standard sample, the origin of which is known without doubt. No two objects are exactly alike, but a given population of objects can be divided into groups according to similarities and differences between individuals.

5.1.1.1 Color

The color of the crude drugs may indicate the origin of the drug, for example, material derived from the aerial part of the plant is usually green and the underground plant material is usually devoid of green color. For proper examination, untreated samples are examined under diffused sunlight or an artificial light source with wavelengths similar to those of daylight. The color of the sample should be compared with that of the reference material.

Examples: Talka gum, which is used as a substitute for acacia gum, may be identified by its color. Talka gum is usually brown in color and otherwise colorless, whereas acacia is white to yellow.

5.1.1.2 Size

The size of the crude drug, including its length, width, and thickness, are of great importance when evaluating a crude drug. A graduated ruler in millimeters is adequate for this measurement. Small seeds and fruits may be measured by aligning 10 of them on a sheet of calibrated paper.

Examples: Senna is one of the most commonly used laxative drugs. Commercially available species consist of the dried leaflets of Alexandria senna (*Cassia acutifolia* Delile) or Tinnevelly senna (*Cassia angustifolia* Vahl) belonging to the plant family Leguminosae. Variation of the size of the leaves are observed in both the species, e.g., *Cassia angustifolia* is 2–4 cm long, 7–12 mm wide, while *Cassia acutifolia* has the leaf size of 2.5–6 cm long, 7–8 mm wide.

5.1.1.3 Odor and Taste

Odor and taste of a crude material are important sensitive criteria based on an individual's perceptions. If the material is expected to be innocuous, a small portion of the sample can be examined by slow and repeated inhalation of the air over the material. The strength of the odor—weak, distinct, or strong—is first determined and then the odor sensations, such as musty, moldy, rancid, fruity, or aromatic, are determined.

Examples: Capsicum consists of dried ripe fruits of *Capsicum minimum* and *Capsicum annuum* Linn. belonging to the family Solanaceae. This is now being allied with Japanese Chillies and Bombay Capsicum. The original Capsicum has a characteristic odor and an intense pungent taste. The allied species has less pungency (Martín et al., 1998).

5.1.1.4 Surface Characteristic: Texture and Fracture

The texture is best examined by taking a small quantity of material and rubbing it between the thumb and forefinger. It is usually described as "smooth," "rough," or "gritty." Touching the material determines its softness or hardness. The bending and rupturing of the sample provides information on the brittleness as does the appearance of the fractured plane as fibrous, smooth, rough, or granular. All of these characteristics are valuable in indicating the general type of material and the presence of more than one component.

5.2 MACROMORPHOLOGICAL EVALUATION

Based upon the anatomical structure of the plant, the groups or the parts of the plant commonly used for different therapeutic categories are leaves, bark, flowers, fruits and seeds, wood, herb or aerial parts, subterranean organs, and others.

For each group or category of objects, there is a character or group of characters by which the members of the group are related and that distinguish them from the members of other groups. Each group is usually given a name that indicates the sum total of these characters. The actual characters used depend on the objects under consideration, but obviously the larger the number of parameters that are compared, the more accurate the comparison becomes. The simplest example of an authentication procedure is a direct comparison of test and standard samples. This has the advantage that there is little need to define the parameters used with great accuracy. It also has the disadvantage that it requires each analyst to keep a complete reference set of standard samples, and also requires a completely nondestructive examination method of the standard samples, which are to be kept intact.

The application of morphology in drug analysis lies in the field of crude drugs, in which the material is known to occur in a particular form. It can be studied for the whole drug, that is, macromorphography or gross morphology, the study of cell characteristics, that is, cytomorphology, or the study of the morphological character at a particular level. The majority of natural products used as drugs are derived from plants or the parts of plants. Characteristically, the higher plants consist of the vegetative phase of roots, stems, leaves with flowers, and fruits and seeds in the reproductive cycle. Modification of the above structures are frequently present, such as rhizomes (underground stems), stipules, bracts (modified leaves), tendrils (modified stem), and stolons (runners with a stem structure). The interpretation of the morphological characteristics based on different parameters for all the plant organs listed above gives us a first-hand tool to know the features of whole or powdered drugs and adulterants of commercial significance. Some drugs constitute more than one morphological part, for example, whole herbs or roots that constitute both rhizomes and roots. Most species of plant are readily distinguishable in their natural state, but collection, preparation, and drying produce distortion of that natural form, making recognition more difficult. Natural variations in size and shape are common because of environmental factors in which macromorphology plays an important role for preliminary evaluation of the crude drugs (Bauer, 1998).

5.3 MACROMORPHOLOGY OF CRUDE DRUGS FROM BARKS

Bark may be collected in various ways, such as the Felling method (cutting the bark near the ground), the Uprooting method (collecting the bark of both the stem and root), and the Coppicing method (collecting the bark repeatedly). The morphology of a bark is composed of several parts, such as cork (phellum), phellogen, phelloderm (collectively known as periderm), cortex, pericycle, primary phloem, and secondary phloem. The macromorphology of bark drugs for identification and proper authentication can be explained based on the following concepts.

The bark structure shows many cell characters, including cork, pericycle, phloem, and cortex, as follows:

- *Cork cells*	In transverse section they are often tangentially elongated and arranged in regular radial rows. In a surface view, they are frequently polygonal
- *Cell wall*	The cell contents frequently give a positive reaction of tannin
- *Cortex*	This is usually composed of a ground mass of parenchyma; an outer band of collenchyma may often be present

Besides these, sieve tubes, companion cells, phloem parenchyma, and medullary ray cells are always present in the phloem. The cortical cells often contain starch or other typical cell inclusions, such as calcium oxalate crystal. For the cytological evaluation of bark drugs, the following parameters should be noted:

- The presence or absence of outer bark structures, such as cork and phelloderm, along with the site of origin of the cork; the extent, cell structure, and cell content of the cortex have to be noted.
- The presence or absence of sclereids, if present, the distribution, size, and form of sclereids, phloem fibers and secretion cells have to be studied.
- The width, height, distribution, cell structure, and cell content of the medullary rays have to be studied.
- The presence of calcium oxalate crystal, including its crystal form and its distribution, has to be examined critically.
- Transverse and longitudinal sections should be prepared. A preparation treated with cellulose, lignin, starch, oil, and tannin stain should be examined.

In comminute form, the bark always contains sieve tubes and cellulose parenchyma, cork, fibers, calcium oxalate, starch, and other secretary tissues. Xylem tissues are usually absent or present in a small amount, but chlorophyll and aleurone grains are completely absent (Shah, 2009).

5.3.1 Nature of Curvature

(i) *Flat*: When a large piece of bark is collected from an old trunk and dried under pressure, the bark is flat, for example, Quillaia and Aarjuna bark.
(ii) *Curved*: Here, both sides of the bark are curved inside, for example, Wild cherry, Cassia, and Cascara barks.
(iii) *Recurved*: Both sides of the bark are curved outside, for example, Kurchi bark.
(iv) *Channeled*: When the sides of bark are curved toward the inner side to form a channel, for example, *Cascara*, *Cassia*, and *Cinnamon barks*.
(v) *Quill*: If one edge of the bark covers the other edge, it is called a quill, for example, Ceylon, Cinnamon, and Cascara barks.
(vi) *Double quill*: Here, both the edges curve inward to form a double quill, for example, Cinnamon and Cassia barks.
(vii) *Compound quill*: When quills of smaller diameter are packed into larger quills, they are called compound quills. Compound quills are formed to save space in packing and transportation, for example, Cinnamon bark.

According to the extent of curvature, different shapes are assumed for bark and, to explain this, some special terms are used (Fig. 5.1).

5.3.2 Surface Characteristics

Epiphytes, such as lichens and mosses, may appear on the bark surface and the lichens are seen as silver gray patches. The bark may be rossed or scrapped during preparation to give a smooth surface. For the outer surface, the presence of lenticels, cracks or furrows, lichens and moss, and the color before and after scraping should be noted. For the inner surface, the color, striations, and furrows have to be noted. The color and condition of the inner surfaces are of diagnostic value.

(i) *Striations*: When parallel longitudinal ridges are formed during drying, they are called striations and they may be fine or coarse, for example, Cascara bark.
(ii) *Corrugations*: They are the parallel transverse wrinkles formed due to longitudinal shrinkage, for example, Cascara bark.

5.3.3 Characteristics of Fracture

When broken transversely, the behavior of the bark and the appearance of the exposed surfaces are known as "fracture." The fracture depends largely on the number and distribution of the sclereids and fibers. This characteristic of a bark provides us

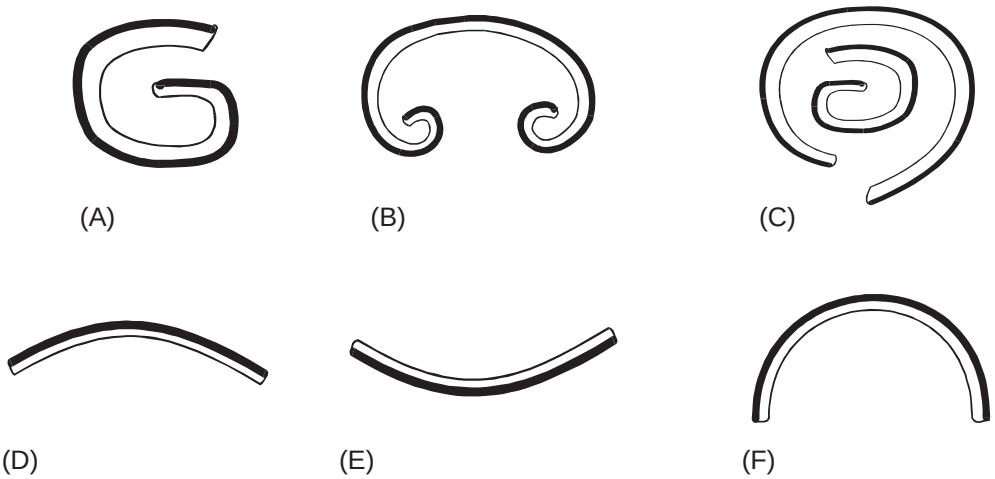

FIG. 5.1 Different curvatures of barks containing herbal drugs. (A) Quill; (B) double quill; (C) compound quill; (D) channeled; (E) recurved; and (F) curved.

with very useful diagnostic features based on the arrangement of various tissues in the bark. The terms commonly used to describe these diagnostic features are as follows:

(i) *Short*: The fractured surface is smooth.
(ii) *Granular*: The fractured surfaces exhibit small, rounded protuberances.
(iii) *Splintery*: Jagged projecting points are formed at the surface.
(iv) *Fibrous*: If fibrous threads extend from the broken surface.
(v) *Laminated*: If the fracture region breaks into tangentially arranged layers.

5.3.4 Characteristics of Transverse Surface

When stained with phloroglucinol and hydrochloric acid (HCl), a smooth transverse surface usually shows the general arrangements of the lignified elements, medullary rays, and cork. The cork cells in a transverse section are often tangentially elongated and arranged in regular, radial rows. Thus, the macromorphological diagnostic features of bark include the shape and the mean dimensions and size of the bark pieces, including the appearance of both the inner and outer surfaces and the form of fracture.

(i) *Smooth*: When development of the cork is even, for example, Arjuna bark.
(ii) *Lenticels*: They are transversely elongated holes formed on the outer surface because of lateral pressure, for example, Wild cherry and Cascara barks.
(iii) *Cracks and fissures*: They are formed due to an increase in diameter, for example, Cinchona bark.
(iv) *Longitudinal wrinkles*: They are formed because of the shrinkage of soft tissues, for example, Cascara bark.
(v) *Furrows*: If the troughs between wrinkles are wide, they are called furrows, for example, *Cinchona calisaya* bark.
(vi) *Exfoliation*: Sometimes the cork of a bark flakes off, exposing the cortex, for example, in Wild cherry bark.
(vii) *Rhytidoma*: This is composite dead tissue consisting of alternate layers of cork, cortex and/or phloem, for example, Quillaia and Tomentosa barks. Sometimes it is removed during peeling.
(viii) *Corky warts*: They are small circular patches that are sometimes found in old bark, for example, in *Cinchona succirubra* and Ashoka barks.
(ix) *Epiphytes*: Epiphytes, such as moss, lichen, and liverworts, are sometimes seen in bark, for example, Cascara bark.

Depending upon the type of incision made in removing them from the tree and the subsequent shrinkage, the shape of the bark varies. Young bark is composed of epidermis, primary cortex, endodermis (which frequently contains starch), pericycle (which usually contains fibers), and phloem (containing sieve tubes). Because of the activity of cork cambium or phellogen, the structure of commercial bark changes. The phellogen may arise in the epidermis, as in a Willow tree, the primary cortex, or the pericycle.

5.3.5 Histology of Barks

The histology of barks may be characterized by the presence of cork cells, parenchymatous phellogen and phelloderm, collenchymatous and/or parenchymatous cortex, parenchymatous or schlerenchymatous pericycle, primary and secondary phloems (sieve tubes, companion cells, phloem parenchyma, phloem fibers, and stone cells), medullary ray cells, starch, calcium oxalate, oil cells, mucilage, etc. (Shah, 2009).

5.4 MACROMORPHOLOGY OF CRUDE DRUGS FROM ROOTS

The root is the descending portion of the axis of the plant and develops from the radical of the embryo. Crude drugs from underground organs usually include stem structures, such as corms, bulbs, stem tubers, rhizomes, and root structures, such as root tubers or adventitious roots. There are three types of root systems.

Tap root system: The radicle grows into the soil and forms the main axis of the root, known as the tap root. It grows further to produce branches in the acropetal manner, known as secondary roots, which further branch to give tertiary roots. These are all true roots. This system is characteristic of dicotyledons.

Fibrous root system: In monocotyledonous plants, the primary root is short-lived and is replaced by a cluster of thin fibrous roots. These roots originate from the base of the stem and constitute a fibrous root system, as seen in the *Oryza sativa* plant.

Adventitious root system: The roots that develop from any part of the plant other than the radicle are called adventitious roots. They may develop from the root base nodes or internodes. This type of root system is found in monocots and in pteridophytes.

5.4.1 Modification for Storage of Food

This type of modification is shown by both types of roots, that is, tap roots and adventitious roots. They store carbohydrates and are used during the early growth of the following season.

(a) *Tap roots* show the following three types of modifications:
- **(i)** *Conical*: These are cone-like, broader at the base and tape-ring at the tip, for example, carrot.
- **(ii)** *Fusiform*: These roots are more-or-less shaped like a spindle, that is, having a taper at both the ends, for example, radish.
- **(iii)** *Napiform*: These are spherically shaped and very sharply tapered at the bottom, for example, beat and turnip.

(b) *Adventitious roots* show the following types of modifications. They store carbohydrates, but do not assume any special shape.
- **(i)** *Tuberous roots*: These get swollen and form single or isolated tuberous roots that are fusiform in shape, for example, sweet potato, jalap, and aconite.
- **(ii)** *Fasciculated tuberous roots*: When several tuberous roots occur in a group or cluster at the base of a stem, they are called fasciculated tuberous roots, as in dahlia, asparagus.
- **(iii)** *Palmated tuberous roots*: They appear like a palm with fingers, as in the common ground orchid.
- **(iv)** *Annulated roots*: The swollen portion is in the form of a series of rings called annules, as in ipecacuanha.
- **(v)** *Nodulose root*: Here, a slender root suddenly becomes swollen near the apex.

5.4.2 Modifications for Support

The plant develops special aerial roots to offer additional support to the plant by way of adventitious roots.

- **(i)** *Clinging or climbing roots*: These types of roots are developed by plants, such as black pepper, for support or for climbing purposes at nodes.
- **(ii)** *Stilt roots*: This type of root is observed in maize and screw-pine. They grow vertically or obliquely downward and penetrate into the soil to give additional support to the main plant, for example, sugarcane, maize.
- **(iii)** *Columnar roots*: In certain plants, such as banyan, additional support is given by specially developed pillars or columnar roots. They even perform the function of regular roots. These are also known as prop or pillar roots, for example, banyan tree.

5.4.3 Modifications for Special Functions

- **(i)** *Respiratory roots or pneumatophores*: The roots of plants growing in marshy places on seashores are unable to respire properly due to continuous water logging. They develop some roots growing against the gravitational force (in the air) with minute openings called lenticels. With the help of lenticels, they carry on the exchange of gases. They look like conical spikes around the stems. This type of root is observed in the case of plants called mangroves that are found in creeks, avicinnia.
- **(ii)** *Sucking roots or Haustoria*: Plants that are total parasites on the host develop a special type of root for the purpose of absorbing food material from the host. These roots neither possess root caps nor root hairs, and are known as sucking roots, for example, cuscuta, striga, and viscum.
- **(iii)** *Photosynthetic roots*: Aerial roots in some cases, especially in leafless epiphytes, become green in color on exposure to sunlight and perform photosynthesis and are known as photosynthetic roots, as in the case of *Tinospora cordifolia*.
- **(iv)** *Epiphytic or Assimilatory roots*: Plants growing on the branches or stems of plants without taking any food from them are called epiphytic and the roots developed by them are called epiphytic roots. They consist of the following:
  - Clinging roots by which they become fixed to the host.
  - Aerial roots that hang freely in the air, which are normally long greenish white in color and absorb moisture from the atmosphere with the help of porous tissue.

(v) *Nodulated roots or Root tubercles*: Plants belonging to the Leguminosae family develop nodules or tubercles. They are formed by nitrogen fixing bacteria and getting carbohydrates from the plants. Roots and bacteria are symbiotic to each other. These swellings developed by roots are nodulated roots.

5.5 MACROMORPHOLOGY OF CRUDE DRUGS FROM STEMS

The ascending part of the plant axis, which bears branches, leaves, flowers, and fruits, is called the stem. It grows above the ground and hence is considered to be the aerial part of a plant. The region bearing the leaves, present at regular intervals on the stem, and its branches are called nodes and the part of the stem that is present between two nodes is called the internode.

The stem of some plants is modified to adapt to present environmental conditions. These modifications are discussed below.

5.5.1 Underground Stem

This modification of the stem generally occurs to store food material where it penetrates the soil and lies below it.

(i) *Rhizome*: Grows parallel or horizontal to the soil surface.
(ii) *Tuber*: Underground stem branch swollen for accumulation of food material.
(iii) *Corn*: Unbranched stem grows vertically under the soil surface.
(iv) *Bulb*: Reduced and disc-shaped stem bud surrounded by many concentric stem leaves.

5.5.2 Stem Tendrils

Auxiliary buds present in the stem to form long, thin thread, like spirally coiled tendrils.

5.5.3 Thorn

Axillary buds lose their ability to grow and are turned into hard, woody, pointed structures.

5.5.4 Aerial Stem

Stems are modified into flattened or fleshy cylindrical structures in arid regions to enhance photosynthetic ability.

5.5.5 Weak Stems

When the stems are thin and long, they are unable to stand erect, and hence may be one of the following types:

(i) *Creepers or Prostate stems*: When they grow flat on the ground with or without roots, for example, grasses, gokharu, etc.
(ii) *Climbers*: These are too weak to stand alone. They climb on a support with the help of tendrils, hooks, prickles, or roots, for example, *Piper betel*, *Piper longum*.
(iii) *Twinners*: These coil the support and grow further. They are thin and wiry, for example, ipomoea.

5.5.6 Subaerial Weak Stem

(i) *Offsets*: Aquatic plants bearing short internodes.
(ii) *Suckers*: Lateral branches grow below the surface of the soil and emerge obliquely and also produce lateral branches originating from the basal and underground portion of the main stem.
(iii) *Runners*: Elongated, prostrate branches with a long internode.
(iv) *Stolons*: Slender lateral branches arising from the base of the main axis and arching downward to touch the ground.

5.5.7 Herbaceous and Woody Stems

These are normal stems and they may be soft or hard and woody, for example, sunflower, sugarcane, and mango.

5.6 MACROMORPHOLOGY OF CRUDE DRUGS FROM RHIZOMES

These are like stem structures growing in a slanted direction at the surface of the ground and for which much of the lower part is surrounded. The surface bears scale leaves with occasional buds in their axils and is often marked with encircling scars of fallen aerial leaves. The lower surface of horizontal rhizomes and the whole surface of vertical and oblique rhizomes bear the roots, which are usually slender and are adventitious. A rhizome appears superficially similar to a root, but it has internal structural arrangements similar to a stem. There are many drugs that are commonly classified as roots, but which actually consist wholly or partly of rhizomes, for example, *Gentian* and *Rhubarb*. As both roots and rhizomes have their own unique problems when diagnosing a crude drug from underground organs, it is better to have a clear idea about their distinguishing characteristics, which will help in the identification and authentication procedure. Rhizomes can be classified based on their form of growth:

(i) *Vertical and oblique rhizomes*: Plants, such as Male Fern, White Hellebore, Green Hellebore.
(ii) *Horizontal rhizomes*:
Dicotyledonous plants—Podophyllum, Arnica, Indian Valerian, Indian Podophyllum, etc.
Monocotyledonous plants—Turmeric, Ginger, Acorus, etc.
(iii) *Corms*: Plants, such as Colchicum and Indian colchicum.

For describing a crude drug from an underground organ, the diagnostic features are given below.

5.6.1 Occurrence or Form of the Rhizome

This includes whether the crude drug is a root, rhizome, or both and whether it is whole or in peeled or sliced form.

5.6.2 Shape of the Drug

It is described by terms such as straight, branching, tortuous, cylindrical, and conical.

5.6.3 Surface Characters

These include the presence of scale leaves, root, color, cracks, wrinkles, scars, lenticels, and annulation.

5.6.4 Transverse Section

The transversely cut surface of a rhizome is always characteristic and is also an aid in the botanical classification of drugs. In rhizomes, the transverse surface never shows a central solid mass of xylem, a useful character that helps to distinguish rhizomes from roots. The color and the distribution of lignified and secretary elements (e.g., in ginger), as well as any abnormalities, such as the absence of a lignin reaction in rhubarb, should be noted. Observation of a transverse surface by a hand lens will give information about tissue arrangements within the structure, for example, the absence of pith, whether the wood is markedly radiate, or any other abnormalities that are found in Jalap and Senega roots. Thus, based on the above characteristics, subterranean organs used as crude drugs can be authenticated or identified and diagnosed properly.

5.7 MACROMORPHOLOGY OF CRUDE DRUGS FROM WOODS

Wood is generally the tissue that lies inside the cambium and chiefly comprises xylem along with other cell types, such as vessels, tracheids, wood fibers, and parenchyma. It has two parts, namely, the outer part, known as sap wood, and the inner part, called heartwood. A transverse section of wood shows annual rings, each of which represent seasonal growth, except in some tropical species in which it is not well marked. There are false annual rings, for example, in the stem wood of *Picrasma excelsa* or *Aeschrion excelsa* of the family Simaroubaceae, commonly known as Quassia. This wood has irregular rings formed by alternating zones of wood parenchyma and fibers. The width and height of medullary rays are of diagnostic importance, as seen in *Jamaica quassia* and Rhubarb spp. The grain of wood primarily results from the arrangement of the annual rings and medullary rays, but is modified by the wavy course of wood elements, which causes the wood to split irregularly. There are only a small number of drugs derived from woods. The description of wood is mostly similar to that of underground organs.

The major studies on the various characteristics of wood include the following parameters.

5.7.1 Size and Color

The central region of wood is called heartwood, while the outer wood that retains its normal functions and appearance is called sapwood. The difference between these two types is very important in studies of crude drugs as the heartwood may not be colored uniformly.

5.7.2 Fracture and Hardness When Split

This characteristic is same as that for underground organs or the barks described earlier.

5.7.3 Transverse Surface

The cells that compose the inner tissues of a stem are not always lignified (and in some cases, the nonlignified elements predominate, for example, wood of Belladonna root). The distribution of the lignified elements is ascertained by treating the smooth transverse, radial, or tangential surfaces with phloroglucinol and hydrochloric acid. The cells of the old wood in trees frequently become colored as they are filled with waste products, for example, resins, tannins, and coloring matter. The distribution of wood fibers and parenchyma and that of true and false annual rings need to be studied.

5.8 MACROMORPHOLOGICAL DESCRIPTION OF THE ENTIRE AERIAL PARTS

When dealing with herbal drugs, sometimes the entire aerial parts of a plant are used as the drug. This consists of the stems and leaves often associated with flowers and young fruits, together called herbs. These materials need to be readily detected in any herb, based on the following descriptions.

For an herb sample, characteristics to study include the shape, color, dimension, whether it is upright or creeping, smooth or ridged, and whether hairs are present or not. Studies on the arrangement of tissues for the presence of structures, such as epidermis, cortex, medullary rays, endodermis, and the presence of pericycle, are required. The vascular bundles of the dictostele are usually collateral or, in some cases, bicollateral, as in curcurbitaceae, solanaceae, and the convulvulaceae family. The differentiation in dicotyledons is usually incomplete as a zone of meristematic cells separates the primary vascular tissues.

5.9 MACROMORPHOLOGY OF CRUDE DRUGS FROM LEAVES

The leaves or leaflets constitute a major portion of the herbal drugs presently used in modern herbal drug development. Leaves are flat, thin, green, appendages to the stem, containing supporting and conducting strands in their structure. They develop in such a way that older leaves are located at the base while younger ones are located at the apex.

5.9.1 Types of Leaves

Taking into consideration the nature of the lamina of the leaves, they are classified into two main groups:

(i) *Simple leaves*: A leaf that has only one leaf blade or lamina is called a simple leaf. It may be stipulate or exstipulate, petiolate, or sessile, but always possesses an axillary bud in its axil. It may have an undivided lamina or may be lobed, for example, vasaka, digitalis, eucalyptus, datura, carica, castor, and argemone.

(ii) *Compound leaves*: A compound leaf consists of more than one leaf blade or lamina and a compound leaf is divided into several segments called leaflets or pinnae, for example, senna, tamarind, and acacia.

Compound leaves have been further classified as follows:

(a) *Pinnate compound leaves*: These are subclassified depending upon the number of rachis (an axis bearing the leaflets in a pinnate compound leaf is known as a rachis).

(b) *Unipinnate compound leaves*: Only one rachis bearing the leaflets is present. When an even number of leaflets is present, it is known as paripinnate, for example, tamarind, gul mohor; if the number of leaflets is odd, it is described as imparipinnate, for example, rose and margosa.

(c) *Bipinnate compound leaves*: These consist of a primary rachis and a secondary rachis. Only the secondary rachis bears the leaflets, for example, acacia.

(d) *Tripinnate compound leaves*: These contain primary, secondary, and even tertiary rachides. Only the tertiary rachis bears the leaflets, as in moringa and oroxylon.

(e) *Decompound leaf*: A compound leaf is irregularly divided, as in coriander, carrot, and anise.
(f) *Palmate compound leaves*: In this type, the leaflets are born by the petiole of the leaf. Depending upon the number of leaflets in a compound palmate leaf, they are further divided as:
- *Unifoliate compound leaf*: Lemon.
- *Trifoliate compound leaf*: Bael and wood apple.
- *Multifoliate compound leaf*: Bombax and alstonia.

5.9.2 Structural Distribution of Leaves

The leaf structure is usually examined in the transverse section of the midrib and lamina. Two types of arrangements are usually observed in the lamina of normal leafy herbal drugs as follows:

(i) *Dorsiventral leaf*: These have a palisade layer below the upper epidermis and a spongy mesophyll above the lower epidermis.
(ii) *Isobilateral leaf*: These leaves have a group of palisade cells inside each epidermis, which are separated from each other by spongy mesophyll.

Beside these, surface characters, such as the presence of trichomes, have to be noted. The mid-ribs and the main veins show vascular tissues in a particular arrangement. The cytological characters, which are consistently present in a leaf, are the epidermis with stomata, cellulose parenchyma, vascular elements, and chlorophyll. Frequently present structures include epidermal trichomes, glands, palisade cells, crystals of calcium oxalate, pericyclic fibers, and collenchyma. For differentiating closely allied leaves, the determination of differential characters, such as the vein islet number, stomatal number, palisade ratio, and stomatal index, play a major role.

5.9.3 Arrangement and Position of Leaves

Through these characters, one particular variety of an herb can be detected. Various terminologies are used to describe the various leaf arrangements as follows:

(i) *Cauline*: Leaves arising from the aerial stem.
(ii) *Radical*: Leaves arising from the crown of the shoot.
(iii) *Adnation*: The fusion of a part of the leaf with the stem takes place here.
(iv) *Opposite, decussate*: The leaves arise in pairs alternately at right angles to the stem.
(v) *Alternate*: The leaves arise from the stem in an alternate manner, for example, *Lobelia* species.

The following features need to be studied when dealing with leaf drugs.

5.9.4 Surface Appearance and Texture

The drugs that are supplied and used in herbal drug industries are in whole or broken form. The texture may be described in different ways, such as "shriveled," "brittle," "leathery," or "coriaceous," "papery," and "fleshy." The color of both the surfaces, upper and lower, including any obvious differences between them, has to be studied based on the following characters:

(i) Leaf surface
It may be of the following types:

(a) *Glabrous*: The surface is smooth and free of hair or any outgrowth, for example, vasaka and datura.
(b) *Rough*: Harsh to touch, for example, digitalis.
(c) *Glutinous*: Covered with a sticky substance, for example, tobacco.
(d) *Glaucous*: Covered with a waxy coating, for example, castor.
(e) *Pubescent*: Covered with straight, short hair, for example, senna.
(f) *Hispid or glandular*: Long and distinct or glandular hairs are present.
(g) *Punctate*: The surface is dotted with oil glands.

(ii) Structure of lamina
(a) Shape of the lamina of leaves

Various shapes of the leaves are due to various types or shapes of lamina. They may be one of the following:

- *Acicular*: Needle-like, for example, *Pinus*.
- *Subulate*: An *acute apex and recurved point, for example, Ephedra sinica.*
- *Linear: It is long, narrow, and flat, for example, grasses.*
- *Oblong: Broad leaves with two parallel margins and an abruptly tapering apex, for example, banana.*
- *Lanceolate: They look like a lance or spear, for example, nerium and senna.*
- *Ovate: Egg shaped or having a broad base and narrow apex, for example, China rose and Buchu.*
- *Obovate: Broad apex and narrow base, for example, Jangali badam.*
- *Obcordate: Inversely heart shaped, that is, the base is narrow but the apex is broad, for example, Oxalis.*
- *Spathulate: Spatula or spoon shaped as in calendula and drosera.*
- *Cuneate: Wedge shaped as in pista.*
- *Cordate: Heart shaped, such as in betel.*
- *Sagittate: Arrow shaped, such as in arum.*
- *Hastate: When the two lobes of a sagittate leaf are directed outward, as in ipomoea.*
- *Reniform: Kidney shaped as in Indian pennywort.*
- *Auriculate: The leaf has ear-like projections at its base.*
- *Lyrate: When it is lyre shaped or the blade is divided into lobes with a large marginal lobe, as in radish mustard.*
- *Runcinate: With the lobes convex before and straight behind, pointing backward like the teeth of a double saw, as in the dandelion leaf.*
- *Rotund (Orbicular): The blade is circular or round, for example, lotus.*
- *Elliptical or oval: The leaves are narrow at the base and apex but broad in the middle, such as in guava and vinca.*
- *Peltate: The* lamina is shield shaped and fixed to the stalk by the center (Hudaib et al., 2002).

In the case of dried leaves in which the original shapes are obscured, they should be soaked in warm water and spread. Common descriptive terms used for describing any lamina shape are given in Fig. 5.2. To describe the intermediate or variable structures of lamina, these terminologies are combined together, for example, ovate to obovate, linear-lanceolate.

The lamina is the flat part of the leaf, which constitutes the major portion of leaf drugs. It can show a very wide variation in its form.

(iii) Composition of lamina

Herbal drugs containing leaves may include true leaves and the individual leaflets of compound leaves. They can be easily distinguished if the attachment of the leaf to the stem can be examined. Various compositions of leaves are shown in Fig. 5.3. It is determined whether the leaf is simple, whether it is pinnate or palmate, and, in a compound leaf, paripinnate (with an equal number of leaflets) or imparipinnate, which depends on the presence of a terminal leaflet.

(iv) Leaf margins

Leaf margins may be of the following types:

- *Entire*: When it is even and smooth, for example, senna and eucalyptus.

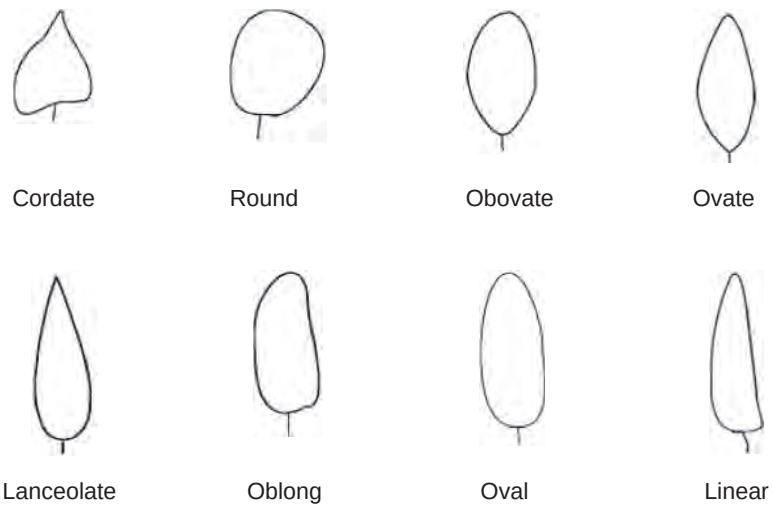

FIG. 5.2 Various shapes of Lamina in leaf drugs.

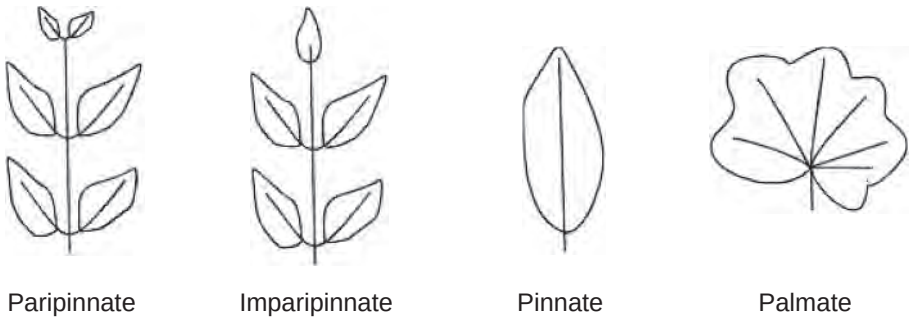

FIG. 5.3 Composition of Lamina in leaf drugs.

- *Sinuate or Wavy*: With slight undulations, as in Ashok.
- *Crenate*: When the teeth are round, as in digitalis.
- *Dentate*: Toothed margin, teeth directing outward, such as margosa and melon.
- *Serrate*: When it is like the teeth of a saw, as in rose and China rose.
- *Ciliated*: It is fringed with hairs.
- *Biserrate*: Lobed serrate margin.
- *Bicrenate*: Lobed crenate margin.

The edge or the margin of a leaf may be entire or with some indentation. These marginal indentations are much smaller than those considered as an incision. The usual terms to describe the different margins are shown in Fig. 5.4.

(v) Incision of leaf

The leaf may be more or less cleft and incision refers to the occurrence of clefts in the edge of the leaf. An increasing depth of incision is indicated by adding -fid, -partite, or -sect as a suffix to the composition terms, for example, palmatifid. The various suffixes used for incision are shown in Fig. 5.5.

(vi) Apices of leaf

The tip of the lamina may be symmetrical or asymmetrical in nature and can appear in a variety of shapes as described in Fig. 5.6. The apex of the leaf may be one of the following kinds:

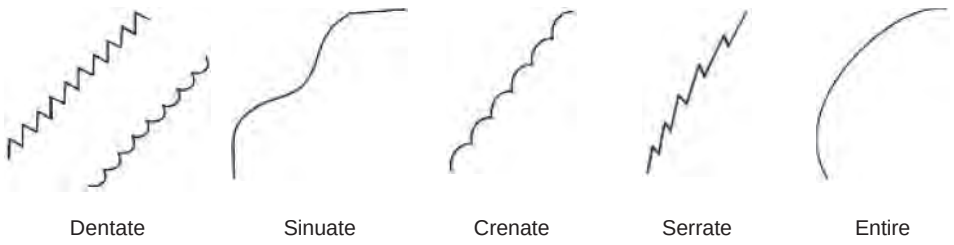

FIG. 5.4 Usual terms used to describe different margins of Lamina in leaf drugs.

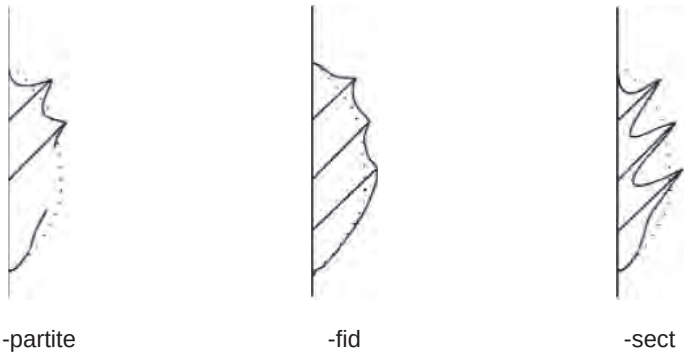

FIG. 5.5 Depth of Incision of Lamina in leaf drugs.

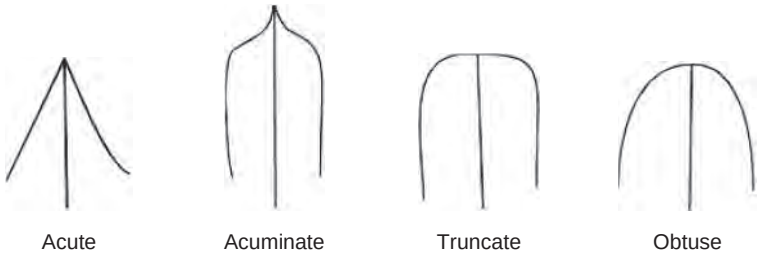

FIG. 5.6 Various shapes of Apex of leaf.

- *Obtuse*: Rounded tip, banyan.
- *Acute*: It is pointed to form an acute angle, but not stiff, hibiscus.
- *Acuminate*: Pointed tip with much elongation, peepal.
- *Recurved*: The apex is curved backward.
- *Cuspidate*: It has a spiny tip, as in the date palm.
- *Mucronate*: A rounded apex ending abruptly in a short point, vinca, and ixora.
- *Retuse*: Broad tip with a slight notch, pistia.
- *Emarginate*: The tip is deeply notched, as in bambinia.
- *Tendrillar*: Tip forming a tendril, such as in Gloriosa—superba.

(vii) Leaf bases

The lower extremity of the lamina of a leaf may exhibit one of the shapes described below. This is the lower extremity of the lamina and it may be symmetrical or asymmetrical with a variety of shapes, such as cordate and decurrent. The various shapes of laminae are shown in Fig. 5.7:

- *Symmetrical*: Equal, as in vasaka.
- *Asymmetrical*: Unequal, as in senna or datura.
- *Decurrent*: As in digitalis.
- *Cordate*: As in betel.

(viii) Venation in leaf

This includes the arrangement of the veins (vascular tissues) on the lamina. Four different types of venation are usually available in commercial leaves, namely, parallel, pinnate (feather like), palmate, and reticulate. The leaf is built up of a protective epidermis, a parenchymatous mesophyll, and a vascular system. The shape, size, and wall structure of the epidermal cells, including the form, distribution, and relation of the stomata to the epidermal cells and the form, distribution, and abundance of epidermal trichomes, are important characteristics in evaluating a leaf drug. Water and minerals absorbed by roots are conveyed to various parts of the leaf by veins and the food synthesized by the leaf through photosynthesis is translocated to other parts of the plant through the veins only. Veins also offer strength, support, and shape to the lamina of the leaf. The prominent vein in the center of the leaf is known as the midrib. In flowering plants, two types of venations exist.

a) *Reticulate venation*: This type of venation is characterized by the fact that many veins and veinlets in the lamina of the leaf are arranged in the form of networks or reticulars. This type of venation is characteristic of dicotyledonous leaves. It is further subclassified as:

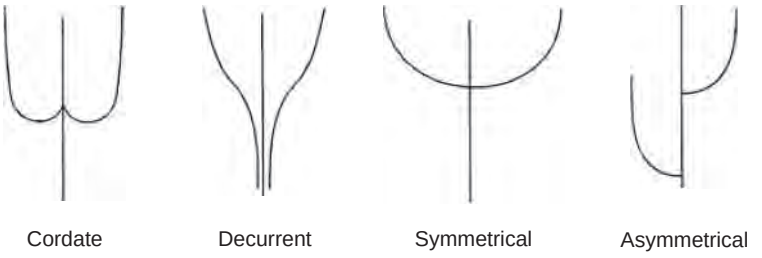

FIG. 5.7 Different bases of leaf drugs.

- *Unicostate-reticulate venation*: The leaf contains only one midrib and several veins are given out on both sides to form a network, such as in henna, eucalyptus, and peepal.
- *Multicostate-reticulate venation*: In this type, many veins of equal strength arise from the end of the petiole. Each vein further branches to give rise to veinlets that form a network. The veins may be convergent (meeting at the apex) or divergent (diverging toward the margin), as in castor, carica, and cucurbita.

b) *Parallel venation*: In this type, the vein and veinlets in the leaf blade are arranged parallel to one another. It is characteristic of monocotyledonous plants with a few exceptions, such as dioscorea and sarsaparilla.

Similar to reticulate venation, there may also be unicostate parallel venation or multicostate parallel venation.

- *Unicostate parallel venation*: Wherein the leaf consists of only one midrib running from the apex to the petiole of the leaf. The veinlets and veins arise parallel to one another on each side, as in banana and canna.
- *Multicostate parallel venation*: In the case of multicostate parallel venation, many main veins of equal strength arise from the tip or the petiole and run parallel to each other. They may be convergent, as in the case of several grasses and bamboo, or divergent, as in the case of the fan palm.

5.10 MACROMORPHOLOGICAL DESCRIPTION OF HERBAL DRUGS CONTAINING FLOWERS

The flower is actually a modified shoot meant for the production of seeds. It consists of four different circles (whorls) arranged in a definite manner. A flower is built up on a stem or pedicel with the enlarged end known as the thalamus or receptacle. Each flower has definite cytological characters, which help in the identification of crude drugs obtained from this organ. The bracts, calyx, and corolla have a leaf structure and yield different cytological characters, such as an epidermis with stomata, glandular and covering hairs, mesophyll cells, oil glands, and various crystals. The epidermal cells of the corolla often have a papillose or striated cuticle. Delicate colored fragments of the corolla can often be distinguished in coarsely powdered drugs. Beside these, the pollen grains, fibrous layer of the anther wall, and the papillose epidermis of the stigmas show some obvious characteristic features for identification.

In a commercial sense, herbal drugs containing flowers include true flowers, buds, and other inflorescence. Floral structure is a complex subject in botanical approaches. Floral drugs of importance are more concerned with production of volatile or essential oil with some specific uses in drugs and cosmetics. The floral drugs of interest can be described as follows.

5.10.1 Receptacle of Floral Drugs

Every flower consists of a receptacle, which is the extremity of the peduncle, on to which four sets of organs are inserted. On the outside, the organ is called a sepal, which is collectively known as calyx, and is usually green; these sepals give a protective function to the flower in the bud stage. Within the sepals, the petals (collectively known as corolla) are present, which are usually brightly colored and conspicuous. Stamens, the male reproductive organs, are present within the petals. Gynoecium, the female reproductive organ, is usually present in the center of the flower and it usually bears a conspicuous style at its apex. When the receptacle is elongated below the calyx, it is called the hypanthium; when it is below the ovary, it is a gynophore (e.g., clove).

5.10.2 Characteristics of Inflorescence

The whole flowering structure of a plant is known as inflorescence. The main axis of inflorescence is called the rachis, while the branches bearing individual flowers or flower clusters are known as pedicels or peduncles, respectively. The inflorescence characteristics of individual flowers vary according to the structure and arrangement of the individual flower to the plant axis. The largest family of flowering plants of therapeutic importance is Compositae. Plants belonging to this family are characterized by a capitulum inflorescence in which a large number of flowers are grouped into a single head. The individual flowers may be identical or the capitulum may bear florets of more than one type. For example, in Pyrethrum there is an outer single row of flattened elongated ray florets surrounding a central mass of squat tubular florets; the apex of the corolla has three teeth, the central one of which is suppressed. These features distinguish Pyrethrum from other related species. Different types of inflorescence in floral drugs are shown in Fig. 5.8. Thus, inflorescences play a major role in characterizing a floral drug and help to differentiate it from other types of adulterants, including flowers of other varieties as follows.

(i) Racemose or indefinite inflorescence:

(a) *Raceme*: In this type of inflorescence, the peduncle is long. Flowers are stalked and born in acropetal succession and the peduncle has indefinite growth and goes on producing flowers, as in mustard, radish, and dwarf gold mohor.

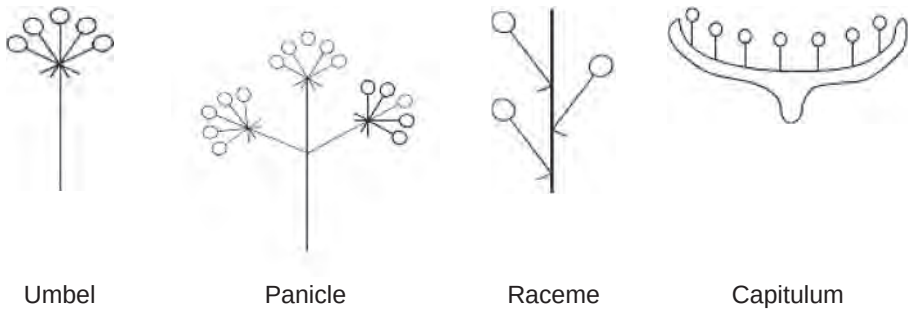

FIG. 5.8 Different types of inflorescence that occur in floral drugs.

When the main axis is branched and the lateral branches bear the flowers, it is said to be a compound raceme or panicle or branched raceme, as in gul mohor, peltophorum, and yuchr.

(b) *Spike*: This is similar to raceme, with flowers as in the Rangoon creeper and vasaka. A branched spike of the polyanthes and terminalia species is known.

(c) *Spadix*: In this inflorescence, the peduncle is short with numerous small unisexual flowers, which are sessile and covered with boat-shaped bract, known as Spathe. Examples of compound spadix include banana, arum, palms, and coconut.

(d) *Catkin*: A spike with unisexual sessile flowers on a long peduncle, as in mulberry and oak.

(e) *Umbel*: The axis is shortened and bears flowers at the top which have an equal stalk and are arranged in centripetal succession. A whorl of bracts is present at the base of the inflorescence, as in coriander, caraway, cumin, and fennel.

(f) *Spikelet*: It is present in the family *Graminae* and is characterized by small and branched spikes. Spikelets are provided with two bracts at the base, known as glumes, and bracteole called palea.

(g) *Corymb*: The peduncle is short, the flowers bracteate, the bisexual oldest flower is at the bottom and the youngest at the apex. The lowermost flower has the longest stack and the youngest has the shortest, lying at same level.

(h) *Capitulum or Head*: In this type, a flattened and expanded peduncle is present, called a receptacle. The base of the receptacle is covered with bracts. The flowers are small and sessile (florets). The flowers toward the periphery are older, while at the center they are younger and open later. Two types of flowers are present, namely, ray florets (strap shaped) and disc florets (tubular shaped), for example, zinnia, cosmos, sunflower.

(i) *Capitate*: Inflorescence similar to the umbel type, except that the flowers are sessile, as in acacia.

(ii) *Cymose inflorescence*: In this type, the growth of the main axis or peduncle is stopped by producing lower. The opening order is centrifugal. Its types are given below:

(a) *Solitary cyme*: Here the inflorescence ends in a single flower, as in datura, capsicum, and China rose.

(b) *Uniparous or Monochasial cyme*: In this type, the axis ends in a flower only; one branch arises just behind and ends in a flower. Uniparous, depending upon the type of branching, is again subdivided into
Helicoid uniparous, which is characterized by branching on one side only.
Scorpioid uniparous, while cyme, characterized by branching on alternate sides.

(c) *Biparous or Dichasial cyme*: This type of inflorescence is characterized by having a flower at the end of the main axis, which is followed by two lateral branches ending again in flowers. Actually, this is a true cyme, as in the cases of Ixora, Teak, and Jasmine.

(d) *Multiparous or Polychasial*: The main axis ends in a flower and a number of flowers are produced laterally in the same manner, as in nerium and calotropis.

(e) *Special type*: This type may include hypanthodium (e.g., Peepal and Figs), verticillasters, such as sacred basil, mentha, and coleus blumi, and cymose-umbel (onion). In some cases, the individual has special characters, which are not covered in any of the types described above.

(iii) *Calyx and corolla*: Based on corolla or petals, the flowers may be Polypetalous or Gamopetalous. A floral drug may be Polysepalous or Gamosepalous, Persistent (e.g., Belladonna), or Caducous (e.g., Poppy); the color, shape, absence

or presence of hair, etc. The presence of any special characteristics like venation (Henbane), oil glands (Clove), etc., has to be studied.

5.10.3 Arrangement of Floral Parts on Thalamus

Depending upon the arrangement of the floral parts on the thalamus, the flowers may be of three types.

- *Hypogynous flower (Superior ovary)*: Brinjal, China rose, mustard, etc.
- *Perigynous flower (Half-superior Ovary)*: Rose, strawberry, peach, etc.
- *Epigynous flower (Inferior ovary)*: Sunflower, cucumber, apple, etc.

5.11 MACROMORPHOLOGICAL DESCRIPTION OF FRUIT DRUGS

A fruit consists of the ripened ovary together with the remains of the style. An herbal drug may be comprised of the whole fruit or some part of it (e.g., orange peel) or the separated seed. The variation in the structure of fruits is very complex. For a morphological description, the following points need to be considered.

5.11.1 Shape and Size

The shape and the general arrangement of the fruit and its external surface play a major role in its identification and authentication. It depends on the arrangement of the cavities in the fruit, the number and position of the seeds, and the point of attachment of the stalk, including the remains of the calyx and the other parts. Based on these criteria, fruits may be classified into different types.

- *Simple fruits*: These are formed from a gynaeceum with one pistil.
- *Aggregate fruits*: These are formed from more than one pistil and thus form and aggregate, for example, Aconite.
- *Collective fruit*: These are formed not only from one flower but from an inflorescence. The family of the Umbelliferae provides a number of therapeutically important components, which are harvested as fruits. The typical inflorescence (Umbel or Panicle type) of this family causes fruit formation.

5.11.2 Types of Fruits

Based on their nature, fruits are of different types, simple, dry, or dehiscent fruits (in which the fruit is formed from one carpel split along both the dorsal and ventral sides [e.g., senna]). This type is called a "legume." Fruit from follicles is found in aggregates and belongs to the follicle type (e.g., Aconite and Strophanthus). The capsule types are the drug dehiscent formed from two or more carpel bearing some special names, such as the silicula of the Crusiferae family or the Pyxis or Pyxidium found in Henbane.

(i) *Simple, dry, and indehiscent fruit*: They consist of different types:

- *Achene*	It is a small indehiscent fruit in which the fruit is formed from one carpel only. When the fruit is formed from two carpels, it is called cypsela, for example, the fruits of the Compositae family
- *Caryopsis*	In this type of fruit, the testa and pericarp are fused together, for example, the Cereals
- *Nut*	This is similar to Achene but typically formed from two or three carpel
- *Schizocarpic or Splitting fruits*	These are bicarpel fruits of the umbeliferae family, usually divided vertically into two halves known as mericarps, each of which contains one seed, which has the outer layer (the testa) fused to the pericarp (fruit wall). The apex of the fruit retains the base of the style with the pericarp bearing a number of ridges running along its length. Each mericarp has five prominent ridges. Thus, distinguishing between the umbelliferous fruits and others, even in the intact form, is not too difficult due to these obvious variations

(ii) *Succulent fruits*: Fruits under this classification are of two types:

Berry	These are formed from one or more carpels and the pericarp is entirely fleshy. They contain many seeds, for example, colocynth, orange, lemon, and capsicum
Drupe	These fruits are typically formed from one superior carpel, the inner part of the pericarp, which is called the endocarp, is hard and woody and usually encloses one seed, for example, almonds and prunes (Horwath et al., 2008)

5.12 MACROMORPHOLOGICAL EVALUATION OF CRUDE DRUGS OBTAINED FROM SEEDS

The seed is a fertilized ovule and is a characteristic of Phanerogams. The parenchymatous body of the ovule, known as the nucellus, contains an embryo sac in which the fertilization of pollen cells takes place, giving rise to an embryo. The seeds are characterized by the presence of three parts, known as the embryo, endosperm, and the seed coat. Many drugs are derived from fruit seeds, so full descriptions of the seed characters are essential. Seeds may be produced from orthotropous, campylotropous, or anatropous ovules. There are several morphological descriptions of seeds and they are given below.

5.12.1 Shape of the Seeds

The shape depends to some extent on the manner in which the ovule was suspended in the ovary. This also affects the position of certain characters of the seeds, which include the following:

- *Hilum*	This is the scar left by the detachment of the seed stalk, where it separates from the funicle
- *Raphe*	This includes the vascular strand that connects the ovary to the ovule
- *Micropyle*	This is a minute pore through which the fertilizing pollen tube enters into the ovule
- *Caruncle or Strophiole*	These are the protuberances arising from the testa near the hilum
- *Testa*	This is the outer layer of the seed, which is very hard. Testa may contain several layers, at least one of which has a definite protective function. The testa may carry various outgrowths, such as aril (a fleshy layer partly covering the seed) or a caruncle (e.g., an oil-containing area near the micropyle in castor oil seed). Different surface characters of seed has been shown in Fig. 5.9
- *Kernel*	The kernel may consist of the embryo surrounded by endosperm or perisperm or both (e.g., albuminous seed). The endosperm and perisperm are tissues containing food reserves inside and outside the embryo sac
- *Embryo*	The internal structure of a seed consists of the embryo, which is usually small and contains some form of food reserve. This may be stored in the swollen cotyledons of the embryo (e.g., exalbuminous seed) or may occupy a separate endosperm layer surrounding the embryo (e.g., albuminous or endospermic seed). Seeds contain many materials, including fixed oil, starch, other carbohydrates, and proteins

Anderson and Smith (2002).

Thus, the description of seeds should include size, shape, color, and general appearance of the seed, and the occurrence of any testa outgrowth. It should also include the distribution of the hilum, raphe, and micropyle, including the embryo and nature of the food reserve.

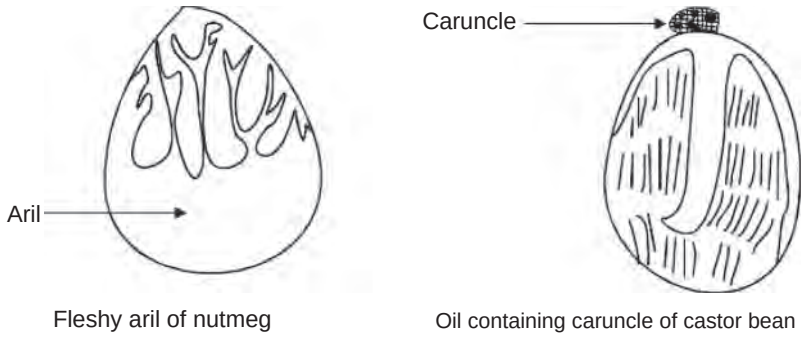

FIG. 5.9 Surface characters of some seed drugs showing testa outgrowth.

5.12.2 Seed Coat

The seed coat is the outermost layer of the seed, which provides necessary protection to the embryo inside the seed. In the case of dicotyledonous seeds, normally, they are hard and may contain two layers; the outermost thick layer is known as the testa, while the inner, thin layer is known as the tegmen. In monocotyledonous seeds, it is thin or may even be fused with the wall of the fruit.

(i) Embryo

The embryo is the main part of the seed. It consists of an axis having apical meristem for plumule, radicle for the origin or root, and adhered to it are one or two cotyledons, which differentiate the plants as monocot or dicot.

(ii) Endosperm

The endosperm is the nutritive tissue nourishing the embryo. Depending upon its presence or absence the seeds are classified as follows:

(a) *Endospermic or Albuminous seeds*: In these seeds, part of the endosperm remains even up to the germination of the seed and is partly absorbed by the embryo. Therefore, these seeds are known as endospermic seeds and are seen in Colchicum, Isabgol, Linseed, Nux vomica, Strophamthus, Wheat, and Rice.

(b) *Nonendospermic or Exalbuminous seeds*: During the development of these seeds, the endosperm is fully absorbed by the embryo and endosperm, and is not represented in the seeds; hence, they are known as nonendospermic, for example, sunflower, tamarind, cotton, and soybean.

(c) *Perispermic seeds*: Herein the nucleus develops to such an extent that it forms a big storage tissue and seeds are found to contain embryo, endosperm, perisperm, and seed coat; for example, pepper, cardamom, nutmeg, and guinea grains. There are some characteristic features in seeds, which are given below:

- *Hilum*: This is the point of attachment of the seed to its stalk.
- *Micropyle*: It is the minute opening of the tubular structure, wherefrom water is provided for the germination of seeds.
- *Raphe*: Raphe is described as a longitudinal marking of an adherent stalk of an anatropous ovule.
- *Funicle*: It is the stalk of the ovule that attaches it to the placenta.
- *Chalaza*: This is the basal portion of the ovule where the stalk is attached.

5.12.3 Special Features of Seeds

Sometimes, apart from the regular growth of seeds, additional growth is visible in the form of appendages, which have special features. They are described as:

- *Aril*: Succulent growth from a hilum covering the entire seed, as in nutmeg (mace) and yew seeds.
- *Arillode*: Outgrowth originating from the micropyle and covering the seed, as in cardamom.
- *Arista (awn)*: Stiff, bristle-like appendage with many flowering glumes of grasses and found in strophanthus.
- *Caruncle*: A warty outgrowth from the micropyle, as in castor, croton, and viola moringa.
- *Hairs*: Gossypium and calotropis are examples of this type of outgrowth.

5.13 MACROMORPHOLOGICAL EVALUATION OF CELLULAR MATERIALS OR UNORGANIZED DRUGS

Unorganized drugs are those that have no specific cellular structure. A number of plant-derived drugs containing materials, such as exudates and crude extracts, volatile oils, fixed oils, fats, waxes, oleoresins, and balsams, are in use. Materials obtained from animals and minerals are also important as drug and pharmaceutical adjuvants. They do not have definite forms of structural configurations like other plant drugs, but their form is dependent upon the source materials and preparation procedures, which can be defined within certain limits. As these materials do not contain any cells, they are described as cellular products or unorganized drugs. The majority of cellular products are solid, although liquids, such as fixed oil, are also possible. Unorganized drugs can be classified according to their origin and nature, giving well-characterized groups, such as dried latex (e.g., opium), dried juice (e.g., aloes), extracts (e.g., catechu), gums (e.g., acacia), resins (e.g., colophony), gum resins (e.g., myrrh), oleoresins (e.g., copaiba), waxes (e.g., beeswax), saccharine substances (e.g., honey), oils and fats (e.g., castor oil), and volatile oils (e.g., clove oil).

5.13.1 Evaluation of Physical State

Several characteristic features need to be observed for unorganized drugs. For the solid unorganized drugs, the parameters to be studied are given below (Ariyama et al., 2004).

- *Size and shape*	Tears, lumps, and bulk, along with the approximate size and weight have to studied
- *Color*	The color of the material, whether single or varied, has to be noted
- *Surface appearance*	The appearance of the surface, for example, shiny or dusty, opaque or translucent, dull, smooth, or cracked and whether there is a perceptible fragrance, has to be studied carefully

5.13.2 Vegetable Debris

Depending on the method of collection and preservation, some vegetable debris may be present in the original material, which should be carefully detected.

5.13.3 Effect on Heating

Whether the material melts while heating or chars, sublimes, or burns without leaving any ash has to be studied carefully.

5.13.4 Morphological Characteristics of a Few Unorganized Herbal Drugs

(i) Dried latex

Latex is an emulsion or a suspension, the continuous phase of which is an aqueous solution of mineral salts, proteins, sugars, tannins, and alkaloids, among others, and the suspended particles are oil droplets, resins, gum, proteins, starch, and other substances. This turbid fluid is often white in color, as in the opium poppy, *Papaver somniferum*, but may be yellow or red in color. It occurs in plants in special structures called Laticiferous cells, tubers, or vessels, from which it is obtained by incision into the plant.

(ii) Resins

Resins are a heterogeneous group of materials, which may be associated with volatile oils or gums. They are found in irregular masses that are insoluble in water but soluble in alcohol. They are brittle, amorphous solids that melt to produce an adhesive liquid and burn with a smoky flame. Resins occur together with oleoresins (e.g., colophony); with gums in gum resins (e.g., myrrh); and as a solution in liquid esters in balsams (e.g., balsam of tolu). All of them are usually secreted into secretary cavities or ducts. They also differ in their physical characters, such as liquid ester resins (balsam of tolu, etc.), which are syrupy liquids. From these materials, the more volatile portions evaporate, thicken, and finally solidify.

(iii) Gums and mucilage

They are the polysaccharide complexes formed from sugar and uronic acid units. Usually, they are insoluble in alcohol but they dissolve or swell in water. They are the plant exudates formed from the cell wall. Their form or structure as unorganized drugs depends on several factors, such as the type of incision made to release the material, the nature of the exudate, the drying procedure, and any subsequent treatment. An example of such a type is tragacanth, which exudes rapidly under pressure through a narrow slit and hardens to form a vermiform ribbon with longitudinal striations. Gum acacia, exudes slowly and dries into ovoid tears, which are then usually sun bleached. Thus, the overall macromorphology of these exudates is an important criterion in the quality assessment.

(iv) Saccharine substances

Honey is a saccharine fluid made by the *Apis mellifica* Linn. family Apidae, from the nectar of flowers. In some instances, bees collect other sweet plant juices also, such as the honey dew formed by the agency of aphids from the leaves of trees, such as pine and lime. Honey is a viscid, translucent, nearly white to pale yellowish or yellowish-brown fluid. It becomes partially crystalline and semisolid in storage, due to the separation of dextrose as crystals. It has an agreeable characteristic odor and taste depending upon the nature of the flowers from which the nectar was collected. The specific rotation of honey is from $+3°$ to $-10°$. The most common adulterants are invert sugars, sucrose, and commercial glucose, all of which alter the rotation of the honey.

(v) Dried extracts and juices

This includes a number of herbal drugs of therapeutic importance. Aloe is the dried juice drained from cut leaves of various species of Aloe, such as *Aloe ferox*, *Aloe barbadensis*, and *Aloe perryi*. Opium is dried latex from the capsule of the opium poppy, and catechu is the dried aqueous extract of some tannin-containing plants. All these products differ individually in composition and show variations in their form according to the source of the particular sample.

(vi) Fixed oils and fats

Fixed oils and fats are widely distributed and occur in both vegetable and reproductive structures. They often occur in seeds as reserve food material and are not uncommonly associated with protein reserves. Like lipids, fat forms an essential component of biological membranes. Reserved fats occur as solids, frequently colored, or crystalline masses, which melt on warming. They are soluble in ether and alcohol (with a few exceptions, for example, castor oil is sparingly soluble in alcohol). Fixed oils occur as small highly refractive drops. Fixed oils, when treated with a 1% solution of osmic acid, acquire a brown or black color and they acquire a red color when treated with a diluted tincture of alkanna. Fixed oils and fats are slowly saponified when mixed with a cold mixture of equal parts of a saturated solution of potash and a strong solution of ammonia. Sometime after such a treatment, characteristic soap crystals may be observed.

(vii) Volatile oils

Volatile oils occur as droplets in the cell. They are sparingly soluble in water but dissolve in alcohol. They resemble fixed oils in their behavior toward osmic acid and alkanna, but are not saponified when treated with ammoniacal potash. Detailed characteristic studies, including chemical, instrumental, and other parameters, will be discussed later in quality control and standardization of cellular products.

5.14 CYTOMORPHOLOGICAL EVALUATION OF HERBAL DRUGS

Cytomorphology includes the examination of the cell form and the arrangement of different cells in a drug. Plant drugs are generally used in powdered or comminuted form in which the macromorphology is destroyed, so that the evaluation of the microscopical cell characters is essential. Consideration must therefore be given to the types of cells and cell inclusions and the manner in which they are distributed in different organs and drugs. Cytomorphological characters play a major role in drug identification. The plant drugs contain some basic cell types, for example, parenchyma, collenchyma, sclerenchyma, epidermis, xylem, phloem, and periderm, along with some cell inclusion characteristics, such as the presence of ergastic substances, such as starch, calcium oxalate, calcium carbonate, aleurone, silica, and various other cell contents. Analysis of plant drugs based on the distribution of these various cell types within different organs is important to ensure the identity and quality of herbal drugs (Bridson and Forman, 1992).

5.14.1 Analytical Application of Cytomorphology

The basis of the analysis using cytomorphological characters is that there are always sufficient differences in the same type or in different types of plants as far as cell characteristics are concerned. Such differences may be very prominent, for example, the presence or absence of a particular specialized cell (such as sclereid) or it may be so marginal that they need to be established by optical micromeritics.

Standardization profiles are not available for most herbal drugs. As with any comparative procedure, the more information that is available to build up a profile of the sample, the more accurate the analysis becomes. Cytomorphological information of a particular drug can be stored and retrieved in various ways. Different pharmacopoeial monographs, textbooks, and atlases of powdered drug provide detailed information on many herbal drugs. Often, these descriptions of the whole drug and powder differ in detail, which is due to the relative resistance of the individual components during the powdering process. For example, sieve tubes are not found in intact powders, whereas sclereid groups are frequently present. However, a considerable aggregation of cellular components is usually found in powders and, thus, cytomorphological study enables us to give a picture of the tissue distribution in many plant drugs (Pferschy-Wenzig and Bauer, 2015).

It is not true that all the features in a particular drug's morphology are of equal value in its identification. Only those features that are usually unique are of the greatest value, as they differ from adulterants, and only the evaluation of the cytomorphological characters can provide such information. From an evaluation of the general types of cells and cell inclusions, it is not too difficult to classify a given powder into a particular morphological group (e.g., the presence of starch is an indication of an underground storage organ and aleurone grains are an indication of a seed). After evaluating cell characters within each morphological group, the characters of the most distinctive types of tissue in different drugs are

then compared, which gives the proper identity of a particular crude drug. In this way, the parameters are carefully selected so as to allow the maximum number of materials to be accurately identified by a minimum number of criteria.

It is obvious that any system of identification of herbal drugs cannot be foolproof. In the case of a completely unknown drug, the cytomorphological descriptions applied to another drug can be used and, thereby, a conclusion can be achieved as to a relative pattern. To remove any doubt and to maintain the validity of the analysis, observation of a number of microscopical preparations has to be performed and analytical observations have to be repeated. Thus, by using cytomorphological parameters (which may be based on minimal information), the final identification of any crude drug is possible based on a comparison of available parameters. Dermal tissue systems help in characterizing crude drugs in different ways, as described later.

5.14.1.1 Epidermis

This layer is the outermost layer in the plant body, which comprises a single layer of flattened cells. The cell walls may be straight, wavy, or beaded, and often covered with a layer of cuticle made up of cutin.

5.14.1.2 Stomata

Stomata, or "*stoma*" as referred to in different literatures, are minute openings in the epidermis of plants. They may occur in leaves, flowers, young green stems, and fruits also. They are generally surrounded by a pair of kidney-shaped "guard cells." These stomata serve as identifying symbols for a particular plant. According to the arrangement of the epidermal cells surrounding the stomata, they have been grouped as follows:

(i) *Diacytic or Caryophyllaceous (cross celled)*: The stoma is accompanied by two subsidiary cells, the long axes of which are at right angles to that of the stoma. This type of stoma is also known as the Labiatae type, as it is found in many plants of the family Labiatae, such as vasaka, tulsi, spearmint, and peppermint.

(ii) *Anisocytic or Cruciferous (unequal celled)*: The stoma is usually surrounded by three subsidiary cells of which one is markedly smaller than the others. This type of stoma is also called the Solanaceous type as it is found in many plants of the family Solanaceae, such as Belladonna, Datura, Hyoscyamus, Stramonium, and Tobacco; it is also found in many plants of the family Compositae.

(iii) *Anomocytic or Ranunculaceous (irregular celled)*: The stoma is surrounded by a varying number of cells that in no way differ from those of the epidermal cells, as in digitalis, eucalyptus, henna, lobelia, and neem.

(iv) *Paracytic or Rubiaceous (parallel celled)*: The stoma is surrounded usually by two subsidiary cells, the long axes of which are parallel to that of the stoma, as in senna and many rubiaceous plants.

(v) *Actinocytic (radiate celled)*: The stoma is surrounded by a circle of radiating cells, as in *Uva ursi*.

5.14.1.3 Trichomes

Trichomes are more elongated outgrowths of one or more epidermal cells, and consist of two carts, a foot or root embedded in the epidermis and a free projecting portion called the body. Trichomes usually occur in leaves, but are also found to be present on some other parts of the plant. Trichomes are rarely present in several plants, such as bearberry, buchu, and henna. Trichomes are absent in glabrous leaves, such as coca, hemlock, and savin. Trichomes occur in different plant organs, such as:

- Seeds of kurchi, Nux vomica, and strophanthus.
- Stems of andrographis and belladonna.
- Fruits of cumin and lady's finger.
- Ubiquitously present in leaves of plants.

Types of trichomes: Depending upon the structure, shape, and number of cells, they are further classified as follows:

(i) Covering trichomes which can be unicellular or multicellular
  (a) Unicellular trichomes
    - Linear, strongly waved, thick-walled trichomes—*Yerba santa*
    - Linear, thick-walled, and warty trichomes—*Damiana*
    - Short, conical trichomes—*Tea*
    - Short, conical, warty trichomes—*Senna*
    - Large, conical, longitudinally striated trichomes—*Lobelia*
    - Long, tubular, flattened, and twisted trichomes—*Cotton*
    - Lignified trichomes—*Nux vomica*, *strophanthus*

- Short, sharp, pointed, curved, conical trichomes—*Cannabis*
- Unicellular, stellate trichomes—*Deutzia scabra*

(b) Multicellular branched trichomes
- Stellate (star shaped)—*Hamamelis*, *Kamala*
- Peltate (shield-like structure)—*cascarilla*
- Candelabra (branched)—*Rosemary*, *Verbascum thapsus*
- T-shaped trichomes—*Pyrethrum*
- Multicellular unbranched trichomes
- Uniseriate, bicellular, conical—*Datura*
- Biseriate—*Calendula officinalis*
- Multiseriate—*Male fern*

(ii) Glandular trichomes

(a) Unicellular glandular trichomes
- Sessile trichomes without stalk—*P. betel*, *vasaka*

(b) Multicellular glandular trichomes
- Unicellular stalk with single spherical secreting cell at the apex—*Digitalis purpurea*
- Uniseriate, multicellular stalk with single spherical cell at the apex—*Digitalis thapsi*
- Uniseriate stalk and bicellular head—*D. purpurea*
- Multicellular, uniseriate stalk, and multicellular head—*Hyoscyamus*
- Biseriate stalk and biseriate secreting head—*Santonica*
- Short, unicellular stalk, and head formed by a rosette of two to eight club-shaped cells—*Mentha*
- Multiseriate, multicellular cylindrical stalk, and a secreting head of about eight radiating club-shaped cells—*Cannabis*.

5.14.2 Basic Cell Types and Cell Inclusions Present in Herbal Drugs

When considering the cytomorphological aspects, in the case of whole drugs, the cell distribution can be determined by sectioning and in powders some degree of cellular aggregation and organization is retained. To evaluate all the cell parameters, the distribution of the basic cell types, as well as cell inclusion, has to be studied. The basic cell types include different cellular parameters of plant cells as detailed in the following.

5.14.2.1 Parenchyma

It occurs as general ground tissue in most plants. These are usually isodiametric and thin walled and the simplest type of cell. By the time of maturity they may have intercellular spaces. Secondary thickening may be present in reticulate or pitted form, which can be lignified. The cytomorphology of different types of parenchyma is shown in Fig. 5.10.

5.14.2.2 Sclerenchyma

This is hard supporting tissue with heavy secondary thickening.

- Long, narrow, thick, and lignified cells, pointed at both ends.
- Fiber-like in appearance.
- The middle lamella is conspicuous.
- They give the requisite strength, rigidity, flexibility, and elasticity to the plant body.

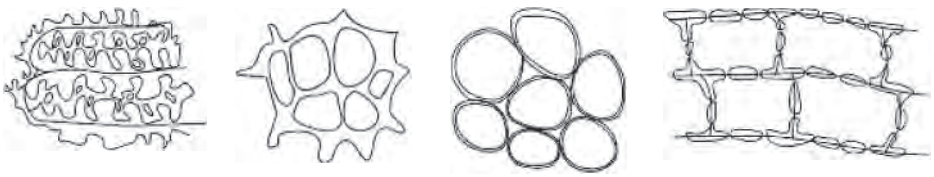

FIG. 5.10 Cytomorphology of different types of parenchyma present in herbal drugs.

They are generally divided into two categories as follows:

(i) Stone cells or sclereids

They are roughly isodiametric, although an elongated and branched form may also occur. They are found singly or as a complete layer or in groups with pitting and stratification often accompanying them. They commonly occur in the hard outer coat of seeds and fruits and the pericyclic regions of woody stems.

(ii) Fibers

They are thick walled, long, and wide. Fibers are classified based on the area in which they occur as pericyclic, xylem, or phloem fibers. Crystal sheaths are sometimes formed and this feature, together with a different size frequency and distribution, plays an important role as a diagnostic feature. The ground mass of the secondary xylem of *Picroena excelsa* is built up of compactly arranged thick-walled wood fibers. The secondary xylem of licorice contains wood fibers arranged in bundles. The phloem fibers of licorice resemble those of the xylem in being enclosed in a crystal sheath. The distribution, abundance, size, and shape of the phloem fibers constitute important characters for the differentiation of medicinal bark. Phloem fibers occur isolated or in irregular rows in the bark of *Cinnamon*, *Cassia*, and *Cinchona*, which constitutes a prominent feature of those powders.

5.14.2.3 Collenchyma

It is a supporting tissue directly derived from parenchyma with greater mechanical strength. Secondary thickening is much greater and composed of cellulose. The thickening may be stratified or unevenly distributed around the circumference of the cell. Collenchyma is present above and below the midrib bundle in many leaves, for example, *Hyoscyamus*, *Digitalis*, *Senna*, and *Belladonna*. The cells in collenchyma are four to six sided in the transverse section, axially elongated. The wall of this tissue has considerable plasticity and so it constitutes the typical mechanical tissue of herbaceous stems and the petiole or mid-ribs of leaves. Collenchyma tissues are present in the cortex of many barks (*Cascara sagrada*) and in the wings of stems (*Lobelia*).

5.14.2.4 Xylem

This is the principle water conducting tissue of a plant. They have lignified secondary thickened walls that can show a variety of forms. Secondary growth in the thickness of stems and roots is usually accompanied by the formation of secondary xylem. Xylem or wood is a conducting tissue and is composed of different kinds of elements: (1) tracheids, (2) vessels or tracheae, (3) wood fibers, and (4) wood parenchyma. Xylem, as a whole, is meant to conduct water and mineral salts upward from the root to the leaf to give mechanical strength to the plant body.

(i) Tracheids

These constitute the basic cell type of xylem tissue. They are elongated, tube-like cells with hard, thick, and lignified walls and large cell cavities. Their ends are tapered, either rounded or chisel-like and, less frequently, pointed. They are dead, empty cells and their walls are provided with one or more rows of bordered pits. Tracheids may also be annular, spiral, scalariform, or pitted (with simple pits). In transverse section, they are angular—either polygonal or rectangular. Tracheids (and not vessels) occur alone in the wood of ferns and gymnosperms, whereas in the wood of angiosperms, they are associated with the vessels. Their walls being lignified and hard, their function is to conduct water from the root to the leaf.

(ii) Vessels or tracheae

Vessels are cylindrical, tube-like structures. They are formed from a row of cells placed end to end, from which the transverse partition walls break down. A vessel or trachea is, thus, a tube-like series of cells, very much like a series of water pipes forming a pipeline. Their walls are thickened in various ways, and vessels can be annular, spiral, scalariform, reticulate, or pitted, according to the mode of thickening. Associated with the vessels are often some tracheids. Vessels and tracheids form the main elements of the wood or xylem of the vascular bundle. They serve to conduct water and mineral salts from the roots to the leaves. They are dead, thick-walled, and lignified, and as such, they also serve the mechanical function of strengthening the plant body. They constitute the fundamental conducting elements of the xylem in angiosperm. The most primitive types of vessels consist of a vertical series of tracheid-like segments, whereas the advance types of vessels show complete dissolution of the end walls to give a slit-like opening. The essential difference between tracheids and vessels is that the former are imperforate, whereas the latter have pores at each end that are connected to form a continuous file or tube. They are present in gentian, clove, squill, and most leaves of herbal interest, for example, stramonium and belladonna.

(iii) Xylem (wood) fibers

Sclerenchymatous cells associated with wood or xylems are known as wood fibers. They occur abundantly in woody dicotyledons and add to the mechanical strength of the xylem and of the plant body as a whole.

(iv) Xylem parenchyma

These cells are generally thin walled, alive, and axially elongated, sometimes thin walled but often with thickening and lignification. They are also known as wood parenchyma. The wood parenchyma assists, directly or indirectly, in the conduction of water, upward through the vessels and the tracheids. It also serves to store food and in some cases the cells are blocked with starch (e.g., *Cephaelis ipecacuanha*).

5.14.2.5 Phloem

Phloem, as a whole, is meant to conduct prepared food material from the leaf to the storage organs and growing regions. The phloem or bast is another conducting tissue and is composed of the following elements:

(i) Sieve tubes:

Sieve tubes are slender, tube-like structures, composed of elongated cells, placed end to end.

- The walls are made of cellulose, with perforations.
- At the end of a growing season, the sieve plate is covered by a layer of carbohydrates, resulting in a layer called the callus pad.
- Through these pores, the tubes have cytoplasmic connections.
- Used for the longitudinal transmission of prepared food materials.

(ii) *Companion cells*: Associated with each sieve tube and connected with it by pores is a thin walled, elongated cell known as the companion cell. It is living and contains protoplasm and an elongated nucleus. The companion cell is present only in angiosperms (both dicotyledons and monocotyledons). It assists the sieve tube in the conduction of food.

(iii) *Phloem parenchyma*: There are always some parenchymatous cells forming a part of the phloem in all dicotyledons, gymnosperms, and ferns. The cells are living, and often cylindrical. They store food material and help to conduct it. Phloem parenchyma is, however, absent in most monocotyledons.

(iv) *Bast fibers*: Sclerenchymatous cells occurring in the phloem or bast are known as bast fibers. They are generally absent in the primary phloem, but occur frequently in the secondary phloem.

This compound tissue is responsible for the transport of food. It contains companion cells, sieve tubes, phloem parenchyma, and secretary cells. The sieve tube is the conducting element in phloem. The sieve elements are highly specialized cells in phloem and the main morphological characteristic is the occurrence of sieve in the walls, which may often be detected by recognition of the callus pad that shows some of the following staining characters:

- Chlor-zinc-iodine solution stains callose to a reddish brown color.
- Aniline blue stains it to callose blue.
- A solution of ammoniacal copper nitrate (BP) does not dissolve cellulose.

Though the sieve elements are usually broken during powdering of herbal drugs, they may still be detected in a few powders. In *Cascara* bark or in powdered Gentian they can often be detected when stained with corallin soda. Beside sieve tubes, phloem possesses some other characters as follows:

Companion cells: They are intimately associated with the sieve tube both structurally and functionally. They are characterized by the presence of dense protoplast, a thin cellulose wall, and a well-developed nucleus.

Phloem parenchyma: They are usually thin walled and axially elongated. They may remain isodiametric and can be arranged in a linear series.

5.14.2.6 Epidermis

The epidermis consists of a single layer of cells covering the whole plant, that is, the outermost layer of the plant structure. The epidermis of the root constitutes the piliferous layer, shoots contain a compact layer of cells that in contrast to the stomatal guard cells, are often devoid of chloroplast. In many cases, the epidermal cells of the two surfaces of a leaf differ in form. Various diagnostic features, including the shape of the anticlinal (vertical) and periclinal (horizontal) wall (straight or wavy), the presence of thickening and the occurrence of striations on the surface cuticle, can play a major role in detecting the epidermis. The epidermis has specialized structures, the most universal of which are stomata, which control water loss from the plant. They occur most frequently on young leaves and stems. The structures of the epidermis and stomata are of first importance in the microscopical identification of leaves (Mukherjee, 2002). Stomata are specialized plant structures responsible for allowing the influx of CO_2 and O_2 into the leaves. These structures are present in the epidermis of leaves. The predominant function of these structures is to control the rate of photosynthesis and the level of transpiration. The

evolution of stomata has been elucidated at the genetic level, where it has been found that they developed in an effort to adapt the plant body during migration from water to an aerobic environment. The stomatal cells develop through three types of cell divisions, namely, the meristemoid mother cells, meristemoids, and the guard mother cells. These mechanisms were first elucidated in the plant *Arabidopsis thaliana*. Finally, the stomatal development involves differentiation of the stomatal cells into different types of cells, that is, the guard cells. The stomatal cells are separated from each other by at least one epidermal cell. It is also evident that ion exchange is necessary for the regulation of the aperture width. Stomata function as a protective mechanism, as previously mentioned. If the plant is under water stress, that is, in a dry and arid region, then the plant should conserve moisture. As a result, the guard cells undergo an efflux of ions and electrolytes so that they shrink and the aperture of the stomata becomes narrower. Conversely, when the transpiration rate needs to be increased, under any circumstances, the guard cells become swollen, and the stomatal aperture is broadened so that the moisture may escape through it. The classification of stomata is shown in Fig. 5.11.

There may be straight-walled epidermal cells, as seen in Jaborandi, Coca, and Senna leaves; wavy walled epidermal cells in stramonium, hyoscyamus, and belladonna; beaded wall cells in *Digitalis lanata* and *Lobelia inflata*; and papillose epidermal wall cells in coca leaf. The arrangement of epidermal cells around the stomata falls into four main types is shown in Fig. 5.11.

Trichomes are usually a variable outgrowth from epidermal cells, which occur in all parts of a plant. They are of value in the analysis of herbal drugs, particularly in leaf drugs. Several types of trichomes may occur on one herbal drug with different distribution and frequencies. They are particularly useful in the examination of fresh material where the stomata and epidermal cells are not readily visible (Dutta, 1997; Thangaraj, 2016).

5.14.2.7 Periderm

This is a protective tissue that replaces epidermis in stems and roots. Typical periderm is usually present in roots, in aquatic and subterranean stems, and in the aerial stems of plants belonging to families, such as Labiatae and Cucurbitaceae. Usually the periderm is formed from the cork cambium (phellogen) on the inside. The presence or absence of cork or secondary

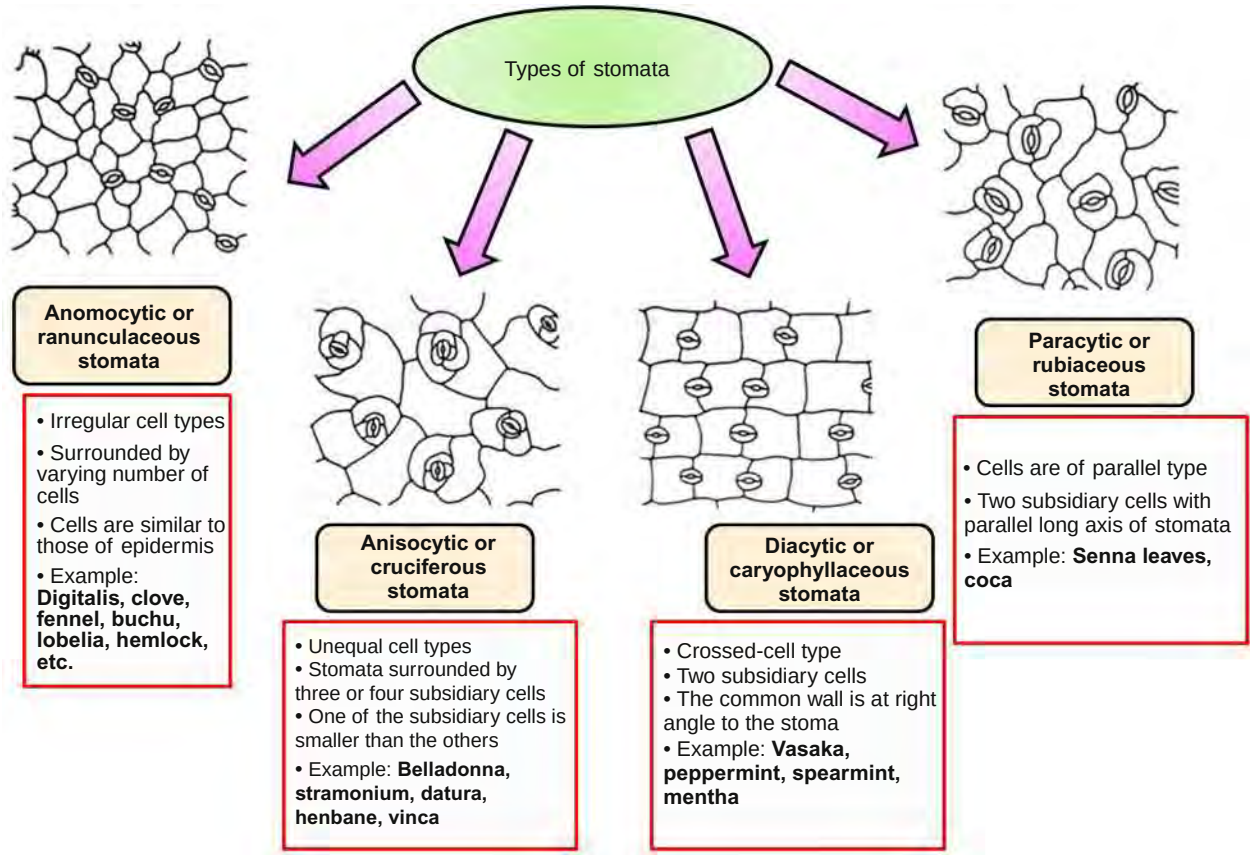

FIG. 5.11 Different types of stomata.

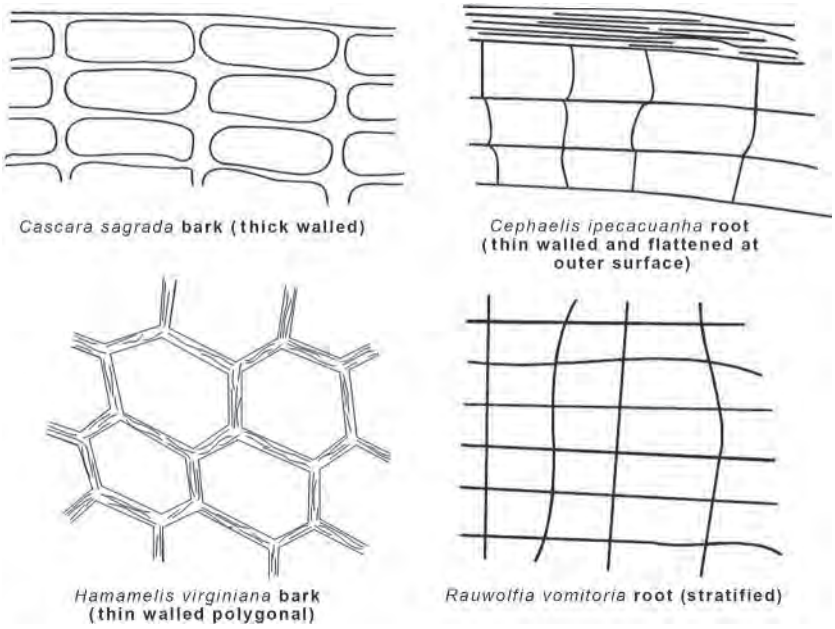

FIG. 5.12 Types of stratifications present in cork cells in periderm in different herbal drugs.

cortex is important in those drugs that are prepared by removing the outer layers from a bark, for example, peeled ginger and cinnamon. Cork cells are not living at maturity and may be lignified. They are usually brown or yellowish in color with some stratification, as shown in Fig. 5.12.

5.14.2.8 Secondary Tissues

These include secretary cells, cavities or sacs, and secretary ducts or canals. These are important features of certain drugs whose therapeutic constituents are contained in the secretions. Oil cells occur in ginger, pepper, cardamom, and cinnamon. Cells containing resins, oleoresins, and mucilage are common. Storage cells, crystal cells, and tannin cells are also considered under this tissue. Individual oil or resin cells may occur as idioblasts in the parenchyma, combined in groups or as an entire layer.

They may be formed into cavities known as glands or ducts by splitting apart different cells. Different secretary tissues are shown in Fig. 5.13. Beside the studies of the basic cell types mentioned in the evaluation of the ergastic substances or cell inclusions, the bodies that are present in the cell cytoplasm can be readily identified by their characteristic form and chemical nature. The evaluation of cell inclusion characteristics plays a major role in diagnosing a crude drug, as they have a fairly constant shape and size range. They are either food storage products or the byproducts of plant metabolism and include carbohydrates, proteins, lipids, calcium oxalate, calcium carbonate, tannins, and resins. Some of these cell contents are of diagnostic importance are briefly described in the following (Vagionas et al., 2007).

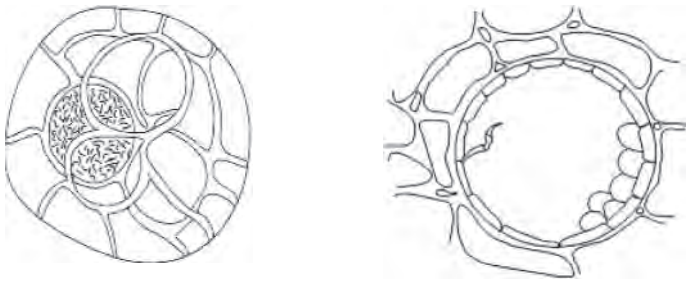

FIG. 5.13 Secondary tissues with secretary structures present in herbal drugs.

5.14.2.9 Calcium Oxalate

Calcium oxalate occurs most abundantly in plant tissues. It is usually present to the extent of about 1% in plants but in some structures, for example, the rhizomes of rhubarb, it may exceed 20% of the dry weight. It often forms a character of considerable diagnostic importance in the evaluation of herbal drugs. It is usually dimorphous and is found either as the trihydrate belonging to the tetragonal system of crystals or as the monohydrate of the monoclinic system. These crystals vary widely in size and appearance, upon which they can be grouped into six classes as follows:

Cubic or isometric	They have three equal axes, at right angles to one another in the form of a cube
Tetragonal	They have three axes, all at right angles to one another, two axes are equal and known as lateral axes, like the shape of a prism
Hexagonal	They have four axes, three of which are equal, in the same plane at an angle of 60° to one another similar to the form of a hexagonal prism
Rhombic	They have three axes with unequal length at right angles to one another, having the shape of a right rectangular prism
Monoclinic	They have three unequal axes, in which two lateral axes are at right angles to one another, but one only is at a right angle to the third or principal axis, obtaining the shape of an oblique rectangular prism
Triclinic	This is also known as an anorthic system, which has three axes of unequal length and with none at right angles to another, having the shape of a doubly oblique prism

Calcium oxalate usually occurs in two forms, namely, tetragonal crystal (CaC_2O_4, $3H_2O$) and monoclinic crystal (CaC_2O_4, H_2O). Tetragonal crystals are formed as a result of super saturation of the cell sap with calcium oxalate with the shape as discussed earlier. In addition to these forms, tiny sandy crystals or microcrystals are found in the herbal drugs of Solanaceae and other families. Tetragonal crystals are present in *Allium cepa*, *Begonia* spp., *Hyoscyamus* spp., and others. Another form of crystal found abundantly in plants is the monoclinic crystal, in which two series of arrangements exist. In the first series, the crystals are inclined toward the longer lateral axis and in the second series toward the shorter lateral axis. These crystals are usually present in such plant species as *Quercus infectoria*, *Veratrum* spp., *Quillaja saponaria*, and *P. excelsa* (Gemperline, 2006).

Calcium oxalate is very useful in the identification of plant drugs, as quite closely related species commonly differ in the form and arrangement of the crystal, which makes identification easier. For the microscopical examination of calcium oxalate crystals, the section to be examined may be cleared with chloral hydrate or caustic alkali, as these reagents dissolve the crystal very slowly. A polarizing microscope will often assist in the detection of small crystals. They can be identified as calcium oxalate if they are insoluble in acetic acid and caustic alkali, soluble in hydrochloric and sulfuric acid without effervescence and, when mixed in a 50% sulfuric acid solution, showing a gradual separation of needle-like calcium sulfate crystal. Calcium oxalate is anisotropic and can be differentiated from carbonate and silica by its insolubility in acetic acid and a 5% KOH solution and solubility in hydrochloric acid (Grubesic et al., 2005; Kim et al., 2010).

5.14.2.10 Calcium Carbonate

It occurs as cell inclusions, occasionally found embedded in or encrusted in the cell wall. The concentration of calcium carbonate, formed on outgrowths of the cell wall are called cystoliths. An example of this is the incrustation in the trichomes of the lower epidermis and enlarged upper epidermal cells of *Cannabis sativa*. It can be differentiated from calcium oxalate by the fact that it dissolves with effervescence in acetic acid, hydrochloric, and sulfuric acid.

5.14.2.11 Starch

This is the most common carbohydrate reserve and is found in varying amounts in almost all plant organs. It occurs in granules of varying sizes and is found most abundantly in roots, rhizomes, fruits, and seeds—where it occurs as larger grains. Small granules are formed in chloroplasts by the condensation of sugar, which are afterward hydrolyzed, so that they may pass into solution to storage organs where, under the influence of leucoplasts, large grains of reserve starch are formed. Starch is of considerable pharmaceutical importance. Various such starches include maize (*Zea mays*), rice (*O. sativa*), wheat (*Triticum aestivum*), and potato (*Solanum tuberosum*) starch. The position and form of the hilum, and the presence or absence of well-defined striations are the most important features in diagnosing starches of different categories. Between crossed polarizers, the granules appear bright on a black background and each usually shows as a dark Maltese

cross due to the spherulitic structure of the granule. This type of appearance is completely specific to starch, although it is also shown by granules of inulin.

Starch occurs as an irregular, angular mass, or as a white powder. It is insoluble in cold water but forms a colloidal solution on boiling with 15 parts (w/w) of water; on cooling, this solution forms a translucent jelly. When heated with water, the granules first swell and then undergo gelatinization. Starch granules also undergo gelatinization when treated with caustic potash, a concentrated solution of calcium or zinc chloride, or one of chloral hydrate. For microscopical examination of starch in herbal drugs, the sample has to be mounted in glycerol or cresol, but not in chloral hydrate as starch is soluble in it. Starch is identified by its characteristic appearance in natural and polarized light and by the formation of a bluish-black-colored compound (starch iodide) with N/50 iodine solution; whereas inulin does not stain with this iodine.

Starch granules usually contain two carbohydrates, amylopectin or α-amylose and amylose or β-amylose. About 80% of starches are composed of amylopectin, and the binding property of starches, useful in tablet technology, is mostly for the presence of this constituent. Beside these ergastic substances, other cell inclusions also exist, which are semisolid or liquid and, therefore, tend to have little characteristic form. These include fixed oils, fats, essential oils, mucilages, and tannins. Oils appear microscopically as small refractive globules and can be stained by oil-soluble dyes, such as Sudan red. Mucilages present in herbal drugs can absorb some stains, such as methylene blue and ruthenium red, and become intensively colored relative to the rest of the mountant. Tannins are usually detected by the green blue color seen in treatment with ferric chloride solution (Labra et al., 2004).

5.15 MICROSCOPICAL TECHNIQUES

Microscopical techniques provide detailed information about crude drugs by virtue of their two main analytical uses. First, magnification permits the fine structures of minute objects to be visualized and thereby confirms the structural details of the plant drugs under evaluation. Second, these techniques can be used in the determination of the optical as well as the microchemical properties of the crude drug specimen under study. Microscopical observations are based on optical phenomena, which are governed by the optical system of the microscope and the nature of the light passing through it. Microscopical inspection of crude drugs of plant origin is essential for the identification of ground or powdered materials. Though microscopy alone cannot provide a complete evaluation profile of an herbal drug, it can still provide supporting evidence, which, when combined with other analytical parameters, can be used to obtain the full evidence for the standardization and evaluation of herbal drugs. To establish the microscopical characters of any herbal drug, considerable skill and experience in microscopy of crude drugs is important. Once the plant material has been examined and grouped according to external characteristics, inspection by microscopy can be carried out as the next step.

In analytical investigations of herbal drugs, a microscopical study often yields information that cannot be obtained by any other method. For this reason, an acquaintance with the microscopic structures of various sections of herbal drugs and the working procedure is of value to analysts. In this type of investigation, one must remember that the study ultimately depends on one's power of sight, which can be increased with various adjustments. Hence, microscopy is unable to give any effective assistance in cases in which the quantities visible to the unaided eye can be made to yield the same results. Therefore, the practical utility of the microscopical methods is limited to some extent and the results obtained are generally considered in connection with important factors obtained by other methods, so that one must be on guard against expecting too much from a particular working method. This may sometimes result in disrepute when it is found that the performance does not meet the expectation of the analyst. Crude drugs, when supplied in intact form, can be identified by morphological characteristics. However, in case of doubt, they can be investigated for histological characteristics to confirm the identity of the supplied drugs. Although there are similarities between two crude drugs in their morphological characters, for example, *Phytolacca* used as a substitute for *Belladonna*, there are sufficient histological differences between the two to allow distinction. In crude drugs supplied in powder form, most of the histological arrangement of the whole drug is lost. However, in spite of these facts, microscopy is of great necessity in the identification of powders. Powdered crude drugs can be identified based on their form and the presence or absence of different cell types based on their cytomorphological characters, for example, parenchyma, collenchyma, fibers, stone cells, vessels, trichomes, secretary cells, and epidermal cells. The same may sometimes be achieved by evaluating the cell inclusion characteristics for some unorganized crude drugs, such as starch grains, aleurone grains, gums, mucilage, and calcium oxalate crystals (Tyler, 1999).

5.15.1 Instruments for Microscopical Study

To perform microscopical evaluation of any crude drug, the first essential step is to examine the particular microscope and to identify its different components. For pharmacognostical studies, the microscope is usually composed of two objectives

of 16 and 4 mm, 2 or 3 eyepieces and a condenser. In microscopical measurements and examinations, the following instruments are helpful.

5.15.1.1 Camera Lucida

This is the simplest method for reproducing a microscopical image for the observer to make a proper drawing of the specimen under study. The magnified image of the object under the microscope can easily be traced on a paper by this instrument. When measuring the image of the specimen, the left eye is used to observe the specimen, while the right eye is kept open and focused on the paper placed on the right side of the microscope. When using this instrument, the illumination of both object and paper must be suitably adjusted and the paper should be tilted at the correct angle to avoid distortion. The magnification is determined by measuring the distance between the selected lines on the drawing paper and dividing by the distance between the corresponding lines on the stage micrometer. For curved and elongated objects, a measurement of the length can be made by the use of a microscope equipped with a camera lucida, which can reproduce the microscopical images.

There are many types of this instrument; it is preferable that the microscope can be used in a vertical position. The swift-ives model of camera lucida is most convenient and is commonly used in such studies. It is quite small and may be attached to the cap of a capped eyepiece or can be adapted to fit on the upper lens of an ordinary eyepiece and then clamped in position by a small screw. When in use, light from the object passes directly to the observer's eye through an opening in the silvered surface of the left hand prism; at the same time light from the drawing paper and pencil is reflected by the right hand prism and by the silvered surface, so that the pencil appears to be superimposed on the object, which may thus be traced. Beside this, there is another model called the "Abbe" model in which a plane mirror is fixed on a side arm and the mirror is at 45° to the bench surface without any inclined board. The specimen crude drug sample is placed on the microscope stage, the image of the object is traced on paper and the length is measured.

Thus, a camera lucida enables an accurate reproduction of microscopical images. The instruments have a microscope eyepiece and a side arm carrying a mirror or prism. By reflecting the image of the object, a camera lucida enables accurate tracing of the outline of a microscopic object.

5.15.1.2 Photomicrography

A photomicrography record of the crude drug specimen under study can be produced if a camera is set up above the microscope. This photographic documentation is advantageous when the specimen is very thin and the relevant structures are obvious. In photomicrographical identification, the optical part used should be of very high quality as any defects are accentuated in the final print of the photomicrograph. The photographic records contain everything visible in a particular field of view, much of which is usually uninformative. Photomicrography is usually not very useful for powdered drugs as rarely does a single field of view in the photomicrograph show all the characteristic features of such drugs (Moreda-Piñeiro et al., 2003).

5.15.1.3 Modified Light Microscopy

If the object under examination is transparent, then normal bright field illumination is not suitable for detecting the microscopical characteristics. In such a case, modified light microscopy is useful, as in dark field illumination in which the object is illuminated by a hollow cone of light that is so oblique that no light can pass directly into the object. However, scattering of light occurs at the boundaries of the specimen objective and these scattered rays are collected by the objective so that the features of the object are seen as bright structures against a dark background. The main disadvantage of dark field microscopy is the production of numerous points of light produced by minute dust particles in the optical path (Pilgrim et al., 2010).

5.15.1.4 Polarizing Microscopy

This apparatus consists of two prisms, one below the microscope stage, called the polarizer and another similar prism fitted above the objective called the analyzer. Both of the prisms are Nicol prisms. When the polarizer and the analyzer have their parallel surface diagonal, a ray of plain polarized light is transmitted by the analyzer. If the polarizer is resolved, the light diminishes in intensity until, at a position of 90°, the polarizer light is totally reflected by the analyzer. This position, at which the diagonal surfaces of two Nicol prisms are at right angles, is called crossed Nicols. Isotropic substances are monorefringent, that is, they have only one refractive index. These substances in no way affect the polarized light passing through them from the polarizer. Anisotropic substances have more than one refractive index and they exhibit different physical properties according to the direction along which they are examined. Many crystalline substances show brilliant

colors when examined in polarized light. Starch grains often show a black cross due to the crystalline refraction of the material. Thus, polarizing microscopy is useful for the detection of calcium oxalate, and, for cases in which small quantities of material are present in the tissues under examination.

5.15.1.5 Phase Contrast Microscopy

This method is useful for examining living cell constituents that show little differentiation. In this system, a diffracted beam of light is further retarded by one quarter of a wavelength so that the beams are one-half of a wavelength out of phase. This change is accomplished by a technique that separates the direct and defracted beams. Monochromatic light is directly transmitted through the sample, which is reduced in intensity, and the deflected light is shifted half a wavelength out of phase with the transmitted light. In this way, a strong contrast in the material under observation is obtained without a reduction in the resolving power of microscope.

5.15.1.6 Electron Microscopy

Here the main principle is based on a stream of electrons, which can be focused by an electromagnet acting as a lens. The object, when placed in the path of the electrons, produces an image that is recorded either on a fluorescent or on a photographic plate. Good stabilization of the lens current is essential for the best lens performance.

5.15.1.7 Ultraviolet Microscopy

This type of microscopy is based on the principle that shorter wavelengths can resolve smaller objects. The lenses are made of fused quartz, which transmit radiation down to a wavelength of 240 nm. The images produced are recorded photographically. These instruments are very useful for studying cell division and cell differentiation.

The equipment required for routine examination of crude drugs is as follows:

- A microscope equipped with lenses providing a wide range of magnifications, a substage, a graduated mechanical stage, color filters of ground glass, objectives with a magnification of 4× and 10×.
- A set of polarizing filters.
- One set of a stage micrometer and eyepiece micrometer to be inserted into a 6× eyepiece.
- A lamp incorporated into the microscope or with a separate attachment.
- A set of botanical dissecting instruments along with slides, cover slips, etc.

5.15.2 Microchemical Testing of Herbal Drugs

In a general sense, microchemical testing is carried out in a way similar to ordinary quantitative chemical analysis in which micro amounts of the substances being tested are used. It is usually recommended only in very special cases in which the amount of available material is very small and in circumstances in which the exact test procedure of the material under test has not been properly established. One can, therefore, test the compound boldly when a preliminary identification has been made microscopically. This technique is also employed for ascertaining the crystalline or amorphous nature of a sublimate or precipitate or of a residue left after evaporation of the solvent. In this analysis, a microscope is of great value as an aid to ordinary chemical analysis. It has special importance in toxicological investigations and other related instances in which only a minute amount of material is available. For this purpose, different chemical reagents and solutions of specific concentrations are used. Among the more important are those known as clearing reagents or bleaching agents, most of which have been discussed earlier. Other substances are generally used for testing widely distributed cell contents, which are identified by adding the test solution in a specific manner or by acquiring some distinguishing color. Some of the reagents and test solutions will be explained in the following sections. Reagents that produce precipitate and color reactions may be useful in particular instances, especially in the examination of the color of tissues and related items based on their chemical nature, but they may not be useful in analytical investigations.

(i) *Phloroglucinol*: This is benzene-1,3,5-triol dehydrate in the form of a white or yellowish white crystalline powder (melting point 218°C) that is soluble in ethanol and water. To prepare its reagent (TS), 1 g of Phloroglucinol (R) is dissolved in 100 mL of ethanol (750 g/L). It stains all lignified walls pink or red. It is not only useful for staining sections but also for vegetable powders and deposits. The section under study is mounted with phloroglucinol and allowed to stand for a few minutes to allow any remaining alcohol to evaporate. Concentrated hydrochloric acid is added drop-wise, covered, and examined under a microscope. When examining powders, a small quantity is mixed with a few drops of Phloroglucinol solution and allowed to almost dry by evaporation of the ethanol. Strong hydrochloric acid (specific gravity 1.16) is then added and a cover glass is applied; all woody structures, ground

olive stones, and coconut shells, are stained red. An aqueous 1% solution of the same works equally well, but then it is necessary to dry the powder mixed with the solution by heating the slide in a steam oven before adding the hydrochloric acid. Other acids besides hydrochloric acid produce this reaction equally well and one may substitute with sulfuric acid made by diluting one volume of strong acid to five with water, or a slightly diluted nitric acid. Hydrochloric acid is usually preferred because it is less likely to yield insoluble products, which may obscure other important features.

(ii) *Iodine water*: Iodine is the most important of all the reagents used in microchemical testing. Generally, a solution of iodine is prepared by diluting 5 mL of 0.1 N iodine to 100 mL by addition of distilled water, or may be diluted further by adding an equal amount of water if required by specific test methods. Iodine colors starch blue and imparts a yellow color to aleurone grains. When examining water deposits, a portion should be mounted and irrigated with iodine solution, which drains away more starch grains and slows down the movement of infusions, thus making them much more visible. Iodine solution is useful for the identification of apple pulp in jams. In this test, a small portion of the jam pulp is placed on a slide and a small amount of iodine water solution is added. It is covered with a cover glass and examined under a microscope for the presence of blue color, which is usually present in the large apple pulp cells. This may be due to the presence of elongated and lignified scelerenchymatous cells, which are arranged in groups.

(iii) *Acetic acid*: It is a very useful reagent in microchemical analysis, which is frequently employed for the recognition of calcium carbonate and distinguishing it from calcium oxalate. The test is useful for all water deposits containing crystalline matter and cells of foraminifer. The reagent is most useful for the analysis of calcium carbonate or calcium oxalate present in drug preparations.

(iv) *Caustic soda solution*: Details about of this reagent have been given earlier. It is also useful for the microchemical examination of some crude drugs. A 50% solution of soda or potash is used for the test of suberized cell walls, which become yellow when mounted in the reagent, and which, on boiling, are replaced by yellow oily drops. Hesperidin is another substance identifiable by this reagent. It occurs as amorphous masses and crystals in orange peel, buchu leaves, hemlock, and other plants. It is insoluble in water, alcohol, chloral hydrate, and others but soluble with production of a yellow color in a solution of caustic soda or potash, which can be easily utilized for the microchemical examination of this particular substance.

(v) *Iodinated zinc chloride solution*: This is prepared by adding 20 g zinc chloride in 8.5 mL water. This is added dropwise to a solution of potassium iodide and iodine, in which 1 g of potassium iodide and 0.5 g of iodine is added to 20 mL water, until a precipitate of iodine is formed. This is used to stain cellulose walls to a blue or violet color. When added, it slowly stains cellulose walls and lignified or suberized walls to yellow or brown and starch grains to blue.

(vi) *Sulfuric acid*: Strong sulfuric acid (specific gravity 1.843) is the best reagent used for the identification of suberized tissues. In this examination, the section is mounted in acid, which dissolves almost all of the structures except the suberized cells. Therefore, the cuticles and other corky walls present remain intact after the treatment. In conjunction with iodine, sulfuric acid forms an excellent test for cellulose. The acid solution should have a ratio of strong acid and water 2:1 and the iodine solution is prepared at a ratio of decinormal iodine: water (1:5). The section is mounted as before without covering with a cover slip in a drop of iodine solution. After a few seconds, the iodine solution is removed with a blotting paper and a drop of sulfuric acid solution is added and covered with a cover slip. In this condition, the cellulose solution swells up and produces a blue stain.

(vii) *Cuoxam*: This is an ammoniacal solution of copper oxide. It is prepared with 0.5 g of commercial copper carbonate with 10 mL of distilled water to a smooth cream in a mortar and then with the addition of 10 mL strong ammonia solution (specific gravity 0.880) to produce a clear deep blue liquid. This solution dissolves cellulose walls but without any action upon woody or suberized walls of vegetable drugs.

(viii) *Sudan red*: To prepare its TS reagent, 0.5 g sudan red (GR) is dissolved in 100 mL of glacial acetic acid. It is used for staining suberized or cuticular cell walls to an orange red or red color.

(ix) *Alkana tincture*: It is prepared by macerating one part of alkanet root and five parts of 90% alcohol for 1 week with filtering afterward. It stains oils, fats, and suberized and cuticular cell walls.

(x) *Picric acid solution*: This is a saturated solution of picric acid in water (soluble 1 in 50). It is used to stain aleurone grains and animal fibers. This stains animal fibers yellow, while cellulose fibers remain uncolored. This reaction is very useful for examining mixed materials, such as gray filter papers or bandage materials. To search for wool or animal fibers in crude drugs, a small quantity of the material is placed on a glass slide and mounted with a saturated aqueous solution of picric acid and allowed to stand for 5 min. The fiber is to be washed 2–3 times by irrigation with distilled water and the preparation is examined microscopically when all the animal fibers are clearly distinguished by their bright yellow color. It can be confirmed by comparing the observation with good drawings or a standard preparation of authentic fibers.

(xi) *Sudan III solution*: This is a solution of sudan III in equal parts of glycerin and alcohol, which is used to stain secretary cells and ducts.

(xii) *Ruthenium red*: This is prepared by dissolving 1 g ruthenium red in 500 mL of water. It stains many gums, pectin, and mucilage.

(xiii) *Corallin alkaline solution*: It is also known as Corallin—soda solution. It contains a 1% solution of rosalic acid in 4% aqueous sodium bicarbonate. It is used to stain the callose of sieve plates and different gums and mucilages. Beside these, Million's reagent for aleurone grains, Braemer's reagent for tannin, alkaloidal reagents, such as Mayer's reagent, and Dragendroff's reagents are also used for the microchemical evaluation of various herbal drugs. There are two procedures for the application of reagents for microchemical analysis. The first and simplest one is to mount the object in the reagent itself. The second is to irrigate the specimen with the test liquid. For this purpose, a drop of the reagent is placed on the slide against the edge of a cover slip, while blotting paper is applied at the opposite side, thus drawing a stream of liquid across the preparation. In this method, one can observe the whole progress of the reaction through the microscope and thus can have a clear idea about the color changes and reactions taking place before and after the application of the test solution. A modification of the method is possible that does not involve the use of blotting paper, which may cause a strong flow of liquid and thereby produce a disturbance in the observation of the particle. For this purpose, the total process is affected by having a minimum amount of water under the cover glass against the edge of which a drop of the reagent is placed. The drop immediately begins to flow underneath by capillary attraction. By changing the position of the slide under the microscope, one is able to watch the approach of the liquid to any special particles and to observe all the changes taking place. Thus, by this process of microchemical analysis, the investigation of crude drugs (fresh or dry) is easily possible, even though minute amounts of the substance are present (Karadaş and Kara, 2012).

5.15.3 Microchemical Precipitation

Microchemical precipitation is used to make a comprehensive study of a drug when the amount of available material is strictly limited, with a view to obtaining precipitates of characteristically formed microscopic crystals. This can be carried out on phyto-constituents, such as alkaloids, with characteristically formed microscopic crystals. In some cases, the microscopical characteristics of the precipitate are of importance, which is a more reliable method of distinguishing between two or more closely allied substances, for example, lactose and maltose, in which the osazones have a characteristic microcrystalline structure of diagnostic value. To perform the precipitation test on a slide, the following method is recommended: Two drops of reagents and the liquid to be tested are kept side by side upon the slide, and their coalescence under the microscope is tested. In many cases, it suffices to mix the two drops placed closely together upon the slide by lowering a cover glass on to them and then watching through the microscope for the appearance of the precipitate. Another method is to mix the solution in a watch glass and observe the effect in the microscope. When working with crude drugs containing alkaloids, this can be effectively used; the reagent most commonly used is ammonia, potassium, or ammonium thiocyanate in 5% solution and picric acid.

5.15.4 Micro-sublimation

By the method of sublimation, it is sometimes possible to identify a definite substance in crystalline form from a crude drug or from a complex mixture of crude drugs. The microscopical evidence of these sublimates can provide some valuable confirmatory evidence for the characterization and analysis of the parent material. The process is applied to crude drugs and other vegetable powders that contain any volatile crystalline principles. In this context, a useful method can be easily utilized for a micro-sublimation test. Here the crude drug under study is kept in a small thin glass tube that is sealed after introduction. The tube is incorporated in a method of heating in which the temperature can be recorded by a thermometer. Here the sample is sublimed at a tolerably correct temperature and the correct idea about the same can be obtained. This method in certain instances gives very good results for the detection of an exhausted drug in an admixture with the genuine articles. This may offer a valuable indication as to the best way of conducting the analysis, which is very useful for the preliminary examination of a number of samples of the same substance. As recommended by WHO the micro-sublimation procedures as applicable to crude drugs should be as follows:

A small square metal plate of $4 \times 4\,cm^2$ size is mounted on a suitable board from which a center hole of 1 cm in diameter has been cut. A ring of about 1 cm diameter and 8 cm height has to be placed at the center of the metal plate aligned with the whole of the asbestos board. About 0.1–0.2 g of powdered crude drug is placed inside the ring to form an even layer of about 2 mm thickness. The ring containing the material is covered with a clean slide and heated gently and gradually over the

small flame of a micro-burner. The slide is removed from the ring and set aside until the sublimate has dried. Then the slide is to be examined under a microscope without adding any fluid and without a cover glass. For those materials or powdered crude drugs that readily yield a crystalline sublimate, such as tea and gentian root, the sample is placed in a small crucible or a shallow porcelain dish (25 mm in diameter) that is placed upright on a sheet of asbestos board from which a central hole has been cut to receive the button of the crucible or the dish. A small amount of the crude drug is placed in the crucible and covered with a microscope slide. The crucible is gently heated and the slide has to be changed as and when required as the moisture can be trapped over the sample during heating. This process can give an idea about the identity and characteristics of the parent compound. The appearance of the sublimates varies somewhat according to the rate of sublimation, the efficiency in cooling, the amount of moisture, and other factors. Gentian root produces a yellowish sublimate with colorless circular or oval reticulate patches scattered throughout and small needles of gentisin separate from the yellow portion. This can be accepted as a method for the detection of adulterants in gentian roots, when they are adulterated by an exhausted drug, which can never give these crystals upon sublimation. Similarly, Rhubarb root in the same treatment yields a yellow crystalline sublimate from which small yellow crystals of dendritic patterns are deposited. Hydrastis rhizomes produce yellow sublimates, which can give very characteristic colorless crystals of the alkaloid—hydrastine, which can be separated after cooling. Thus, the sublimation procedure may be helpful in different ways for the characterization of herbal drugs either for the detection of the exhausted material or for the isolation and identification of the active principal (Soares and Scarminio, 2008).

5.16 EVALUATION OF CRUDE DRUGS BY MICROSCOPY

5.16.1 Preparation of Crude Drug Sample for Microscopical Examination

The microscopical examination of crude drugs aims to determine the chemical nature of the cell wall along with a determination of the form and chemical nature of the cell contents. Thus, it determines the size, shape, and relative structure of the different cells and tissues in a plant drug. Representative samples of the plant drugs are selected. Dried materials often require softening before preparation for microscopical studies. This may be done by exposing the sample to moist conditions (for leaves and flowers) or by boiling in water (for roots and barks) (Fernández-Cáceres et al., 2001).

Preparing a crude drug sample for microscopical study necessarily depends on the form and nature of the sample. In order to observe the details of the structure, the specimens have to be placed in the proper optical path of a microscope in which the form and condition of the sample play a major role. The main factors affecting the visibility of the object are sample thickness and the presence of other pigments or obscuring materials. The sample thickness can be controlled by proper sectioning of the material or by the selection of a particular portion of powdered material. For small quantities of material, a wad of moistened cotton wool covered with filter paper is placed in a test tube containing the material to be examined. The test tube is stoppered and allowed to stand overnight or until the material is softened and suitable for cutting. Bark, wood, and other hard materials require soaking in water or equal parts of water ethanol and glycerol for a few hours or boiling in water for a few minutes until they are soft enough to cut. Sometimes water-soluble components can be removed by soaking in water, for example, starch grain is gelatinized by heating with water (Trease and Evans, 1983).

To prepare a microscopical specimen of the powdered drug, 1–2 drops of water/glycerol/ethanol/chloral hydrate are placed on a glass slide. The tip of a needle is moistened with this reagent and inserted deep into the powdered sample to be analyzed. The small quantity of the material that adheres to the needle tip is placed on the drop of liquid on the slide. It is mixed thoroughly; a cover glass must be applied. With the handle of the needle the cover glass is pressed slightly and the excess fluid from the margin of the cover glass is removed with tissue paper. To make the specimen completely free of air bubbles, it is boiled over a micro-burner for some time until the air is completely removed. The cover glass must be filled until the operation is completed.

For surface tissues of leaves and flowers, representative pieces of the sample to be examined are selected and cut to a suitable length, cross or transverse sections are prepared by cutting with a razor blade or by microtome at a right angle to the longitudinal axis of the material. Longitudinal sections are prepared by cutting with the same in parallel with the longitudinal axis either in a radial direction or in a tangential direction to prepare a radial or a tangential section, respectively. To render pieces of thin leaves transparent, they are boiled on a slide. A piece of a leaf is cut into portions and one piece is turned upper side down and chloral hydrate solution is added. The specimen is boiled over a micro-burner and, as soon as the bubbles escape, the slide is removed from the flame. When the bubbles cease to appear, the sample is boiled again until it is transparent. For slightly thicker and papery leaves, square pieces are cut about 6 mm from the edge of the leaf if not otherwise specified. The specimen should include a midrib or large vein along with the lamina. For broken or cut leaves, the specimen should be taken in the same way, and placed in a test tube containing

chloral hydrate solution and boiled until it becomes transparent. A small fragment is transferred to a slide and cut into two equal portions. One piece is turned upside down and then the two pieces are aligned so that both upper and lower surfaces can be observed under the microscope. This is followed by the addition of 1–2 drops of chloral hydrate and covering with a cover glass. Thicker leaves do not become transparent enough when treated in the above method; the leaves are clarified from the fragments by boiling with chloral hydrate TS in a test tube. A small fragment is transferred to a slide and cut into two equal portions. One piece is turned upside down. The surface of the two portions should be scraped using a scalpel until a single layer of epidermis remains. The epidermis layer is washed with chloral hydrate or ethanol or glycerol TS to remove any residue.

To prepare the section for routine work, disposable single-edge razor blades mounted in a holder are usually used as they have the advantage of not requiring sharpening. In this simplest system, the thickness of the section depends to a great extent on the skill and judgment of the worker. A hand microtome enables better control of the thickness. This microtome consists of a tube into which the pith and the sample are loaded. A screw-threaded plunger is placed at the base of the tube, which can be raised to extrude the sample to the cutting table. The thickness of a particular section is controlled by the degree of rotation of the screw between sections. For very precise work the sample can be frozen or embedded, and the sections are cut on an automatic microtome. Thick materials, such as wood or woody stems, rhizomes, and roots, can be cut into small pieces by holding the softened material between the thumb and index finger or by holding it with the central hole of the microtome. Thin materials, such as leaves or petals, can be bound between two halves of elder pith or with any suitable support. The sections are cut as thin and even as possible and are transferred with a brush moistened with ethanol TS to a dish containing ethanol. For preparation of the slide, satisfactory sections must be selected. Sometimes, the characteristics of plants are obscured by the abundance of cell contents, shrinkage or collapse of cell walls, as well as the presence of different coloring matter in the plant cells. To restore the original shape of the cell wall, some reagents are used for the removal of the cell content, which hinders microscopical examination. For this purpose, different clearing, defatting, and bleaching agents are used as described in the subsequent section.

5.16.2 Clearing Reagents and Preliminary Treatment

5.16.2.1 Chloral Hydrate Solution

Chloral hydrate is a colorless hygroscopic crystal with a melting point of 55°C. To prepare a test solution (TS), 50 g of chloral hydrate is dissolved in 20 mL of water. It is effectively used as a clearing and bleaching agent to dissolve starch, protein, chlorophyll, resins, and other materials and causes shrunken cells to expand. It does not dissolve calcium oxalate, so it can be used effectively for detecting these crystals. In spite of its use in sections, it can also be used for whole leaves, flowers, and pollen grains. When starch is present, the material should be boiled with chloral hydrate. It is a very delicate reagent and is very useful to determine the structure of different delicate tissues, which, during drying, tend to shrink until the cell lamina disappears. It produces less swelling of cellulose walls and is less destructive in its action upon certain cell contents, for example, calcium oxalate, so this solution is generally preferable for use with caustic soda or potash. It clears and expands tissues without producing marked distortion and can even be useful in exhibiting small crystals, such as those that are found in the parenchyma of gentian root. For all kinds of crude drugs of herbal origin, whether fresh or dried, chloral hydrate can be used most effectively as a clearing reagent.

This reagent can also be used for the identification of extracts made from crude drugs. Here 0.2 g of extract is triturated with 5 mL water in a mortar; the turbid liquid produced is transferred to a conical centrifuge tube and centrifuged for 2 min. The supernatant liquid, if it is still turbid, is poured off, stirred well, and again centrifuged. This solution is then mixed with chloral hydrate solution and once more centrifuged. The final deposits are mixed with one or two drops of chloral hydrate solution or glycerin and small quantities are taken to a slide with a pipette for microscopical examination. Fragments of the tissues of the drug from where it has been prepared are found in the material on the slide and, in this way, the identity of the extract can be established.

5.16.2.2 Phenols

Cresol and liquefied phenol are used, like chloral hydrate, as clearing agents. The advantages of using them over chloral hydrate are that they cause no destruction of starch but cause good penetration and produce less swelling and also render starch particles so transparent as to be practically invisible, while the tissues themselves are clearly defined.

5.16.2.3 Nitric Acid

It is prepared by diluting nitric acid of specific gravity 1.42–5 times its volume by the addition of water. It is used for the separation of the epidermis of leaves and stems of fresh or dried crude drugs. The leaves or the stems of the drugs under study are cut into small pieces and the material is boiled gently with dilute acid until the epidermis of the sample can be easily separated. For this testing, a small portion of the sample under study is removed and an attempt is made to strip the epidermis off from the specimen on the slide. When this can be easily accomplished, the boiled leaves or stems are taken out in a beaker with water. In some cases, this is more preferable than caustic potash or soda as the acid causes less swelling and distortion of the cell wall. It gives more useful results for softening hard woody tissues, such as coconut cells and olive stones. For this purpose, hard woody crude drugs are boiled with the acid solution for a few minutes and then allowed to macerate in the same solution at a gentle heat for about 2–3 h or until a portion removed and washed with water is sufficiently soft to cut off. After this operation is complete, the samples are washed with distilled water several times and reserved for sectioning.

5.16.2.4 Hydrochloric Acid

A test solution of hydrochloric acid is prepared at a concentration of 250 g/L with the density of HCI as 1.12. It is a powerful cleaning reagent. A test solution may dissolve many cell contents, including calcium oxalate. The vegetable debris of catechu stains on a simple application of HCI. HCI vapor may cause damage to the microscope; to prevent this, the slide should be removed as soon as possible from the microscope stage.

5.16.2.5 Hydrofluoric Acid

This is more preferable than nitric acid for softening the hard woody herbal drugs, such as olive stone, coconut cells, peach stone, and hard woods. It acts by dissolving out any minerals and silica present in the cell wall but it does not attack the middle lamella so that the cells of the softened tissue do not fall apart. For this purpose, hard woody drugs are boiled with water for a few hours. Then they are cooled and transferred to commercial hydrofluoric acid for 1–4 weeks depending on the hardness of the drugs. The soaking is continued until they are soft enough to cut into pieces for microscopical studies. This process may be continued up to 6 weeks and, in that case, the hydrofluoric acid solution can be changed once during the whole operation. After this operation is complete, the samples are washed with distilled water several times and stored in a mixture of equal parts of glycerin and 30% alcohol. After a week or so, they will be useful for sectioning. The maceration is carried out in dishes coated with paraffin wax as it is corrosive and should not come into contact with the skin.

5.16.2.6 Ether–Ethanol Reagent

This consists of a mixture of equal parts of ether and alcohol and is used effectively for the removal of oil, fats, volatile oil, resins, tannins, and chlorophyll, among others. It is mostly used as a defatting agent in the case of oily seeds, such as linseed and strophanthus.

5.16.2.7 Sodium Hypochlorite Solution

This is a solution of sodium hypochlorite containing 100–140 g/L of available chlorine. This is useful as a powerful bleaching agent to remove the dark color of many barks, as well as to remove chlorophyll from leaves. On prolonged contact, it may remove starch and lignin from the microscopical mount, which should be avoided when performing the experiments.

5.16.2.8 Caustic Alkali

This includes both aqueous and alcoholic solutions of caustic soda or potash in 2%–5% concentration in either water or alcohol. Potassium hydroxide up to a strength of 50% is sometimes used. A 5% aqueous solution is mostly used as a clearing agent, which rapidly dissolves starch and proteins. A 0.3% solution is useful to dissolve aleurone grains. These reagents have a solvent action upon starch and proteins and a strong softening and disintegrating effect upon cellulose. As lignified and cuticularized walls are much less readily attacked by this reagent, they are more useful in isolation of such elements when they occur in association with cellulose tissues and with starch and proteins. Barks, which consist mainly of phloem tissues, seeds, and leaves, are the most important plant organs that exhibit such characteristics.

It is for the isolation of the more resistant parts, such as fibers, sclerenchyma, laticiferous tissue, and cuticles, that caustic alkali is most often employed. The swelling action of this reagent is most disturbing in the case of leaf epidermis for which nitric acid is used instead. For this purpose, the material to be examined is cut into small pieces and digested in

a beaker with some alkali in a water bath. The operation is continued until a fraction that is removed and dissected shows the characteristics of testing. When this condition is reached, the material is washed several times with distilled water to remove the alkali and used for microscopical investigation.

5.16.2.9 Clove Oil

Clove oil is used particularly in the examination of oily powders, such as pepper, mustard, and linseed. Here the oily matter dissolves completely and the globules do not obscure the other structures present. It causes no swelling, but has rather a tendency to harden and shrink tissues. Because of its great penetrating power, it gives good effect to polarized light. Clove oil does not show any tendency to crystallize, as happens with chloral hydrate and liquefied phenol, especially when it is necessary to warm the preparations to gelatinize starch or to thoroughly expel air. The oil can be effectively cleaned from the slide by wiping both the cover glass and the slide with paper and polishing with a duster. Some ointment formulations can be readily subjected to a preliminary examination. Here, a small amount of ointment is placed on a slide, 1–2 drops of clove oil is added, the sample is covered with a cover slip, and the preparation is gently warmed.

5.17 QUANTITATIVE ANALYTICAL MICROSCOPY

5.17.1 Optical Micromeritics

Optical micromeritics is used for the identification, characterization, and standardization of crude drugs, as well as to gain knowledge of their cellular contents. A material can be authenticated by comparing the characteristics observed in the sample with those of standard materials. Subjective evaluation and correlation of the data by the analyst help to determine the proper identity of the material. This is necessary for a quality evaluation of the drug.

Numerical data obtained through this method provide information regarding the physical properties of a crude drug. The physical dimensions obtained through this method are variable and largely depend on the treatments used on the material. The physical dimensions of a sample obtained through this analysis are variable and depend largely on the exact treatment to which the material was subjected. In any randomly selected sample, the cellular and subculture characters of some materials are constant, which gives a range of values. After a number of determinations, the characteristic features/numbers obtained are noted and compared with the range and standard deviation (SD), and we get an idea about the identity and characteristic features of the drug. For applying quantitative numerical data in the analysis of drugs, the two major factors to be considered are whether or not the sample is representative of the entire sample or consignment and whether or not the estimate to be performed will give sufficient statistically reliable data or not. By controlling these two factors, the reliability of any method can be increased. Two types of quantitative microscopical techniques may be used when assessing an herbal drug. One includes an estimate of the measure of the individual features and the other includes an estimate of the event of a specific component in a particular amount of material (Smith, 2005).

5.17.2 Micrometers

Microscopical measurements are performed with the use of micrometers. Stage and ocular micrometers are needed for quantitative microscopy. For microscopical measurements, the scale needs to be adjusted so that it superimposes on the image of the material. Such scales, known as graticules, can be of several designs. The stage micrometer generally consists of a scale of 1.1 mm length, taking the form of an ordinary mounted microscopic object. The aggregate length is partitioned into 11 equal parts of 0.1 mm (100 μm) and odd 10ths are again subdivided into 100ths, which are made into 10 equivalent amounts of 0.01 mm (10 μm). This scale is engraved or photographed upon, or mounted on a cover glass, just as any other sample that is planned to be examined by a magnifying lens, and is seen through the instrument. Hence, the stage micrometer is a slide that has a progression of lines engraved on it. The second scale, that is, the eyepiece micrometer or visual micrometer (OM), comprises a little circle of glass that is dropped on to the stomach of a Huyghenian eyepiece. Around the center point of the plate, there is an engraved scale, which is separated into units and tenths of units. Despite the fact that the correct size of the selected unit is unimportant, more often than not the scale is 10 mm long and every millimeter is divided into 10ths.

To calibrate the visual micrometer the OM scale is focused by moving the focal point up to the point at which a sharp definition is obtained. To do this the lens of the eyepiece is unscrewed, put on the edge inside and the focal point is supplanted. The stage micrometer is superimposed on the scale of the visual micrometer and lined up on the scale division. By turning the eyepiece, both are set precisely in a parallel position. On the off chance that it is vital, the stage micrometer is moved until the point at which the beginning line of both scales match with each other. The quantity of visual micrometer

divisions relating to a specific length of the stage micrometer is checked so as to decide the length, which is proportional to one division of the visual micrometer scale. For instance, 100 divisions of the visual micrometer scale are equivalent to 30 divisions of the stage micrometer. Because the divisions of the stage micrometer are separated by 0.01 mm, 100 visual micrometer divisions are comparable to 0.30 mm and every little division of the visual micrometer equates to 0.003 mm, that is, 3.0 µm.

A second type of eyepiece micrometer is required for tallying particles under the magnifying instrument, and is additionally helpful for drawing large articles without a camera lucida. This is a squared or net micrometer, which comprises a circular disc of glass having a focal region of 1 cm^2 partitioned into 100 little squares, each of 1 mm^2. For the micrometer scale, it is most helpful to set aside a micrometer scale that remains permanently in position upon the diaphragm within the eyepiece tube. At a point at which the scale in not appropriately centered, it is corrected by alteration.

5.17.3 Micrometry

The scale of an eyepiece micrometer is an arbitrary scale and this has to be standardized before using it for a measurement. This is accomplished by placing a stage micrometer upon the stage of the microscope and focusing on the line engraved upon it, which is done by using either a low-power objective or a high-power objective. The eyepiece is turned a little to place the scales in a parallel position and, if necessary, the stage micrometer is moved a bit until the starting lines of both scales coincide with one another. This is followed by searching as far along the scale as possible for the location at which two other division lines are exactly superimposed. The number of divisions on the ocular micrometer is noted as is the corresponding length on the stage micrometer scale in order to determine the length that is equivalent to one division on the ocular micrometer scale. In Fig. 5.14, the appearance of a field showing both micrometer scales is depicted. In this figure the lines reaching across the field represent the image of the ruling upon the stage micrometer, while the shorter lines of the numbered scale represent the ruling of the ocular micrometer. If there is no exact coincidence between the extremities of the ocular scale and two of the rulings of the stage micrometer, the drawtube of the microscope must be extended until coincidence occurs to determine the number of stage micrometer divisions, which are exactly equivalent to 100 divisions of the ocular scale. In the figure, this number is 3.9 divisions. Because the rulings of the stage micrometer are 0.1 and 0.01 mm apart, respectively, the results can be described as in the following.

One hundred ocular divisions are proportionate to 0.39 mm, or every division of the eyepiece scale represents 0.0039 mm or 3.9 µm with this specific optical mix. In another case, if in a calibration 83 divisions on the ocular micrometer scale are equivalent to 35 divisions of the stage micrometer scale, because the divisions in front of the audience micrometer scale are separated by 0.01 mm (10 µm), 83 ocular micrometer divisions are equivalent to 0.35 mm. In this way, every division on the ocular micrometer scale equates to 0.35/83 = 0.0042 mm = 4.2 µm. As the calibrations apply only for a specific focal point mix, it is best to decide and record the ocular micrometer values for the most often utilized blend.

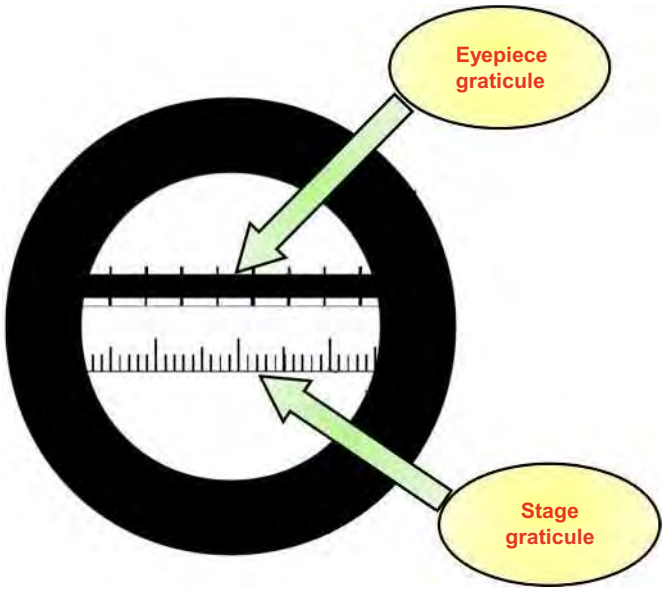

FIG. 5.14 Eyepiece and stage micrometer scale.

For this reason, the example is put on the microscope stage and the object to be observed is focused upon. The ocular micrometer scale is superimposed as previously depicted and the dimensions of the object are measured. The quantity of the scale division by micrometer esteem is multiplied to give a genuine measurement of the object. By this technique, utilizing a 40× objective and 6× eyepiece, estimates are accurate to the nearest 1 μm, that is, a measurement of 10 μm has an error of 1 μm or 10% or ±5%. A measurement of 100 μm is connected with a mistake of 2% or ±1%. In the above case, if the stage micrometer is expelled and any ordinary object is seen through the same lenses without adjustment of the tube length, the measurement of the object can be read off in terms of the eyepiece scale. This number multiplied by 3.9 gives the real measurement in microns. When utilizing camera lucida, estimates can be made either with a phase micrometer or an adjusted eyepiece micrometer. For each situation, the scale is followed off on the paper, and the genuine estimates of these divisions being known, they quickly give the scale by which the measurement of the object can be acquired from the illustration made on the paper. Estimations are often important for the quantitative distinction of closely related substances. Much of the time, these similar substances are blended with the original ones as adulterants or substituents. For instance, Cinnamon bark from *Cinnamomum cassia* Blume has a starch grain in the range of 10–20 μm, while cinnamon bark of *Cinnamomum zylanicum* Breyn has a smaller starch grain of 6–8 μm width; by this means, a blended substituent can be recognized. Thus, Rio or Brazilian ipecacuanha root acquired from *Psychotria ipecacuanha* stokes contains starch grains that never surpass 15 μm in breadth and they can be thus be distinguished from *Psychotria acuminata* Karsten, which contains starch grains between 17 and 22 μm. Wheat starch can be recognized from scarcely starch just by estimating the biggest grain that it contains. Grain starch grains never surpass 40 μm, while wheat starch grains can be up to 50 μm across. Adulterants and substituents can also be recognized by this method.

5.17.4 Leaf Characters for the Evaluation of Impurity

Different leaf drugs can be distinguished by studying the characters of the leaves from species to species based on their specific characters. There are several characters of leaves that should be studied. Two of them are discussed in the following sections.

5.17.4.1 Vein Islet and Veinlet Termination

Vein islet is the term used to denote the fine regions of photosynthetic tissues surrounded by definitive divisions of vascular stands. The zone of the leaf considered is ideally taken from the lamina halfway between the midrib and edge. The number of vein islets per square millimeter is called the vein islet number. This number per unit area of a leaf is constant. It can be effortlessly utilized as an identifying mark to distinguish between various types of the same plant or between different plants. For instance, *Erythroxylum coca* has a vein islet number of 8–12, while the *E. truxillense* has 15–26, thus distinguishing the two. *Cassia senna* has 15–29, though *C. angustifolia* has 19.5–22.5; for this situation, effectively distinguishing between them is difficult with this method.

Veinlet termination—An extreme free end or end of a veinlet is called a veinlet termination and the quantity of them per square mm of leaf surface is called the veinlet termination number. It is determined according to the vein islet number and can be evaluated at the same time. It can be utilized as a identifying character for the leaves of similar species or for a different one, especially when the vein islet number does not give distinguishing results. *Cassia senna* has an estimated number of 32.7–40.2, while *C. angustifolia* has 25.9–32.8; *Atropa belladonna* has an estimated number of 6.3–10.3, though *A. acuminata* has an estimated number of 1.4–3.5, for this situation both the varieties can be recognized effectively with this parameter. The main inconvenience of this method is that it requires quite a large segment of a leaf and is ideally measured on a specific piece of the leaf. Therefore, it is useful for the assessment of intact leaves instead of powders.

Methodology—The methodology is depicted schematically in Fig. 5.15.

5.17.4.2 Stomatal Index

For the identification and characterization of leafy crude drugs, the stomatal number and the stomatal index play a crucial role. Based on the form and arrangements in the surrounding cells, there are several types of stomata that are often available:

Stomatal number and stomatal index:

- *Stomatal number*: It is calculated as the average number of stomata per mm^2 of the leaf epidermis on each surface.
- Each stomatal aperture is considered as a single unit.
- The stomatal number varies depending upon the environmental and geographic effects.

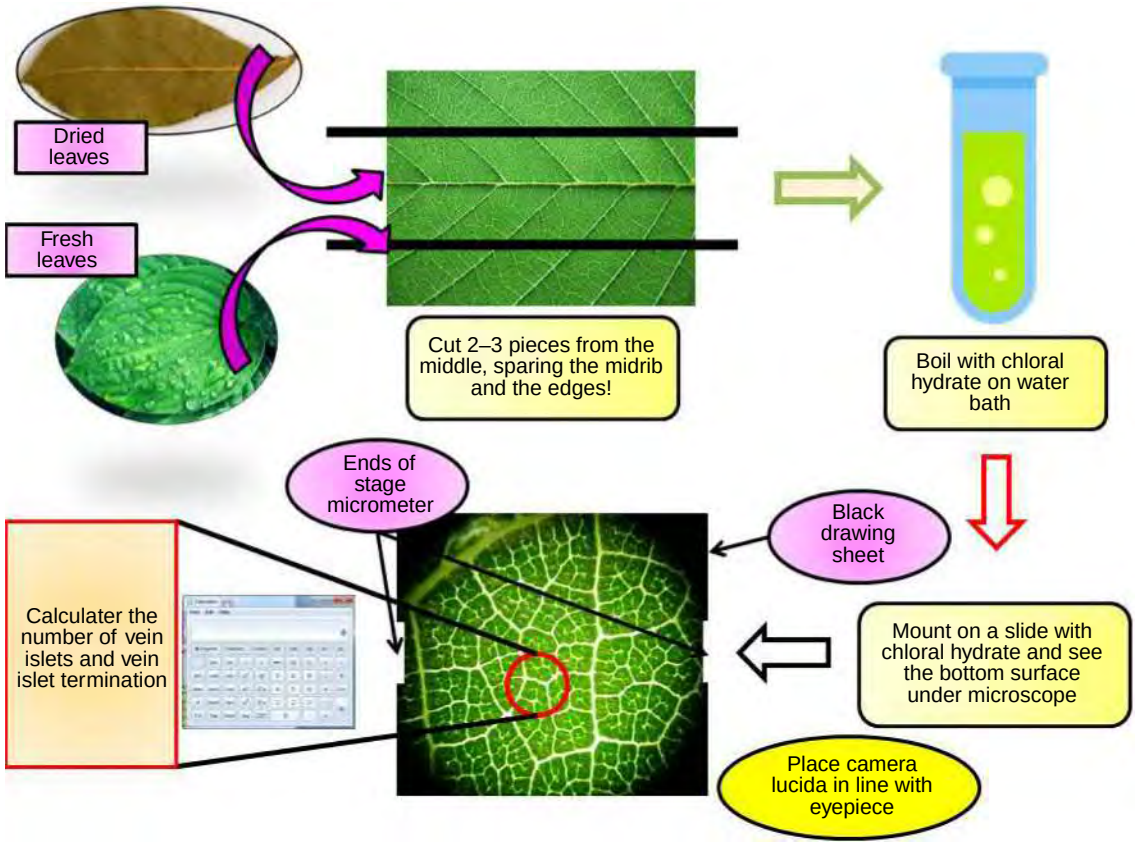

FIG. 5.15 Determination of vein islet and veinlet termination numbers.

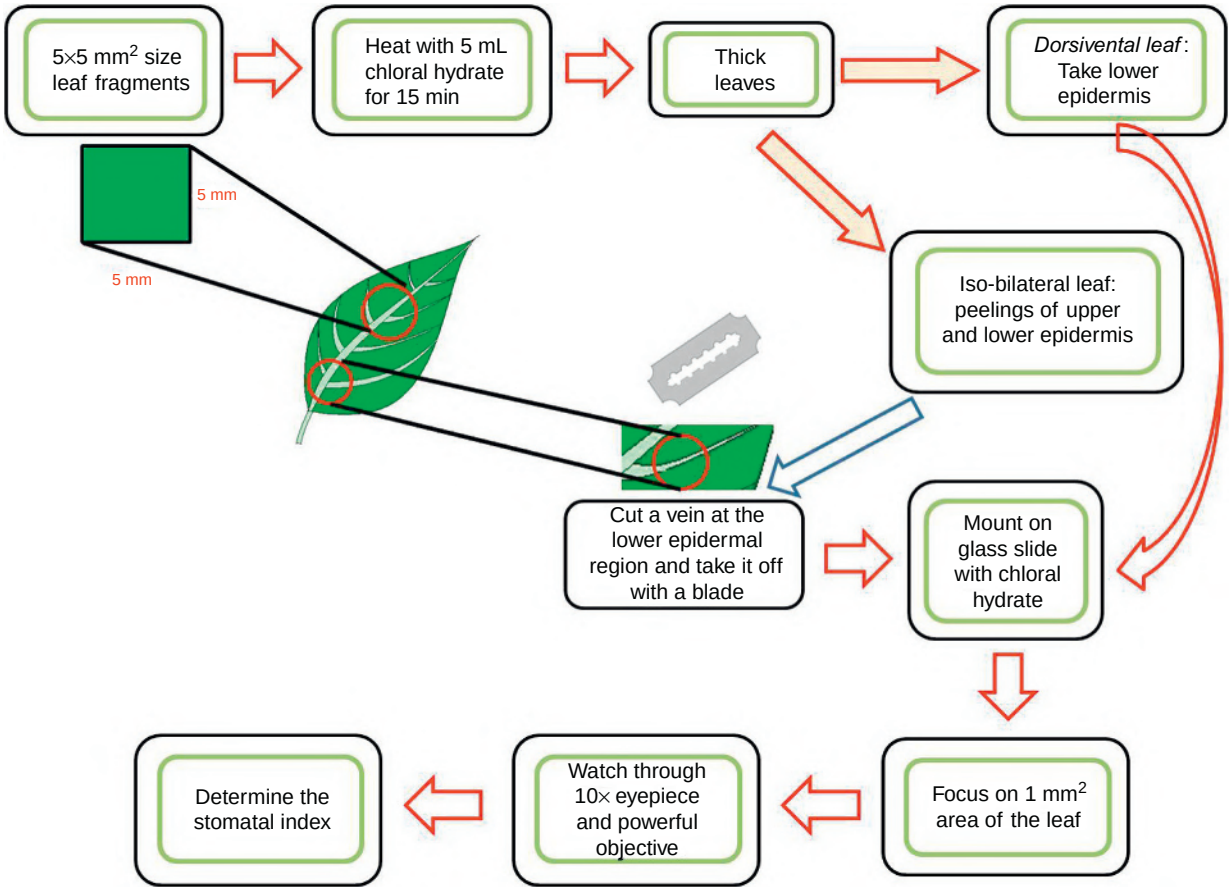

FIG. 5.16 Determination of stomatal index.

- *Stomatal index*: It is the percentage proportion of stomata on one side and epidermal cells plus stomata on the other side. In other words, the stomatal index is defined as the percentage of stomata from the total number of epidermal cells, which can be explained as:

$$\text{Stomatal index} = \left[S / \left(E + S \right) \right] \times 100.$$

where S is the number of stomata in a given area of leaf and E is the total number of epidermal cells, including trichomes, in the same area of the leaf.

For determining the Stomatal index—The procedure for determining the stomatal index has been described in Fig. 5.16.

On tracing paper a circle smaller than the field of view is drawn and the camera lucida is fixed in such a way that the circle/square is clearly visible in the center of the field of vision. For determining the stomatal number, only the stomata are to be marked and, for each leaf sample, not fewer than 10 determinations should be carried out to calculate the average index. By using this parameter, identification and standardization of crude leafy drugs is easily possible.

5.17.4.3 Palisade Ratio

The palisade ratio is another characteristic feature that is quantitatively determined for the quality evaluation of leaf drugs. It serves as a primary means of identification of a sample and can provide very useful supporting evidence, which, taken together with other factors, can make an accurate evaluation and identification.

The average number of palisade cells beneath each upper epidermal cell is known as the Palisade ratio. For a particular species, the value remains more or less unchanged, and it is taken as a hallmark for the quality of that particular plant. The

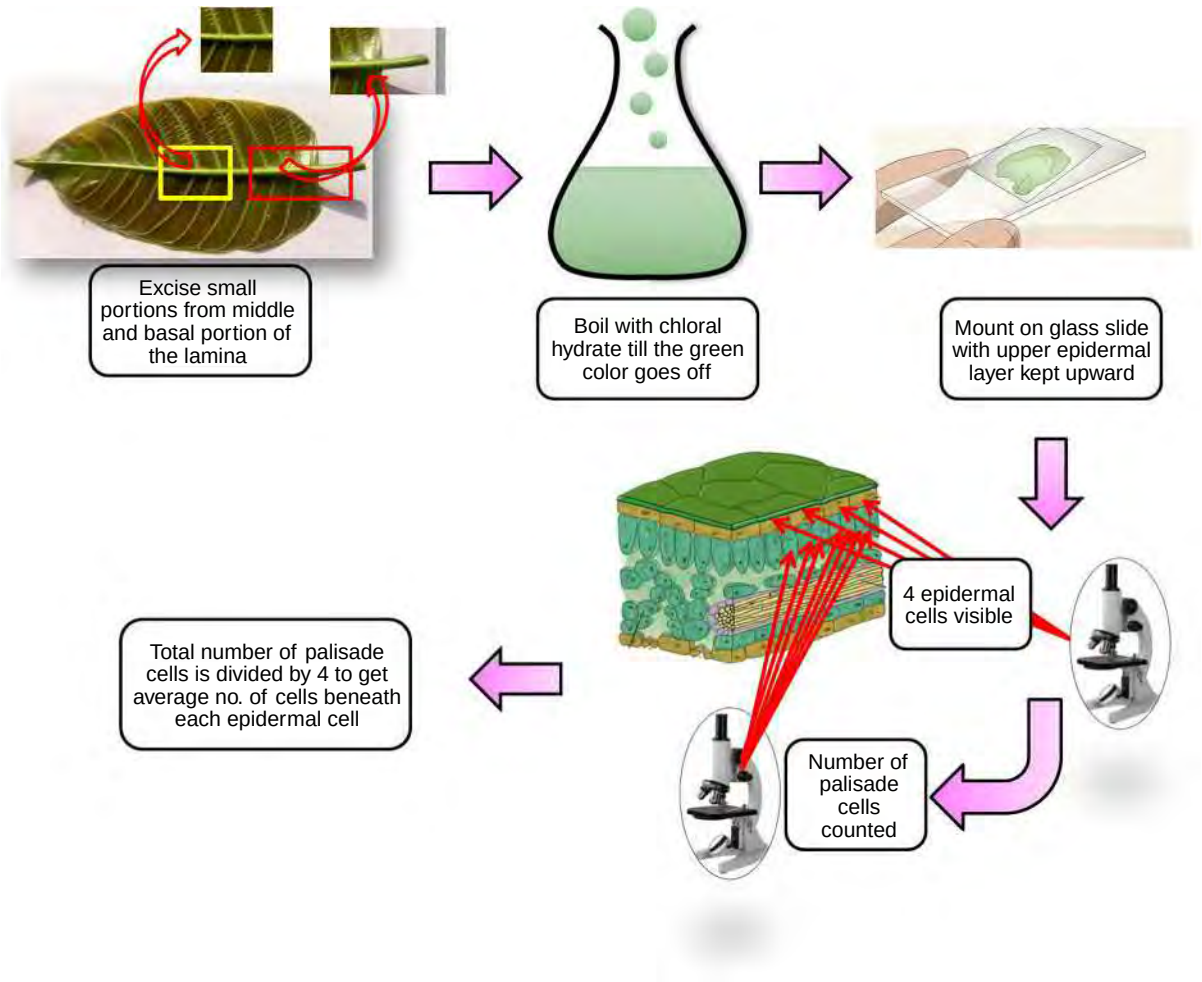

FIG. 5.17 Determination of palisade ratio.

limitation of this method is that it is applicable only to dicotyledonous plants. The differentiation in mesophyll cells is not possible in monocot plants.

Methodology—The Methodology for the determination of the palisade ratio is presented in Fig. 5.17.

5.17.4.4 Lycopodium Spore Method

It is not possible to use a microscope for quantitative estimation without checking the number of particles of a specific sort or estimating the area of a sample of a material having a given thickness and density. In light of this, the Lycopodium spore technique is essential. Lycopodium comprises the spores of *Lycopodium clavatum* Linn. Every spore is tetrahedral in shape, with a rounded base and three level sides that meet to frame three surrounding checked covering edges, which meet at the top. The entire surface of the spore is covered with minute reticulations and the inside is loaded with fixed oil. The spores are outstandingly uniform in size (25 μm), so one can easily realize that a positive number of spores represents a specific weight of lycopodium. The entire procedure can be improved by measuring a solitary spore or it can favorably be communicated as a number of spores per mg. This amount has been resolved and interpreted after various investigations to average 94,000 spores for each mg, which is taken as a consistent value, detailed by Wallis (1985), the well-known pharmacognosist. Utilizing this figure, one can compute the weight of any number of spores under any condition under the magnifying instrument. In the event that the lycopodium has been blended with a particular amount of another substance, one can discover quickly the amount of the second substance added, when analyzed minutely. On the off chance that it is admixed with any fine particles, such as dust grains, starch, and so forth, with trademark countable particles it is conceivable to compute the quantity of such characteristic particles per mg. By this method, it is possible to have a standard value that represents any such unadulterated material. The quantity of specific characteristic particles per unit weight is regularly uniform and is valuable in evaluating the nature of a sample. To utilize such a strategy, the quantity of particles in a decent-quality sample should either be known or first decided.

Some examples of particle properties determined with the lycopodium spore method are given below:

- *Pyrethrum flower*: 1000–2000 pollen grains per mg.
- *Ginger*: 261,400 starch grains per mg.

Comparison with the expected count gives the percentage purity of the observed sample. A suspending agent, such as mucilage of tragacanth, castor oil, olive oil, or a mixture of oils, is necessary for this purpose. A mixture of these two oils give a liquid of suitable viscosity and, depending on the environmental temperature, a suspending agent can be prepared. When oil cannot be used, mucilage of tragacanth (1.22% (w/v) tragacanth in hydro-alcoholic solution) or a mixture of it with glycerin can be used. This method is of particular importance in herbal drug evaluation as it can be extended to cover not only a simple estimate of the frequency of a single type of particle in a sample, but is also useful for the determination of the proportion of different powders in a mixture and the area or length of a particular structure per unit weight of powder.

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Chapter 6

Extraction and Other Downstream Procedures for Evaluation of Herbal Drugs

Chapter Outline

6.1 BASIC PRINCIPLES AND RATIONALE

Extraction is the process for the separation of medicinally active substances from plant parts using a particular solvent(s). These preparations are also known as galenicals, which is a standard medicinal preparation containing usually one or more active constituents of a plant and made by a process that leaves the inert and other undesirable constituents of the plant undissolved (Handa et al., 2008). The extraction of crude drugs aims at extracting out the therapeutically important fractions or single molecules, determining their contents in the particular plant extract, as well as many more purposes, with

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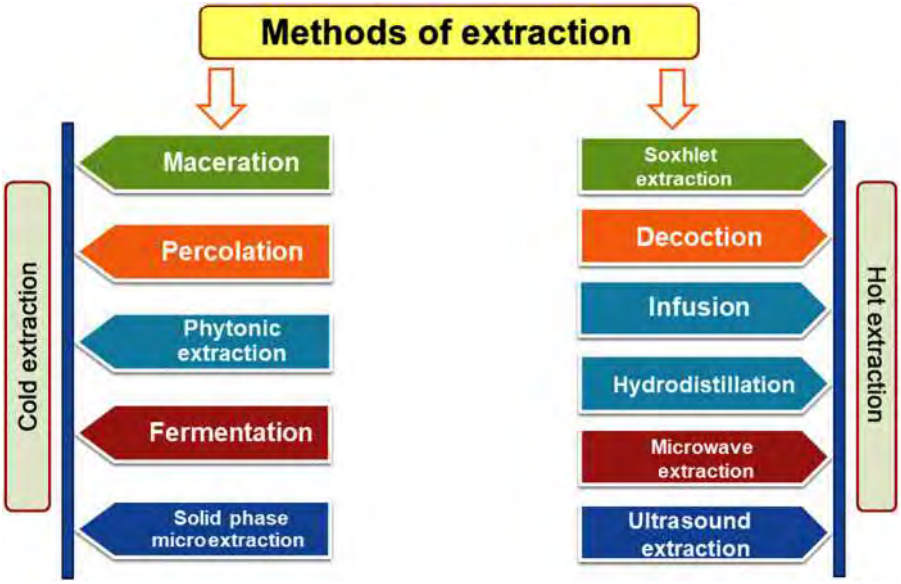

FIG. 6.1 Different types of extraction methods.

the ultimate aim of a bioactivity-guided isolation process (Mukherjee, 2002). An extract, obtained after extracting the crude plant material with a suitable solvent, may be used in several forms, such as a tincture, infusion, decoction, or in a capsule/tablet dosage form. Also, a crude extract may be subjected to fractionation, and isolation of active single molecules to isolate different metabolites from various groups, such as alkaloids, anthraquinones, coumarins, essential oils, flavonoids, steroids, terpenoids, and triterpenoids. The different extraction methods may be classified as cold or hot extraction methods. This classification has been shown in Fig. 6.1.

Process parameters affecting extraction yield include the plant parts used, the nature of the solvent used, the extraction process and equipment, the ratio of the crude drug to the extract, etc. Appropriate selection of all of these parameters is essential in order to extract the desired bioactive constituents (Houghton, 1998).

The use of appropriate extraction technology, plant material, manufacturing equipment, extraction method, solvent and an adherence to good manufacturing practices also affect these parameters. The biodiversity of medicinal and aromatic plants is an important thing for the flourishing of the medicinal plant extraction industry (Handa et al., 2008). For exhaustive extraction of all of the constituents (polar and nonpolar), the solvent of choice is alcohol (methanol or ethanol). The conventional procedure for extraction of plant materials is a successive extraction process using solvents of increasing polarity. First, the most nonpolar solvent, such as petroleum ether or hexane, is used to remove the fats, lipids, waxes, and chlorophylls. After that, solvents of increasing polarities, such as ethyl acetate, chloroform, acetone, and methanol, respectively, are used for extraction. Solvents used for extraction with different polarities are shown in Table 6.1.

Separation, recovery, and purification of active principles from a crude extract, be it the mother liquor or the powdered extract, is done by chromatographic separations, such as column chromatography, flash chromatography, countercurrent extraction, preparative TLC, and high-performance liquid chromatography (HPLC), along with spectroscopic techniques, such as NMR, IR, and mass spectrometry (MS) (Mukherjee, 2002).

An extraction process aims to reach an equilibrium state of the concentration of the extractives between the solid state and the solvent. The extraction process is considered complete when the concentration gradient between the solid and liquid state becomes zero. This state is denoted by a constant, K, as given by the following equation (Mukherjee, 2002).

$$K = \frac{\text{Concentration of extractive substances in the miscella}}{\text{Concentration of extractive substances in drug residue}}$$

There are several determinants of the extraction process, such as the type, quantity, moisture content, degree of comminution, and solvent properties. For a constant quantity of the solvent, a greater amount of crude drug material gives fewer yields. Increased proportions of the disintegrated cells augment the extraction by improving the dissolution from the disintegrated cells. The final step for extraction is the dissolution of the soluble materials out of the cells. This depends on the solubility of the cellular materials in the solvent, the particle size (PS), the degree of swelling, the temperature, the properties of the solvents, and the rate of establishment of the equilibrium changes. This in turn affects the extraction efficiency.

TABLE 6.1 Solvents Used for Extraction and Separation of Herbal Drugs Based on Their Polarity Index

Solvent	Polarity Index	Dielectric Constant DK (20bzw, 25 °C)	Boiling Point (°C)	Solubility in Water (%, w/w)
1,4-Dioxane	4.8	2.2	101.0	100.000
1.1.2-Trichloro-trifluoroethane	—	2.4	47.7	—
1-Butanol	3.9	17.8	117.2	7.810
2-Propanol	4.3	18.3	82.4	100.000
Acetic acid	6.2		118	100.000
Acetone	5.4	20.7	56.2	100.000
Acetonitrile	6.2	37.5	81.6	100.000
Benzene	2.7	—	80	0.180
Butyl acetate	4.0		125	0.430
Carbon tetrachloride	1.7	2.2	76.5	0.080
Chloroform	4.4	4.8	61.7	0.815
Cyclohexane	0.0	2.0	80.7	0.010
Di ethyl ether	2.8	—	35	6.890
Dichloroethane	3.7	10.6	83.4	0.810
Dichloromethane	3.4	9.1	40.0	1.600
Di-isopropyl ether	2.2	—	68	—
Dimethyl formamide	6.4		155	100.000
Dimethyl sulfoxide	7.2		189	100.000
Ethanol	5.2	24.3	78.5	100.000
Ethyl acetate	4.3	6.0	77.1	8.700
Isoctane	0.4	1.9	99.2	
Methanol	5.1	32.6	65.0	100.000
Methyl ethyl ketone	4.7		80	24.000
n-Heptane	0.0	1.9	98.4	0.003
n-Hexane	0.0	1.9	68.9	0.001
n-propanol	4.0		92	100.000
Pentane	0.0		36	0.004
Tert. butyl methyl ether	2.9	—	55.2	4.800
Tetrahydrofuran	4.2	7.4	66.0	100.000
Toluene	2.3	2.4	110.6	0.510
Trichloroethylene	1.0	—	87.0	0.110
Water	9.0	80.2	100.0	100.000
Xylene	2.5	—	139	0.018

Temperature is also an important parameter, as a higher temperature expedites the dissolution of the constituents. The pH value of the solvent also affects the selectivity of the extraction.

Dissolved constituents may adsorb at the surfaces of the inert plant support material. Examples include adsorption of quinine on cinchona bark and strychnine on *Nux vomica* seeds. Such incidences have been attributed to an increased amount of crude drug for less solvent and a lower quantity of yield. Wormwood (Vermuth), lesser centuary, thyme, and also the leaves of henbane (Hyoscyamus) showed similar results. Attempts at a quantitative description of the conditions have led to Muller's maceration isotherms and to Melichar's theory of colloidal dissolution. The degree of lipophilicity (and obviously the hydrophilicity, also) determines the amount and qualitative compositions of extracted substances for a particular solvent (Mukherjee, 2002).

6.2 FACTORS AFFECTING EXTRACTION OF HERBAL DRUGS

The basic parameters influencing the quality of an extract are:

- plant part used as starting material;
- solvent used for extraction; and
- extraction procedure.

The effect of the extracted plant phytochemical depends on:

- the nature of the plant material;
- its origin;
- degree of processing;
- moisture content;
- particle size (PS).

The variations in different extraction methods that affect the quantity and secondary metabolite composition of an extract depend upon:

- type of extraction;
- time of extraction;
- temperature;
- nature of solvent;
- solvent concentration;
- polarity.

Plant-based natural constituents can be derived from any part of the plant, such as bark, leaves, flowers, roots, fruits, and seeds, that is, any part of the plant may contain active components.

6.2.1 Choice of Solvents

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. The factors affecting the choice of solvent are:

- quantity of phytochemical to be extracted;
- rate of extraction;
- diversity of different compounds extracted;
- diversity of inhibitory compounds extracted;
- ease of subsequent handling of the extracts;
- toxicity of the solvent in the bioassay process; and
- potential health hazard of the extractants.

The choice of solvent is influenced by what is intended with the extract. Because the end product will contain traces of residual solvent, the solvent should be nontoxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted.

Variations in the extraction method usually depend upon:

- length of the extraction period.
- solvent used.

- pH of the solvent.
- temperature.
- PS of the plant tissues.
- the solvent-to-sample ratio.

The basic principle is to grind the plant material (dry or wet) finer, which increases the surface area for extraction, thereby increasing the rate of extraction. Earlier studies reported that a solvent-to-sample ratio of 10:1 (v/w) solvent to dry pressure ratio is ideal (Pandey and Tripathi, 2014).

6.2.2 Types of Extracts

Extracts may be of different types, such as aqueous or hydroalcoholic, and may be in the forms of an infusion, decoction, or a tincture. They may further be concentrated in to soft, dry, or liquid extracts.

(a) Aqueous extracts

These are the extracts whose medicinal preparations are to be used immediately after preparation or to be preserved for use. The following methods are generally used for their preparation.

(i) *Decoction*: This is the ancient and popular process of extracting water-soluble and heat-stable constituents from crude drugs by boiling in water for about 15 min. The boiled crude drug–water mixture is then cooled and filtered and a sufficient volume of cold water is passed through the drug to produce the required volume.

(ii) *Infusion*: An infusion is generally a dilute solution of the readily soluble constituents of crude drugs. It is nothing but a type of periodic maceration of the drug with either cold or boiling water. The infusion is filtered to remove crude vegetable material and then produced in the required volume by the addition of water.

(iii) *Digestion*: Digestion is also a type of maceration in which moderate heating is preferred during extraction. Heating causes the digestion of drug material and increases the solvent efficiency. It is preferred for those drugs for which the use of moderately elevated temperatures does not cause degradation of the constituents.

(iv) *Tinctures*: Tinctures are alcoholic or hydroalcoholic solutions prepared from crude drugs or from pure organic or inorganic substances. Tinctures of crude drugs may contain 10–20 g of drug per 100 mL of tincture. The methods used for the preparation of tinctures are maceration and percolation. An iodine tincture is an example of an inorganic pharmaceutical. Belladonna tincture is prepared by percolation, while compound benzoin tincture and sweet orange peel tincture are prepared by maceration.

(v) *Liquid extracts*: Liquid extracts are also called fluid extracts in some official books. They are a liquid preparation of crude drugs that contain ethyl alcohol as a solvent and preservative. A liquid extract may contain active constituents to the extent of 1 g of drug per mL. Pharmacopoeial liquid extracts are prepared by the percolation or modified percolation techniques.

(vi) *Soft extract*: Those extracts produced as semisolids or liquids of syrupy consistency are called soft extracts. These extracts are used in a variety of dosage forms ranging from ointments to suppositories, or they can be used in the preparation of some other pharmaceuticals. Glycyrrhiza extract USP comes in the form of a soft extract.

(vii) *Dry extract*: Dry extracts are also known as powdered extracts or dry powders. The total extracts obtained using a suitable process of extraction, are filtered, concentrated, preferably under vacuum, and dried completely (Shah and Seth, 2012).

Preextraction operations for crude drugs have a great influence on the extraction procedures.

The rate of solid–liquid extraction is determined by several factors, which include postharvesting processes, the characteristics of the crude drug matrix, solvent selection, method of contact, and the extraction temperature.

6.2.3 Raw Materials

Despite the fact that there is usually no choice in the raw material used, there are several factors to consider. The influence of the physical state of the sample (solid, liquid) on the outcome of the extraction is well known. When dealing with solid samples, other factors, such as PS, shape, and porosity, are of crucial importance because they have a direct effect on the mass transfer rate of the process. In order to increase the extraction rate, the solid matrix must be comminuted to increase the mass transfer area. On the other hand, particles that are too small must be avoided. Their use can compact the bed, increasing the internal mass transfer resistance and causing channeling inside the extraction bed. As a result, the extraction rate decreases due to nonhomogeneous extraction. After harvesting, fresh medicinal plant materials may be dried to keep microbial infestation at bay. This may be done by shed drying (a thin layer of finely size-reduced crude drug, air dried under

a shed) or oven drying (with hot air in an oven). The drying conditions should be carefully monitored in order to prevent damage to the active constituents. The extractives in a crude drug may remain adhered to the solid surface or be entrapped in a solid matrix. In the former case, the extraction is a bit easier and size reduction does not need much attention. On the other hand, if the extractives remain entrapped within complex matrix structures, the size reduction should be optimized to such a degree so as to augment the release and dissolution of soluble constituents from the crude drug.

6.2.4 Choice of Solvent

Several aspects need to be considered when selecting a solvent for commercial use, such as the solvent power (selectivity), boiling temperature, reactivity, viscosity, safety, cost, vapor pressure, and recovery. These factors will be detailed in later sections.

6.2.5 Conditions for Extraction

Crude drugs used for extraction should be in the form of coarse granules and not in the form of fine powders. The latter impedes the flow of solvent through the sample bed. The particles of a crude herbal drug should be stirred and dispersed during the extraction procedure. Agitation helps to suspend the particles in the extracting solvent (Handa et al., 2008).

6.2.6 Selection, Collection, and Identification of Plant Material

The selection of plants is accomplished based on random, ethnopharmacological, chemotaxonomic, geographical, or compound structural bases. The selection of plant materials is done by first carrying out a thorough literature survey of the different medicinal plants used and prescribed by local healers in some specific system of medicine, for example, Ayurveda, Siddha, Unani, Traditional Chinese Medicine, or Traditional Thai Medicine. Modern, high-throughput approaches involve the use of databases of traditional medicinal plants, such as the NAPRALERT database, which are explored with the help of software, such as the Literature Information Selection Technique. Such software helps to establish a scientific basis for the selection of plants, correlating the medicinal values, therapeutic uses, and the taxonomic and other factors. Factors affecting the quality of herbal preparations, as well as extracts, need not be discussed in detail in this section as they have already been covered in Chapters 3 and 4. In brief, it may be said that environmental factors (light, soil nutrients, stresses, temperature, and altitude), the plant part selected, the phenological phase, and the preservation of herbarium samples for further authentication and identification are all very important for maintaining the quality of the extract (Mukherjee, 2002).

6.2.7 Drying of the Crude Drugs

Plant materials are dried to prevent microbial growth. In the course of drying, some of the chemical ingredients of the plant also decompose, such as the flavonoids. Thermolabile phenolics remain intact, which has been substantiated by a study on *Moringa oleifera* leaves.

Fungal infestation may occur in a fresh plant material, if it is kept for a long time, thus causing sample deterioration. Also, if the plant material contains some photosensitive material, it should never be sundried and should be stored below 30 °C. In several cases, in which drying of the plant material is not quite feasible, the plant material, immediately after collection, is size reduced and soaked in methanol or ethanol, as these alcohols have antimicrobial properties. Also, they check the endogenous enzymatic activities within the plant tissues. For studying essential oils, the fresh plant material itself is distilled or otherwise extracted, and not dried, to prevent the loss of volatile components, followed by documentation of a good TLC fingerprinting chromatogram of the oil.

- *Air drying* is a time-consuming process, taking days, or even months to be completed. The advantage is that this procedure leaves the thermolabile compounds unaffected.
- *Microwave heating* involves the application of microwaves (1 mm to 100 cm) to a plant material containing a little bit of water or other polar solvents. These polar solvents absorb the microwaves and generate dipolar rotations and oscillations. This results in heating the material from the inside to the outside, decreasing the drying time, though often degrading some of the active molecules.
- *Oven drying* is another preextraction method that uses thermal energy to remove moisture from the samples. This sample preparation is considered to be one of the easiest and most rapid thermal processes that is capable of preserving phytochemicals.

- *Freeze drying* is a method based on the principle of sublimation. Sublimation is a process in which a solid is changed into the gas phase without entering the liquid phase. The sample is frozen at −80 °C to −20 °C prior to lyophilization to solidify any liquid (e.g., solvent, moisture) in the samples. After an overnight (12 h) freezing, the sample is immediately lyophilized to avoid melting the frozen liquid in the sample. Freeze drying yields a higher level of phenolic contents compared with air dying as most of the phytochemicals are preserved using this method (Azwanida, 2015).

6.2.8 Comminution and Classification

Lowering PS increases the surface contact between samples and extraction solvents. Grinding results in coarse smaller samples; meanwhile, powdered samples have a more homogenized and smaller particle, leading to better surface contact with extraction solvents. This particular preparation is important, as for efficient extraction to occur, the solvent must contact the target analytes and a PS smaller than 0.5 mm is ideal for efficient extraction. A conventional mortar and pestle or electric blenders and mills are commonly used to reduce the PS of the sample (Mukherjee, 2005).

Comminution is done to get a narrow particle size distribution (PSD). It helps to avoid the clogging of the column and thereby yields dust-free fractions. Large differences in the PS of the drug also result in long extraction times, as the solvent takes longer for complete extraction of the coarser material. The plant material needs to be shredded and sieved properly.

Plant materials are shredded and thereafter sieved to get a particular grain size. After successive grinding and sieving, we get different types of fractions:

- *Finely shredded fractions*: Used for preparation of teabags or sachets.
- *Semifinely shredded fractions*: For tea or infusion mixtures.
- *Coarsely shredded materials*: Used for ready-to-use drugs or for extraction.

Impurities in a plant crude drug, such as sand, metal, pieces of wood, and dead beetles, may be removed through various procedures. Examples include pneumatic removal of the sand and magnetic removal of the metal. The comminution of different parts of the herbal drug will be explained in subsequent sections.

6.2.8.1 Medicinal Leaves and Herbs

Size reduction in leaf-type materials should be done in such a manner that the sample does not contain any stem or stalk. The size reduction should be performed using a shredding mill, hammer mill, pin mill, fluted rollers, or grinders. Tobacco grinding may be considered as one prototype size reduction mechanism. Plant materials containing a high content of stem and stalk are shredded with shredding mills. The hammer mills are for friable materials. Fluted roller grinders are for drugs having a high content of ethereal oils. In addition to this, ball mills and cutting mills are quite prevalent in industry.

6.2.8.2 Roots and Barks

Roots and barks are reasonably hard or woody; however, now and again fragile and friable plant parts can also be seen. Their condition fluctuates after collection. Fine thin bark (*Cinnamomum*, *Cortex frangulae*) and coarse thick compositions (*Cortex condurango*, *Cortex quercus*) exist. Herbal roots are thin, woody, grouped structures (*Radix ipecacuanha*, *Radix valerianae*). Medicinal plant cutters or shredders mostly have sharp edges for accomplishing an alleged square cut, while shredder mills are utilized for cutting and destroying. Crushing of powdered medications is accomplished with hammer mills and fine mills with sifters.

6.2.8.3 Seeds and Fruits

The comminution of seeds and fruits often proves to be particularly difficult because they contain fats and ethereal oils. Both fine mills with sieve attachments and shredder and pin mills are used. Roller and fluted roller mills are also used, particularly for grinding coffee and cocoa.

6.3 METHODS OF EXTRACTION

In assembling different classes of therapeutic plant extracts, for example, decoctions, imbuements, liquid concentrates, tinctures, semisolid concentrates (pilular), and powdered concentrates (famously known as galenicals), both straightforward conventional strategies and propelled innovations are utilized, conforming with the official methodology and particulars as set down in different pharmacopeias and codices of the world (Handa et al., 2008). Depending on the type(s) of the metabolites of interest, particular extraction procedures are used. Examples that are common in industry include solvent–solvent

extraction for alkaloids, water distillations (hydrodistillation, steam distillation, cohobation, SFME) for the isolation of volatile oil, extraction with polar organic solvents, such as methanol, ethanol, and water for phenolics, flavonoids, and other polar metabolites. Also, the mode of extraction may be different, such as cold maceration, Soxhlet extraction, decoction, or infusion as discussed previously.

Extracting a drug at atmospheric pressure along with the application of heat (infusion or decoction, as may be the case) is considered to be the most popular method of extraction. In addition, different improvised techniques have emerged, such as steam distillation (extraction of essential oils), supercritical fluid extraction (SFE), and extractions using different liquefied gases.

6.3.1 Factors Affecting the Choice of Extraction Process

The following factors affect the extraction yield and the stability of metabolites:

- Nature of the crude drug (fatty, fibrous, oily, soft, hard, rough, etc.).
- Stability profile of the crude drug (thermolabile or thermostable, compatibility with organic solvents, etc.).
- Cost of the crude drug.
- Nature of the solvent.
- Method employed for concentration of the extract.

The decision to utilize maceration or percolation fundamentally relies on the nature and attributes of the unrefined medications to be extricated. Accordingly, information on the kind of organs and tissues in the plant matter is basic for accomplishing the best outcome. A strategic distance ought to be maintained when using persistent hot extraction techniques when constituents of the medication are thermolabile. When the rough medication is costly (e.g., ginger), it is attractive to acquire the finished extraction. In this way, from a monetary perspective, percolation ought to be utilized. For modest medications, maceration, in spite of its lower effectiveness, is worthy because of its lower cost. The choice of the solvent depends on the dissolvability of the desired parts of the material. In the event that the constituents require a solvent other than an unadulterated bubbling solvent or an azeotrope, nonstop extraction ought to be utilized. Weak items, for example, tinctures, can be made by maceration or percolation. For semiconcentrated arrangements, the more efficient percolation process is utilized. Concentrated arrangements, for example, fluid or dry concentrates, are made by percolation (Handa et al., 2008).

6.3.2 Maceration, Digestion, and Remaceration

Maceration, digestion, and remaceration take place based on the principle of leaching, whereby the soluble constituents get dissolved in the solvent and come out of the physical structures of the crude drug (cells or tissues).

Maceration is the process of extracting a drug with a solvent with several daily shakings or stirrings at room temperature. Compared with other methods of extraction, the intensity of movement is so low that we use the term stationary conditions.

Kinetic maceration is carried out at room temperature, like simple maceration, the difference being that the material is kept in constant motion.

Remaceration: In this technique, some of the solvent is added to the drug. After filtration, the residue is extracted with the remainder of the solvent and the drug residue is squeezed out to express as much solvent as possible.

When the maceration process is completed and equilibrium is achieved, the extract solution is filtered through a cloth. For filtration of the marc, a special instrument, such as a filter press, may be employed. A liquid rich in active constituents is referred to as the miscella. The filtered liquid is generally cloudy and contains small particles. It is recommended to provide sufficient time for the liquid to settle and, thereafter, it is filtered and the extract is subjected to concentration (Fig. 6.2) (Handa et al., 2008).

Macerates are officially described as the aqueous extract of the crude plant material, prepared by soaking the plant material in water for 30 min, followed by filtration.

Digestion is maceration at higher temperature, normally at 40–50 °C. In the more important pharmacopeias, maceration has various meanings, which includes macerates, among others, with water, also called "aqueous drug extracts."

One specific case in which maceration is the method of choice occurs when the drug possesses little support material. Some examples as per the British Pharmacopeia are:

- Senna (Liquid extract)
- Squill (Tincture)
- Compound Benzoin tincture
- Catechu tincture
- Opium tincture

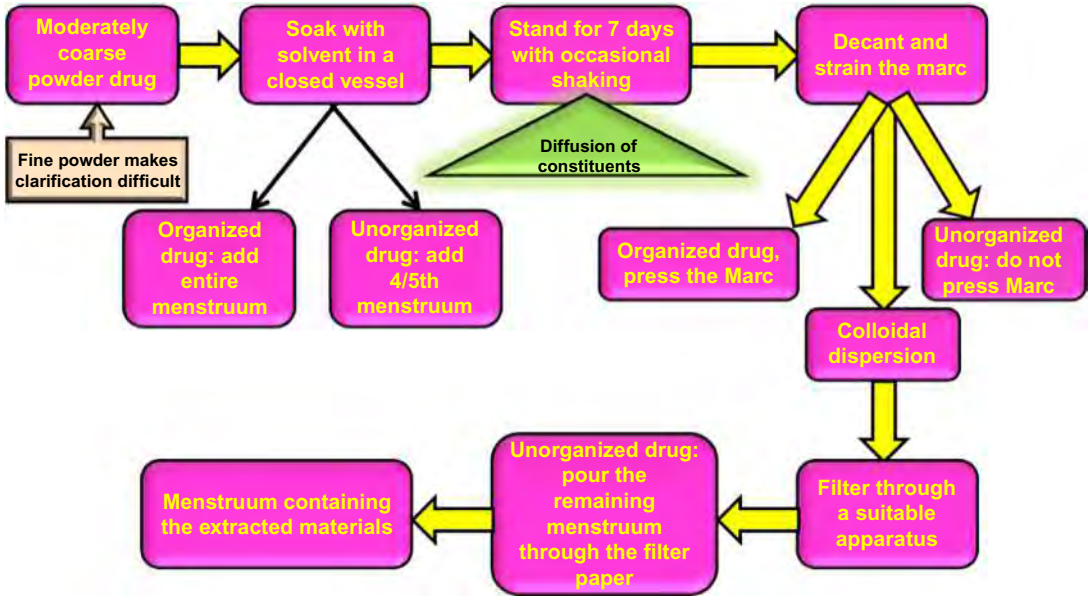

FIG. 6.2 Schematic diagram for extraction of the crude drug using maceration.

6.3.2.1 Advantages and Disadvantages

Advantages

- The preparation of macerates in the pharmacist's laboratory may be easily scaled up to a production scale, with little variation in the operational parameters.
- It does not require heating of the plant material, and so thermolabile compounds may be obtained by this process.
- Highly versatile process.
- Drugs having a high swelling index must only be extracted through maceration.
- Efficiency may be further enhanced through the introduction of agitating mechanisms, such as a magnetic stirrer or homogenizer.
- Moreover, the mother tinctures of homeopathic medicines are also prepared via maceration.
- The methodology is simpler than percolation.

Disadvantages

- Exhaustive extraction is not possible by this method. For exhaustive extraction, we need to decant the solvent and extract repeatedly using fresh solvent, which is economically unacceptable.
- Repeated filtration is necessary for the extraction process. This will cause a loss of solvent, which is again not feasible economically.

6.3.2.2 Percolation and Repercolation

Percolation is an exhaustive extraction procedure, by which all of the soluble constituents are completely removed from a comminuted plant material, by extracting the crude drug by fresh solvent. In the case of repercolation, the percolate is again introduced as the solvent, thus reducing solvent consumption. Continuous countercurrent extraction may be another advancement, whereby fresh solvent and plant material flow in opposite directions, in order to bring already extracted material in contact with fresh solvent. This increases the concentration gradient of the extractives from the plant material to the solvent, expediting the extraction (Dévay, 2013). A schematic diagram of the extraction of a crude drug using percolation is shown in Fig. 6.3.

Percolation may be performed using different types of percolators at both large and small scales. In order to prepare the concentrated extract, after maceration or percolation, the miscella is concentrated under reduced pressure. A percolator is a tapered glass or metallic container with a stopcock at the base, which controls the rate of the solvent elution (Fig. 6.3). There are several special attributes of the percolation process, which include:

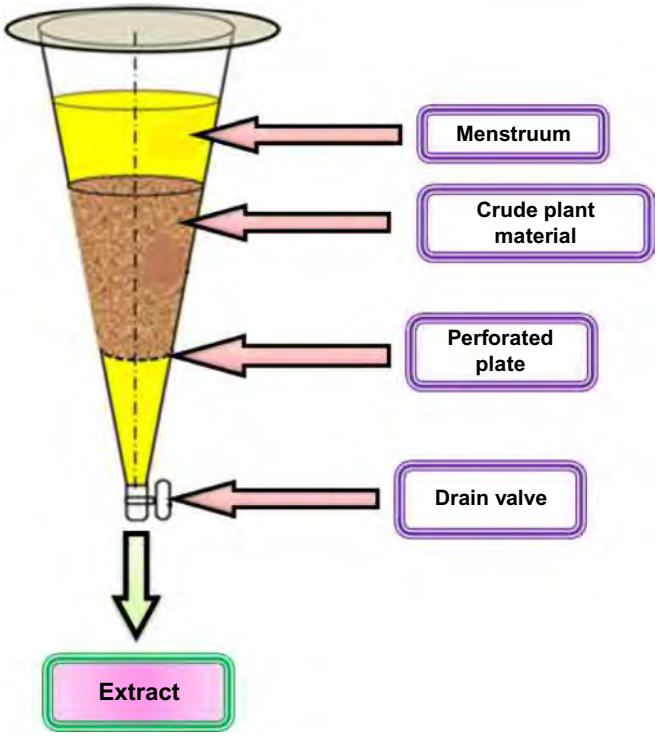

FIG. 6.3 Schematic diagram of the extraction of a crude drug using percolation.

- It is less time consuming and also does not need much manipulation.
- Coarsely fragmented samples, passed through a 3 mm sieve are used for the percolation process. Otherwise, having too large a PS is not suitable for the equilibrium necessary for extraction.
- As the sample gets exposed to fresh solvent repeatedly, this process is more suitable for an exhaustive extraction.
- Hot or cold solvent may be used.
- Very fine powders, resins, and powders that swell or give a viscous eluent cannot be extracted by this method.

Percolation is generally performed as a batch process, rather than a continuous process. In this method, the sample has to be kept in the percolator up to 24 h (repeated up to three times). The eluted materials are then collected and pooled.

The percolation procedure is depicted schematically in Fig. 6.4 (Handa et al., 2008).

6.3.2.3 Examples of Experiments on Percolation and Observations

Example 6.1

Ambrosia sp. has been studied for the presence of secondary metabolites in its extracts. Extracting the plant twice using chloroform removes all of its sesquiterpene content, and consequently, in the third fraction, no sesquiterpene is obtained.

Example 6.2

The presence of a chemical class of compounds may be ascertained using particular qualitative tests of that chemical class. The presence of alkaloid in the crude extract of a plant may be detected using qualitative tests for alkaloids, such as Mayer's test or Dragendorff's test. A precipitation reaction indicates the presence of alkaloids. For the detection of the presence of flavonoids, performing qualitative tests is optional. In the case of cardiac glycosides, Kedde's reaction may be used, while for carbohydrates Molisch's test or Fehling's test may be employed.

Example 6.3

In the case of fatty substances, the plant material should be first extracted with pet ether or *n*-hexane, followed by concentration in vacuum (Mukherjee, 2002).

The percolation procedure is applicable at both the laboratory scale and the industrial scale. The percolation procedure has been schematically described in Fig. 6.4. The raw material used for extraction should be size reduced by comminution or shredding to a coarse PS. If the size is reduced to a fine PS, it may clog the conical percolator and the percolation process will be disrupted. Also, it is desirable that the percolate does not need to be filtered or clarified. In the case of a

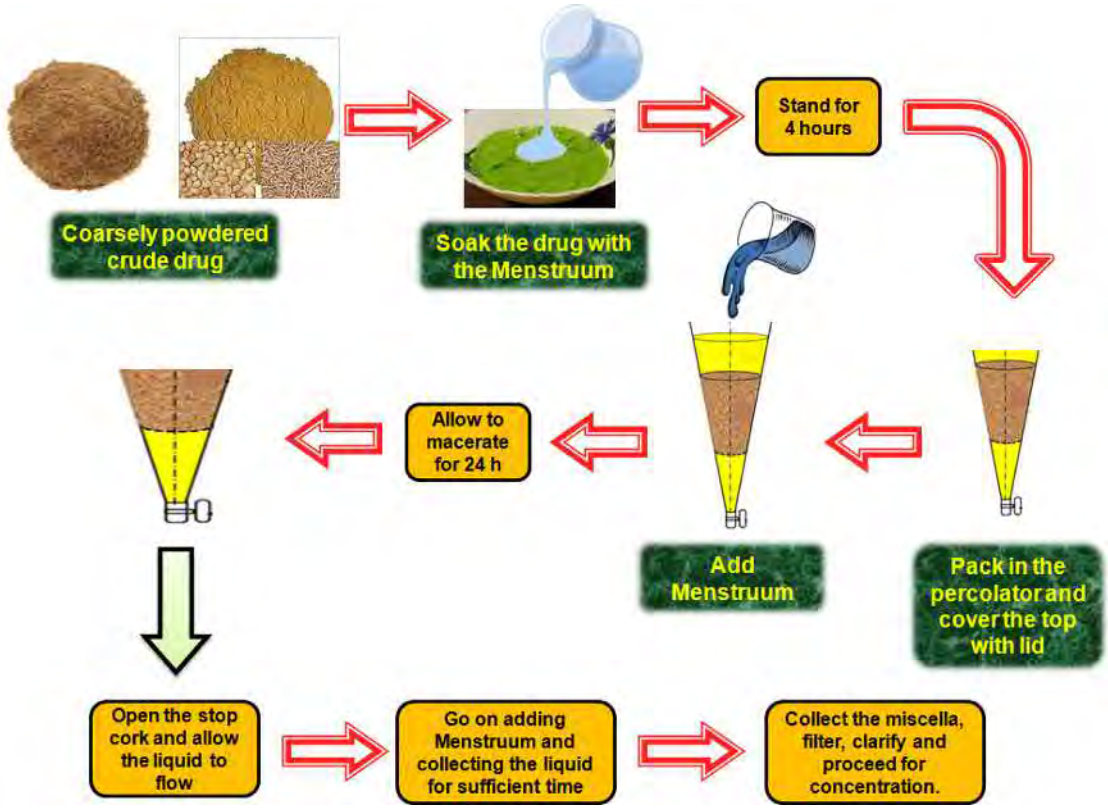

FIG. 6.4 Schematic representation for the extraction of crude plant material using the percolation procedure.

finely powdered crude drug, filtration becomes a must to remove the particles, which is time consuming, as well as providing chances for processing losses. These are unacceptable. Again, a preswelling process has to be carried out outside the percolator first to have a better extraction. The preswelling should never be carried out within the percolator, as this will break the glass percolator. After preswelling is complete, with sufficient solvent, the crude drug is allowed to stand in the percolator for 24 h, called the intermediate maceration.

6.3.2.4 The Factors Affecting the Percolation Process

(A) Selectivity of solvent

The selectivity of the solvent is important, not only for the yield of one or more principal substances but also for the qualitative and quantitative composition of the accompanying substances.

(B) Quantity flow (dropping rate) of the solvent

The flow rate of the solvent is governed by the mixed dropping rate. This therefore determines the contact time between solvent and drug. The drug/solvent ratio is the ratio of the quantity of drug used to the total quantity of solvent consumed. Although temperature is important in percolation, it is a practice only rarely used as a controlling factor.

(C) Temperature

It has an important role to play because there are some phytoconstituents that require a warm environment for more extracting, while some others may be destroyed at higher temperature. So, temperature has to be considered always while planning the percolation process (Mukherjee, 2002).

(D) Modifications to the general process of percolation

Some limitations of the conventional percolation procedure include problems concerning the concentration of extract having thermolabile substances and the concentration of hydroalcoholic mixtures.

(a) Reserved percolation

In this case, extraction is done through the general percolation procedure. At the end, evaporation is performed under reduced pressure in equipment, such as a climbing evaporator, to the consistency of a soft extract (semisolid) such that all the water is removed. It is then dissolved in the reserved portion, which is strongly alcoholic and easily dissolves the evaporated portion without any risk of precipitation.

(b) Cover and run down method

In this method, the sample is repeatedly extracted by maceration. The latter stages of the extraction involve introducing the percolate as a solvent in the percolator, thus achieving a concentrated extract. In this method, we may use methanol (methylated spirit), instead of ethanol (rectified spirit). As a result, the cost decreases. The toxic methanol is removed during the evaporation process.

(c) Large-scale extraction procedures

In the case of large-scale industrial extractions, some modifications in the conventional extraction methods are required. In the case of small-scale extractions, agitation of the extraction vessels is not a problem, whereas in the case of large-scale production, shaking of the vessel is not quite so easy. There are several methods available for agitation, with or without the application of heat. The degree and time of heating applied is another important determinant of the cost, which is quite important.

(d) Circulatory extraction

The effectiveness of extraction during a maceration method may be improved by arranging for the solvent to be continuously circulated through the bed of the crude drug. Solvent is circulated from the bottom of the vessel (through an outlet) and is distributed by spray nozzles over the surface of the drug. The movement of the solvent reduces boundary layers and also the uniform distribution minimizes the local concentration in a shorter time.

(e) Multistage extraction

In the general maceration method, extraction is incomplete because mass transfer ceases once equilibrium is reached. This drawback may be overcome by employing a multistage method. The instrumentation necessary for this technique is a vessel for the crude drug, a circulating pump, spray distributors, and a number of tanks to receive the extracted solution. The extractor and tanks are such that any of the tanks may be connected to the extractor for the transfer of the product. Every batch of drug is treated many times with solvent and, once a cycle is in motion, the receivers contain solution with the strongest in receiver 1 and the weakest in receiver 3 (Fig. 6.5).

(f) Extraction battery

While performing percolation, the percolate may be a dilute solution instead of a more desirable concentered one. To overcome this, continuous extraction devices can be used to handle a greater amount of material through a multistage process.

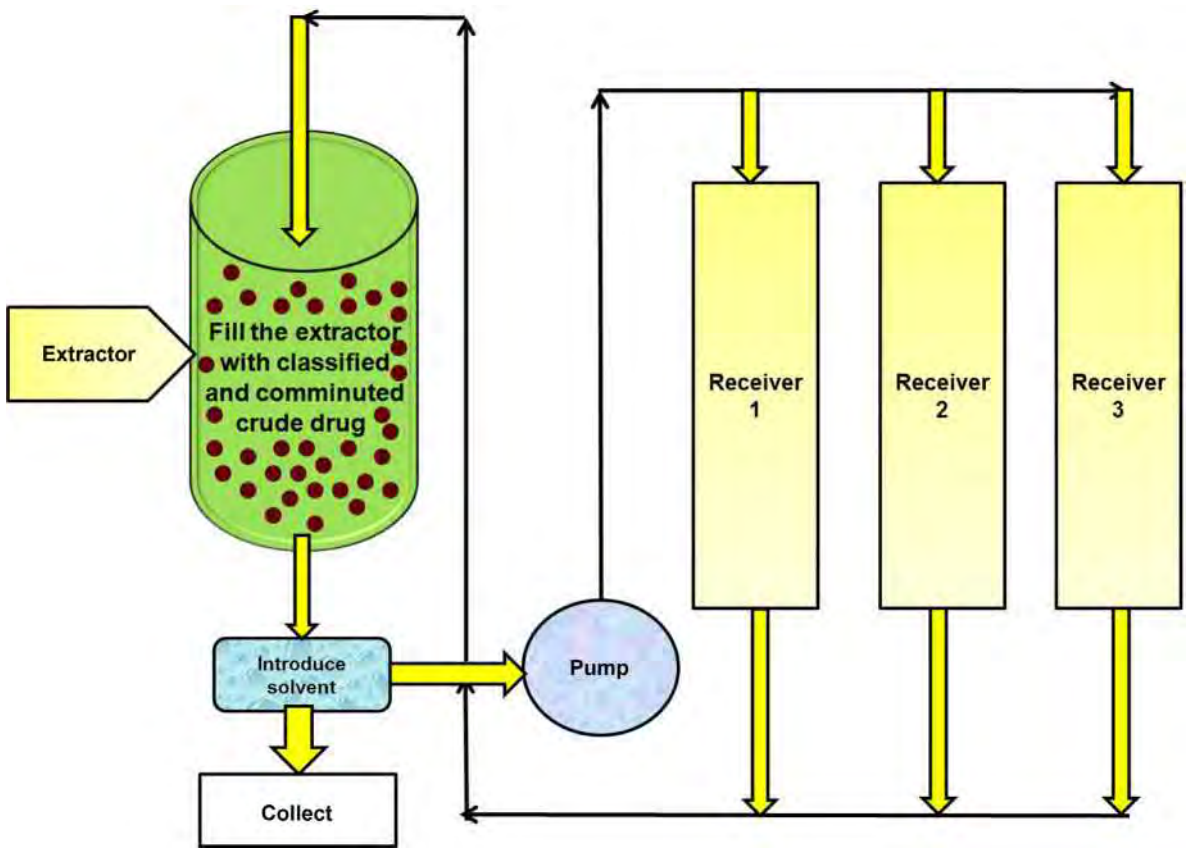

FIG. 6.5 Multistage extraction assembly.

An extraction battery comprises various vessels with interconnected funneling. Vessels are arranged so that solvent can be added to and the item taken from any vessel. These vessels can, consequently, be made into an arrangement in which any of the vessels may be the first of the arrangement. In a typical percolation process, the permeate is dilute and, in that capacity, exceptionally weak. To have the maximum concentration, persistent extraction devices of this composition are utilized when a lot of single material is processed. This can be accomplished by devising a phase-astute process. In this procedure, a progression of vessels are utilized and extraction is semiconstant. The equipment is called an extraction battery and comprises a number of vessels with interconnected pipes. Vessels are arranged to the point that solvent can be added to and the item taken from any vessel (Handa et al., 2008; Singh, 2008).

6.3.3 Infusion and Decoction

Infusions are one sort of official preparation in which the drug is macerated in cold or boiling water for a particularly short period of time, followed by straining through a filter medium. After that, concentration is achieved using a suitable method. The infusion thus obtained is diluted with water and dispensed for therapeutic use. As per official prescriptions, one part of the infusion should be 10 times diluted with water (Handa et al., 2008). These preparations should be used up within 24 h of preparation, as they are quite liable to microbial contamination (Fig. 6.6).

Decoction is an extraction technique, little different than conventional extraction techniques, and generally used for tough and fibrous drugs. In the Indian traditional Ayurvedic system of medicine, this was known as the "Kwatha." Depending on the hardness and toughness of the drug, the volume of the solvent added varies. The crude drug:solvent ratios are 1:4 for soft drugs, 1:6 for moderately hard crude drugs, and 1:8 for hard drugs. After that, the system is boiled, strained, and the extract is concentrated to achieve a concentrated extract (Handa et al., 2008). Though many of the plant constituents are soluble in water, such as carbohydrates, flavonoid polyglycosides, quaternary alkaloids, saponins, and tannins, the plants are generally not extracted with water. Generally, such compounds are extracted by methanol–water extracts. Such factors are further illustrated elsewhere in the chapter. This decoction method may also be replaced by the Soxhlet, pressurized liquid extraction (PLE), microwave, and other methods, which are discussed later (Mukherjee, 2002).

6.3.4 Vortical or Turbo Extraction

Basic maceration is a moderate extraction process. There have been many attempts to decrease the time involved.

Even though motor maceration supplies an identical yield by shaking and mixing, vortical or turbo extraction is faster. Here the drug to be removed is mixed within the solvent with a fast blender or homogenizer. The dispersion of extractive substances through the cell layers is to a great extent supplanted by washing out from the demolished cell tissues. This results in achieving the maceration balance significantly more quickly and, thus, in an impressively short amount of time. The vitality provided by the fast mixing and comminution of the medication material raises the temperature in the extraction, which is undesirable in view of the danger of deterioration of the thermolabile constituents. Hence, the temperature rise should be kept as small as possible. This is accomplished either by stopping the procedure periodically or by cooling the vessel. The further comminution of the drug, which favors quick establishment of equilibrium (see above), carries with it the drawback of making the separation of medication deposits from the miscella more troublesome. The division can

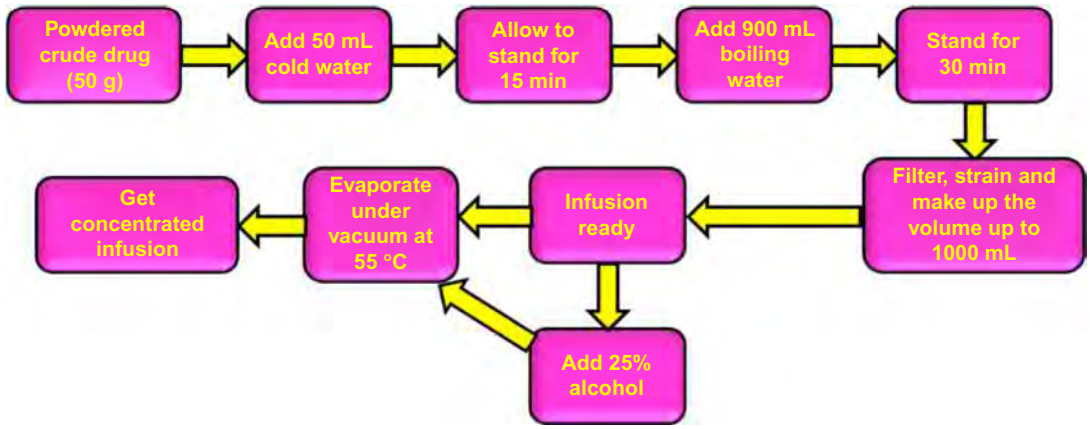

FIG. 6.6 Method for preparation of infusions.

be done by filtration, sedimentation, or centrifugation. While using the vortical technique for cinchona bark, it has been demonstrated that, in contrast with maceration and permeation, turbo extraction is better than maceration and about on a par with permeation of more than six phases. Based on the results of the study, a few vortical extractions of the material, extractions finished with half or 33% of the solvent are proposed for a proficient and quick extraction. The concentrate fluid is centrifuged off between the individual extractions (Mukherjee, 2002).

6.3.5 Ultrasound Extraction

- Ultrasound frequencies, that is, above 20,000 Hz, are generated using magnetostrictive or piezoelectric ultrasonic transmitters.
- In the magnetostrictive case, nickel steel, composed of nickel foil discs isolated from each other to prevent eddy currents, vibrate in an alternating magnetic field produced by a high-frequency alternating current. The change of length undergone by ferromagnetic substances upon magnetization (magnetostriction) is used for generation of the ultrasound wave. Frequencies up to 200 kHz can be produced in this type of instrument.
- On application of an alternating current to a quartz cell, it starts to vibrate and, when such a crystal is placed between the plates of a condenser and an alternating electric current is applied, it begins to vibrate at the frequency of the applied current. Higher frequencies are obtained with piezoelectric ultrasound transmitters.
- The major effects of ultrasound in a liquid medium are attributed to the cavitation phenomena, arising from the physical processes that result in the creation, enlargement, and implosion of microbubbles of gases dissolved in the liquid.

The main effects of ultrasound extraction are often summarized as:

- An increase in the porosity of the cell walls.
- The creation of cavitations (the spontaneous formation of bubbles in a liquid below its boiling point arising from strong dynamic stress).
- An increase in the mechanical stress of the cells (known as interface friction) (Ovadia and Skauen, 1965) (Fig. 6.7).

6.3.6 Extraction by Electrical Energy

In this method, electrical energy is used in the form of an electrical field, an electromagnetic field, and as electrical discharges to accelerate extraction and improve the yield. Extraction of hyoscine from the seeds and capsules of Indian thorn apple has been reported by this method with the help of a plate as a cathode at the bottom of the extraction vessel and several carbon electrodes as anodes at the top. The alkaloid yield was significantly increased by application of a current. The extraction of valerianic acid from valerian root was performed using this method by surrounding an extraction column with an electric coil and producing an alternating electromagnetic field of 50 Hz, which was more effective than simple maceration (Mukherjee, 2002).

6.3.7 Countercurrent Extraction

This is one type of advanced extraction technology whereby a crude drug is transformed into a fine slurry in a suitable solvent using a toothed disc disintegrator. Then, it flows through a metallic cylinder and the solvent flows in the opposite direction to the slurry. As a result, the extract comes out through one end and the Marc is released through the opposite end. The advantage of this over the other hot extraction methods is that comminution of the crude drug and the extraction method are such that they help to avert heat generation (Varma, 2016), thus helping to avoid any decomposition of any thermolabile constituents (Fig. 6.8).

6.3.7.1 Continuous Countercurrent Extraction

Here, the general countercurrent method is modified so that the initial crude drug is exposed to a miscella already containing some of the extractives within it. As a slurry of the drug proceeds through the chamber, the concentration of the extractives gradually decreases as a gradient and, at the last point of the vessel, is exposed to pure solvent. Thus, the yield can be improved.

6.3.7.2 Relative Countercurrent Extraction

The extraction solvent is in motion (one phase) and the solid phase is stationary. It does not really operate as a true continuous countercurrent extraction, although, in practice, this term is also used for it. This is a gradual transition between percolation and continuous countercurrent extraction.

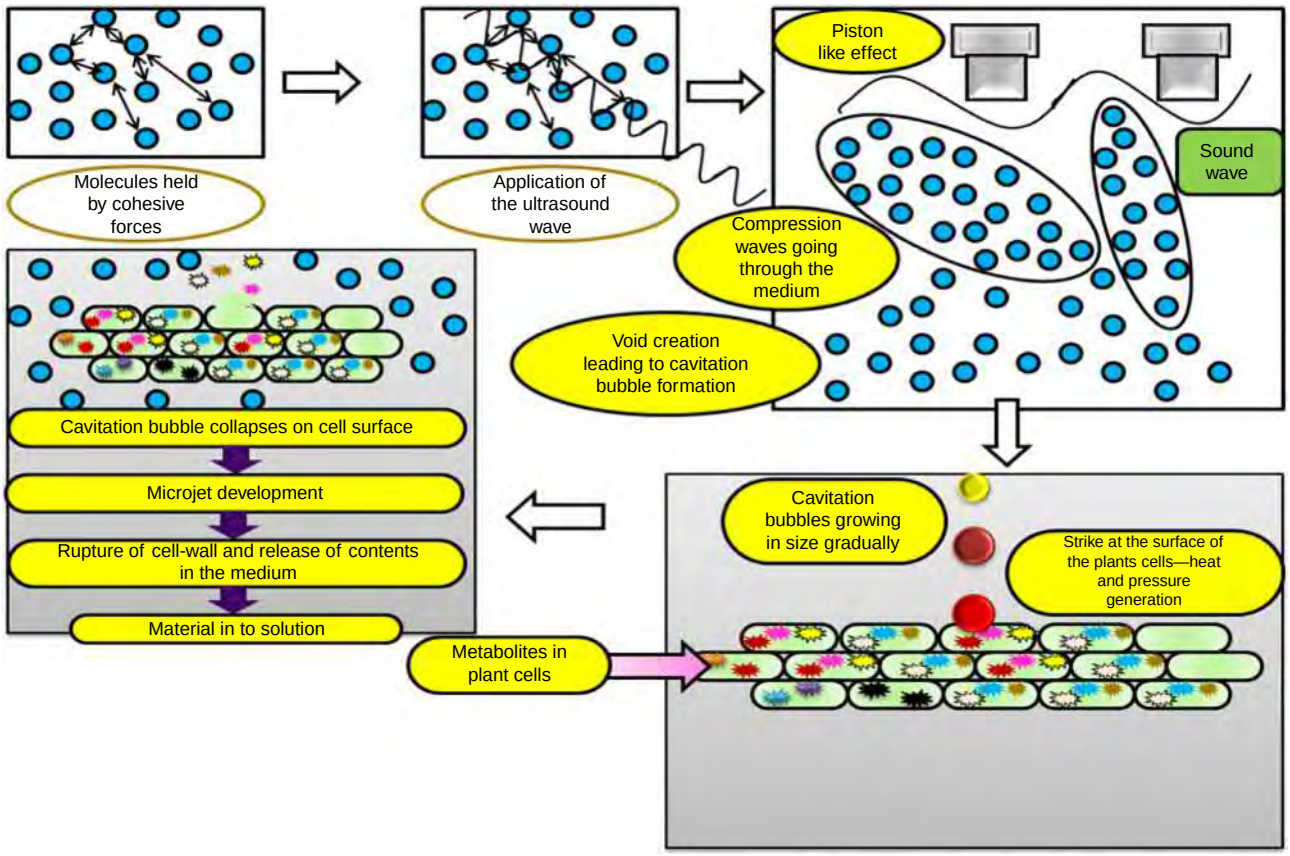

FIG. 6.7 Schematic representation of the cell rupture of basil leaves after ultrasound extraction (Rostagno and Prado, 2013).

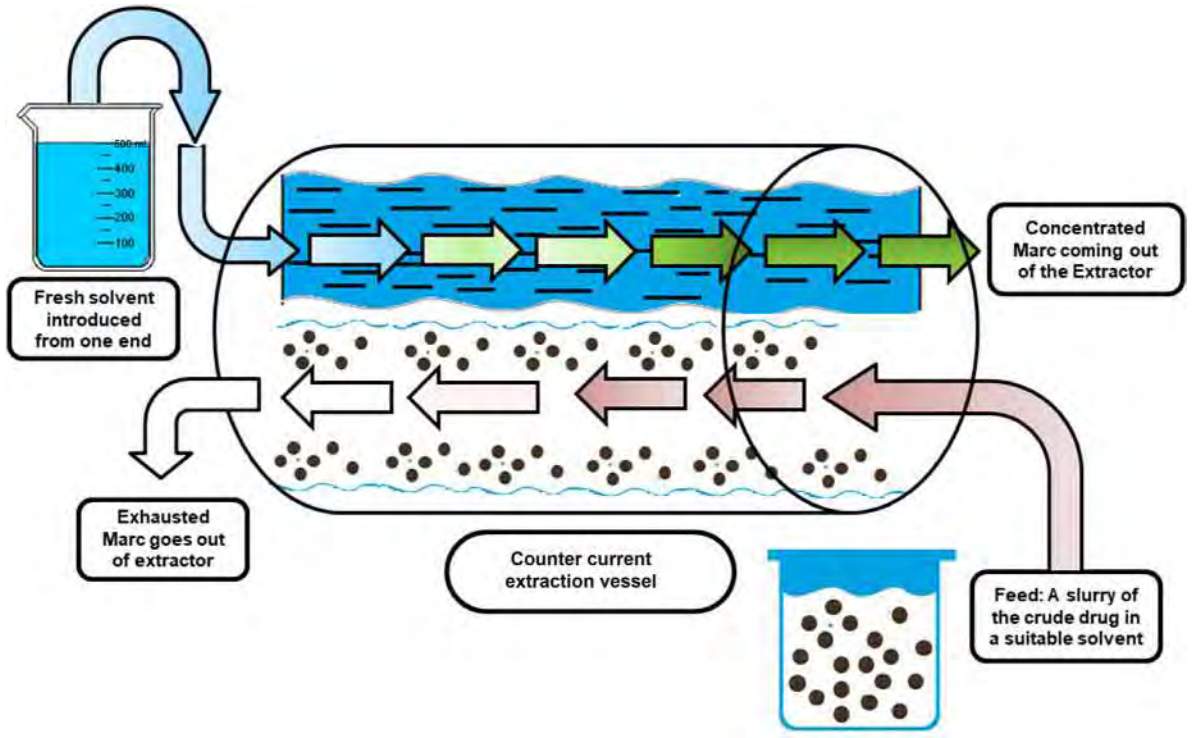

FIG. 6.8 Schematic representation of countercurrent extraction.

6.3.8 Hot Continuous Extraction—Soxhlation

Advancements in hot continuous extraction have led to the development of the Soxhlet extraction technique, which, itself, has further undergone several improvements. The objectives, which were finally achieved, were a shortening of the leaching times with the use of auxiliary energies and automation of the extraction assembly (Castro-Vargas et al., 2010). The main advantages of this technique lie in the fact that the solvent and time consumption are both lower in comparison with the other conventional extraction techniques. It requires 500 mL solvent and <24 h time for the extraction of a 500 g sample. The main disadvantage is that the extract is subjected to heating for quite a long time, which may cause decomposition of many thermolabile substances. Soxhlet extraction may be done with one pure solvent or a mixture of two or more solvents. In the latter case, the problem remains that the two solvents may possess significantly different volatilities and, thus, are distilled at different rates. So, the ratio of the solvents changes with reference to the original one in the thimble (Fig. 6.9).

A Soxhlet apparatus consists of several components, including:

1. A round-bottom flask at the bottom, to be heated by a heating implement, such as a heating mantle. It holds the solvent, and after the start of extraction, the solvents.
2. The central compartment, inside which the crude drug is packed in a cellulose, paper, or cloth jacket (of course, the material must be permeable), called a thimble.
3. A condenser at the top of the whole setup, accompanied by one water inlet and one water outlet.

The round-bottom flask may be of 500, 1000, 2000, or 3000 mL volume, and holds the solvent, which may be petroleum ether, alcohol, water, hydroalcoholic mixtures, or other organic solvents, such as chloroform, acetone, or ethyl acetate. This is connected to the central compartment of the Soxhlet, containing the thimble. The central compartment contains two tubes within it. One tube is the side tube, through which the solvent vapor passes and accumulates in the condenser. There, the vapor gets concentrated and, thus, ultimately accumulates in the central compartment. Once the solvent level in the central compartment reaches the level of the siphon tube, the whole liquid held in the central compartment gets siphoned to the round-bottom flask and thereafter the whole thing starts boiling in the round-bottom flask (Mukherjee, 2002).

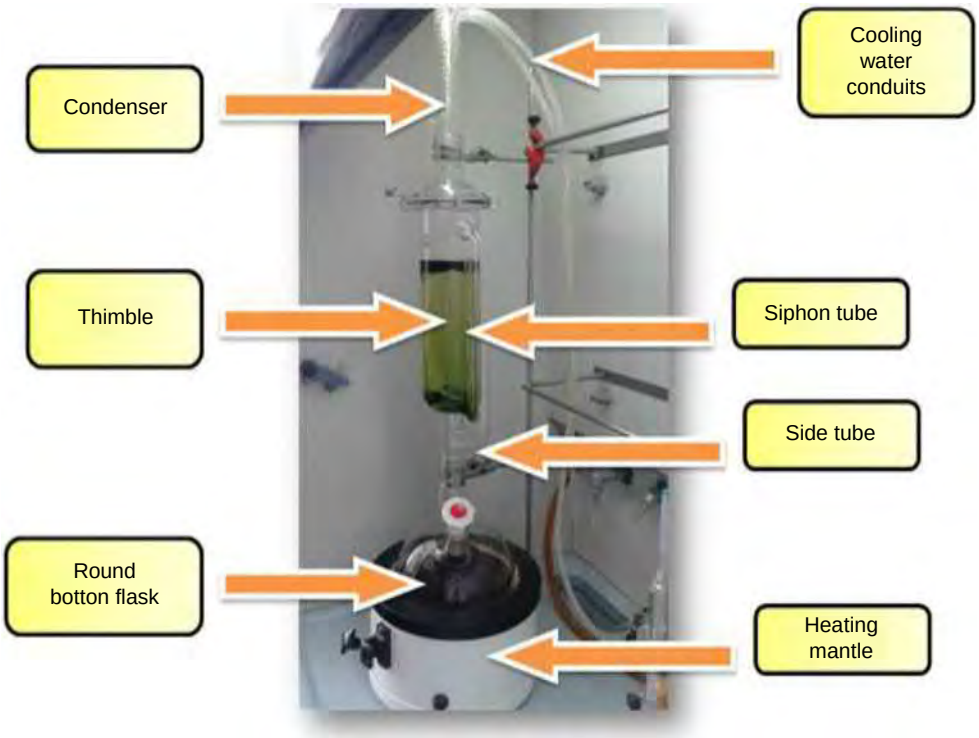

FIG. 6.9 A Soxhlet apparatus with its components indicated.

6.3.8.1 Advantages of a Conventional Soxhlet Apparatus

- The sample is repeatedly brought into contact with fresh portions of extractant, which facilitates displacement of the transfer equilibrium.
- In addition, no filtration is required after leaching.
- Simple methodology requiring little training.
- Can extract more sample mass than microwave-assisted extraction (MAE), SFE, etc. It seems to be subject to no matrix effects.
- There are varieties of official methods involving a sample preparation step based on Soxhlet extraction.
- Involves no agitation, helping to expedite the process.

6.3.8.2 Disadvantages of a Conventional Soxhlet Apparatus

- Long time and large amount of extractant required for extraction.
- Samples are usually extracted at the solvent boiling point over long periods, resulting in thermal decomposition of thermolabile target species.
- In addition, the large amounts of extractant used call for an evaporation–concentration step after extraction.
- Limited by extractant and difficult to automate.
- This methodology is restricted to extraction with pure solvents or azeotropic mixtures and cannot be used for extraction with any solvent mixture, for example, hexane:DCM 1:1.

Most of the modifications reported over the last few decades have been aimed at bringing Soxhlet closer to more recent techniques for solid sample preparation, by shortening leaching times with the use of auxiliary forms of energy and automating the extraction assembly.

6.3.8.3 High-Pressure Soxhlet Extraction

Soxhlet extraction under high pressure is accomplished by placing the extractor in a cylindrical stainless-steel autoclave or through the use of either commercial or laboratory-made supercritical liquid Soxhlet extractors. The character of high-pressure Soxhlet extraction is that the extractants do not achieve supercritical conditions. They can be low-boiling solvents or gases under ordinary pressure and temperature, but in the liquid state under high pressure. The improvement of the Soxhlet procedure under high pressure (1000–1500 psi) should shorten the time required and reduce solvent use. High-pressure Soxhlet extraction has been utilized to detach organochlorine pesticides and polychlorinated biphenyls (PCBs) before assurance in ensured potato, carrot, olive oil, and lyophilized fish tissue tests. In this application, carbon dioxide was used as the extractant medium at the top fame of the extractant. The extraction setup was submerged in a thermostated bath with a framework to direct cooling water (0 °C) to condense the extractant. Another application was fractionation of low molecular weight polyethylene. In this study, liquid carbon dioxide was observed to be a good solvent for the lowest molecular weight hydrocarbons; however, it failed to solubilize hydrocarbons with molecular weights higher than C-40–C-50. Liquid pentane was observed to be an effective solvent for hydrocarbons that are insoluble in liquid carbon dioxide. The primary disadvantage of these extraction frameworks is related to their operational standards. The Soxhlet procedure should not be influenced by execution under high pressure, which includes an additional level of complexity and that diminishes the power of the extractors.

6.3.8.4 Automated Soxhlet Extraction

Automation of Soxhlet extraction was first achieved on the commercial Soxtec System HT, which gave significant savings in time and extractant. This device uses a blend of reflux boiling and Soxhlet extraction (both helped by electrical heating) to perform two extraction steps (boiling and washing), followed by extractant recovery. In light of the utilization of these devices, there are several tested techniques accessible as Application Sub Notes inside the horticultural, food, and modern industries, ranging from total fat extraction in meat to extraction of PCBs in soil and sludge. Soxtec systems have been utilized as a part of formally endorsed strategies, for example, AOAC 2003.05 and 2003.06 (unrefined fat in forage, oat grain, and scavenge utilizing diethylether and hexane extraction techniques), AOAC 991.36 (fat rough in meat and meat items), ISO 1444:1996 (free fat substance in meat and meat items), or EPA 3541 (extraction of PCBs in soil and waste). In spite of the use of commercial extractors in reference investigation strategies, their reduced arrangement does not improve the rare adaptability of the traditional Soxhlet machine.

6.3.8.5 Ultrasound-Assisted Soxhlet Extraction

An extractor using the Soxhlet extraction method assisted by ultrasound has been developed and used by researchers in the extraction of the total fat from oil seeds, for example, sunflower, rapeseed, and soybean. The approach utilizes the

traditional Soxhlet glassware, yet has the Soxhlet chamber situated in a thermostatic bath through which ultrasound is connected by methods for an ultrasonic test. The application of ultrasound to the sample cartridge gives equivalent results, or stunningly better than, those acquired by traditional Soxhlet extraction (official ISO strategy); however, it greatly decreases the number of Soxhlet cycles required in customary methods. Be that as it may, the most essential result of ultrasound application is the decompaction impact it produces, which avoids regular steps of grinding a few times between Soxhlet cycles to decrease the increased compactness delivered by the dropping extractant. Regardless of the detailed oxidative impact of ultrasound under intense conditions, the mild conditions utilized as a part of this extractor do not degrade the separated oil.

6.3.8.6 Microwave-Assisted Soxhlet Extraction

Among the attempts to improve Soxhlet performance, the most successful has been the use of microwaves, which has provided a wider variety of approaches. In fact, microwave-assisted Soxhlet extraction remains the most interesting improvement of conventional Soxhlet extraction. Microwave-assisted Soxhlet extraction differs mainly in some or all of four aspects from other MAE techniques:

- The extraction vessel is an open one, working under normal pressure.
- Microwave irradiation is focused on the sample compartment.
- Exhaustive extraction possible.
- No subsequent filtration is required (Castro-Vargas et al., 2010).

6.3.9 Extraction of Essential Oil

6.3.9.1 Distillation

The distillation method for the determination of the volatile oil content of medicinal plants is quite popular. The hydrodistillation method comprises the following phenomena:

(i) hydro diffusion;
(ii) hydrolysis; and
(iii) decomposition by heat.

6.3.9.2 Hydro Diffusion

For the essential oil in the vacuoles to be distilled with water, the oils must first be freed from the vacuoles. In the case of distillation with water only, the cell membranes become swollen and the oil, dissolved in the water, exits the cells by diffusion and then is vaporized with water. In the case of steam distillation, the soaked plant material swells and the oil leaves the cells and is then vaporized with the steam. The water and oil ultimately are condensed and form separate layers in the graduated tube. After this, the components may decompose due to prolonged heating and they may hydrolyze into simpler compounds. Hydrodistillation may be of three main types, namely: (1) water distillation, (2) water and steam distillation, and (3) direct steam distillation.

6.3.9.3 Water Distillation

In this process, the crude drug to be extracted is immersed in water, the ratio of the drug and water (or water/glycerol mix) being 1:8.

The flask may be heated by direct fire, closed steam jacket, steam jacket, microwave, open steam coil, and other methods.

There are some drawbacks to water distillation, such as (1) ester-type compounds in the oil are susceptible to hydrolysis, and the hydrocarbons and aldehydes are susceptible to polymerization. These may be degraded during the extraction process. (2) Oxygenated parts, such as phenols are inclined to dissolve in water, therefore, their complete removal by distillation is not attainable. (3) As water distillation tends to be a small-scale operation, it takes an extended time to accumulate a lot of oil, therefore, good-quality oil is usually mixed with low-quality oil. (4) The distillation method is treated as an art by local distillers. (5) Water distillation may be a slower method than either water and steam distillation or direct steam distillation.

6.3.9.4 Water and Steam Distillation

In water and steam distillation, the steam is generated either in a satellite boiler or inside the still, though separated from the material. Like water distillation, water and steam distillation is widely employed in rural areas. Moreover, it does not

require any more capital expenditure than water distillation. Also, the equipment used is generally very similar to that employed in water distillation; however, the material is supported on top of boiling water on a perforated grid. In fact, it is common that persons performing water distillation eventually accomplish water and steam distillation (Houghton, 1998).

6.3.9.5 Direct Steam Distillation

Steam distillation is a well-known technique for the extraction of oils from crude drugs. It may be performed in numerous ways. One strategy is to mix the plant material with water and to heat to boiling (distillation with water). The vapors are gathered and allowed to condense, and the oil is isolated from the water. In any case, if the oils are then drawn out with the end product, boiling is maintained at a strategic distance. At that point, steam generated from other sources should be flowed through the plant material suspended in water, but nonetheless not boiled (hydrosteam distillation) or straight through the crude drugs, which are kept between the steam bay and the condenser (coordinate steam distillation).

Mechanical-scale renditions rely upon similar standards but may utilize additional features that allow for the reuse of condensed water. While the steam and oil are consolidated, both layers may be isolated by physical means, while the oil may be the higher or lower layer. For oils with a thickness equivalent to, or more than, the water, a natural solvent, for example, xylene, which is thinner than water, is placed within the accumulation vessel; the unstable oil then breaks down in this higher layer because it consolidates. To increase the yields of the oils, safety measures should be taken to ensure skillful buildup of the steam and volatilized oil and gathering of the condensate so as to anticipate the loss of the unstable material. However, to remove the danger of explosion, a very closed framework should not be used. Steam distillation depends on the physical rule that, once two incompatible liquids merge, each liquid independently applies its own vapor pressure. The aggregate pressure of the effervescent mix is the total of the unfinished pressures, that is, the pressures applied by the individual components (Houghton, 1998). Because boiling starts once the combination pressure is similar to the environmental pressure, boiling starts at a lower temperature than each liquid in its pure state. Typically, steam distillation requires simple hardware, and no additional filtration step is needed to isolate the freed oil. In any case, it cannot be utilized in the case of oil that contains hydrolysable segments, for instance, esters or bodies that change or are decomposed by heat. For these oils, elective methods may well be used, for instance, coordinate extraction from materials with settled oils (enfleurage), solvents, critical, or phytosols (Mukherjee, 2002).

In this case, the steam is generated in a separate boiler and the skillfully sliced crude drug is kept unbroken on a perforated platform. This permits the crude drug to be extracted underneath high with the steam generated, and conjointly facilitates the removal of the crude drug when the extraction is complete. The distillation, that ultimately ends up in the separation of the water and the oil into separate layers, is collected using a Florentine flask, a glass jar, or using a receptacle product of pure steel with one outlet close to the bottom and another close to the top.

6.3.9.6 Cohobation

Cohobation is a procedure that may only be used during water distillation or water and steam distillation. It uses the practice of returning the distillate water to the still after the oil has been separated from it so that it may be reboiled. The principle behind this is to attenuate the loss of oxygenated components, particularly phenols that dissolve to some extent in the distillate water. For most oils, the resolution in water is smaller than 0.2%, whereas for phenol-rich oils the amount of oil dissolved within the distillation water is 0.2%–0.7%. Similarly, if an aerated element is consistently brought in contact with a direct heat supply or facet of a still that is significantly hotter than 100 °C, then the probability of degradation is increased (Houghton, 1998).

The disadvantage is that prolonged heating could trigger a reaction of the compounds. As a result, the use of cohobation is not recommended unless the aerated constituents within the distillation are not exposed to temperatures above 100 °C. The equipment used for laboratory-scale hydrodistillation could be used as a circulatory distillation apparatus that is most typically referred to as a Clevenger apparatus (Houghton, 1998).

6.3.10 Microwave Extraction

Microwave assisted extraction (MAE) is a procedure to use microwave energy to warm solvents in contact with a sample so as to take out the analytes from the sample lattice into the solvent. The capacity to quickly warm the sample solvent blend is innate to MAE and the main preferred feature of this system. By utilizing closed vessels, the extraction can be performed at elevated temperatures, quickening the mass exchange of target compounds from the sample lattice. An average extraction takes 15–30 min and uses only a small amount of solvent in the range of 10–30 mL. These volumes are around 10 times less than the volumes used by regular extraction systems. By and large, recovery of analytes and reproducibility are enhanced, in contrast to regular methods, as has been seen in a few applications (Fig. 6.10).

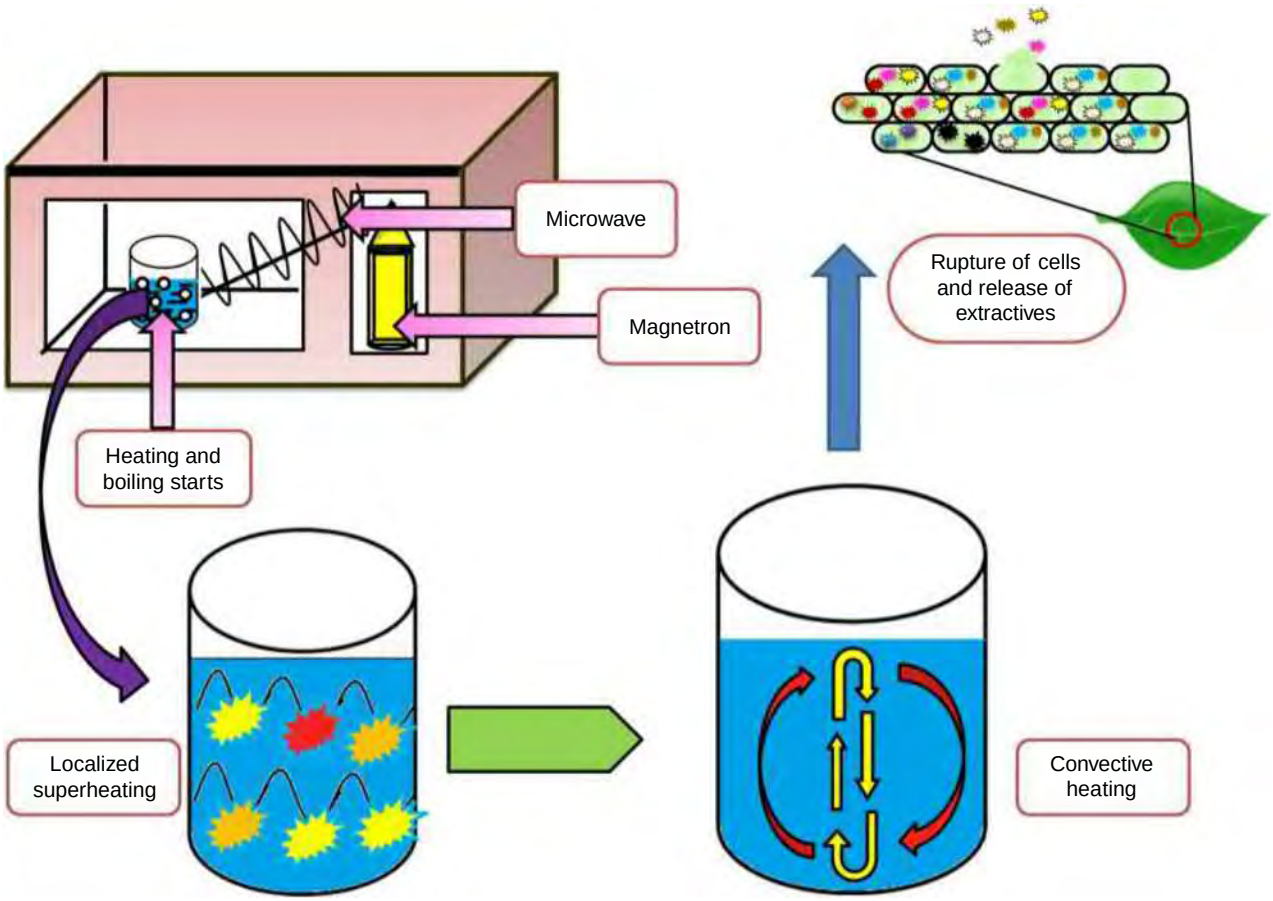

FIG. 6.10 Mechanism of microwave extraction.

6.3.10.1 *Principles*

The standard of heating using microwave energy depends on the immediate impact of microwaves on particles by ionic conduction and dipole revolution. In numerous applications, these two effects happen all the while. Ionic conduction is the electrophoretic relocation of particles when an electromagnetic field is applied. The resistance of the solution to this current will bring about friction and, in this way, heat the material. Dipole revolution implies realignment of dipoles with the applied field. At 2450 MHz, which is the frequency utilized as a part of commercial systems, the dipoles adjust and randomize 4.9310 times each second and this brings about heating. The capacity of a solvent to assimilate microwave energy and pass it on as heat to different atoms somewhat relies upon the dielectric loss tangent (tan ∂). The dissipation factor is given in the following equation.

Meanwhile, the transformation of electrical energy into thermal energy is expressed as:

$$P = K \cdot f \cdot \in' \cdot E^2 \cdot \tan \partial$$

where tan ∂ is called the dielectric loss tangent, E is the electric field strength, $\in'$ is the dielectric constant, f denotes the frequency, K is a known constant, and P represents microwave power dissipation per unit volume (Ekezie et al., 2017). Polar particles and ionic arrangements (typically acids) will retain microwave energy unequivocally on the grounds that they have a perpetual dipole moment that will be influenced by the microwaves. However nonpolar solvents, for example, hexane, will not heat up when exposed to microwaves. A straightforward examination among methanol and water demonstrates that methanol has a lower dielectric constant, yet a higher dielectric loss factor than water. This shows methanol, in contrast with water, has a lower capacity to absorb the microwaves as they go through, yet a higher capacity to disperse the microwave energy into heat. In closed vessels, soluble materials can be heated to well above their typical breaking point, in this manner improving extraction productivity and speed. The way that distinctive compounds retain microwave energy to various degrees suggests that the heating imparted to the surrounding media will change with the

chemical substances utilized. Consequently, for tests with nonhomogeneous auxiliary attributes, or that contain different compound species with various dielectric properties scattered into a homogeneous situation, it is possible to deliver a particular heating to a few regions, or segments, of the sample. This marvel is sometimes called superheating (Eskilsson and Bjorklund, 2000).

At the point at which microwaves interact with modest hints of dampness inside the cell grid, vanishing happens and constructs exceptional pressure on the cell divider, which bursts and causes the arrival of the dynamic constituents. Higher throughputs can be accomplished via a cautious choice of working conditions, for example, temperature, because an increment in temperature promotes quicker infiltration of solvent into the cell framework. In like manner, considering the presence of a changeless dipole moment that associates with microwaves, polar atoms, and ionic blends simply assimilate microwave energy. However, for nonpolar solvents, for example, hexane, the solvents do not heat up immediately when they are in contact with microwaves. Different parameters affecting the execution of MAE frameworks incorporate solid-to-liquid proportion, extraction length, microwave control, nature of tests, and blending. The impacts of these parameters have already been assessed and the points of interest can be found elsewhere (Ekezie et al., 2017).

6.3.10.2 Microwave-Assisted Subcritical and SFE

A supercritical (SC) state occurs when a substance is subjected to temperature and pressure levels above its critical point. Certain improved properties, for example, consistency, surface tension, and solvation limit, makes SC liquids especially favorable for removing sustenance parts, particularly oils and fats. Moreover, SFE leaves no solvent pollution and works at low temperatures. The main disadvantage of SFE is the low polarity of carbon dioxide (the most diffused SC liquid) yet this can be handled utilizing different modifiers. Then again, subcritical water extraction (SWE) utilizes water as an extractant, which is superheated to temperatures in the vicinity of 100 and 374 °C at raised pressure levels that guarantee that liquids stay in their liquid form. It is additionally called pressurized or low-polarity water and throughout the years has seen a great deal of consideration as a reasonable solvent for the extraction of both polar and nonpolar compounds. A contemporary extra to MAE is subcritical or supercritical liquid extraction for expanded extraction efficiency. For instance, one group pretreated *Chlorella vulgaris* with microwaves before supercritical carbon dioxide extraction (MWSC carbon dioxide) and demonstrated that the yield of unsaturated fats from the example network was expanded. The oil throughput from MWSC carbon dioxide extraction was higher and contained critical amounts of oleic, linoleic, α-linolenic, and palmitic acids, when contrasted with supercritical liquid extraction without microwave pretreatment. So also, one group investigated the recovery of alkaloids from Gynura Segetum and found higher yields of alkaloid with MWSC carbon dioxide under the ideal conditions of a soaking time of 10 h and microwave control level of 90% than with supercritical carbon dioxide extraction alone. With respect to MAE extraction utilizing subcritical water, an attempt was made by a few specialists to produce a single-stage microwave-assisted subcritical water extraction (MWSWE) apparatus for simultaneous assurance of inorganic–metallic constituents in reference sample tests.

6.3.10.3 Microwave-Assisted Enzymatic Extraction

Enzyme-assisted extraction (EAE) is a potential panacea for conventional extraction because of its exceptionally favorable properties, including ecological friendliness, reduced solvent use, increased extraction efficiency, and simplicity. The natural properties of compounds, that is, high selectivity, sharp specificity, and capacity to catalyze certain responses, are the mechanical pathways for efficacious extraction. Some phytochemicals in the plant grids are scattered in the cell cytoplasm and a few compounds are held in the polysaccharide-lignin network by hydrogen or hydrophobic bonding, which are not available with a solvent in a standard extraction process. Enzymatic pretreatment has been considered as a novel and a successful method to discharge limited compounds and increase general yield. The addition of particular chemicals during extraction, such as cellulase, an amylase, and pectinase, improves recovery by breaking the cell wall and hydrolyzing the auxiliary polysaccharides and lipid bodies. There are two methods of EAE: enzyme-assisted aqueous extraction (EAAE) and enzyme-assisted cold pressing (EACP). For the most part, EAAE strategies have been used essentially for the extraction of oils from different seeds. In the EACP strategy, chemicals are utilized to hydrolyze the seed cell wall, on the grounds that, in this framework, polysaccharide–protein colloid is not accessible, which is evident in EAAE. Different variables, including enzyme structure and concentration, PS of plant materials, solid to water proportion, and hydrolysis time, are perceived as the key elements of the extraction. The oil separated by enzyme-assisted strategies was found to contain a higher level of free unsaturated fats and phosphorus substances than conventional hexane-removed oil. The EAE is perceived as an ecofriendly innovation for extraction of bioactive compounds and oil because it utilizes water as a solvent rather than natural chemicals.

6.3.11 Supercritical Fluid Extraction

The utilization of Supercritical fluid extraction (SFE) as a general extraction procedure for an extensive variety of dynamic ingredients or analytes from plants and microbial samples is of specific significance in the extraction of obscure items or for preparing entire living being separate for chemical study, bioassay, or screening programs.

SFE offers varied focal points, as well as lower solvent use, manageable properties, additional slender concentrates, and less heat dissipation when contrasted with normal solvent extraction and steam distillation techniques. There has been a spurt of action within the utilization of critical liquid for extraction. Supercritical fluids have prevalent mass exchange properties by ideals of their low thickness and high solute diffusivity alongside the capacity to enter microporous materials. Supercritical carbon dioxide has especially appealing properties, for example, it is nontoxic, nonflammable, not acidic, chemically inert, ecofriendly, low cost, easily accessible, and it has a low critical temperature (304 K) and a reasonably low critical pressure (73 atm). It is the favored solvent for some supercritical extractions (Mukherjee, 2002).

The critical point of a pure substance is defined as the highest temperature and pressure at which the substance can exist in vapor–liquid equilibrium. At temperatures and pressures higher than this time one homogeneous fluid is made and is claimed to be supercritical (Silva, 1998).

A variety of organic compounds can be dissolved in supercritical fluid by virtue of the characteristics of the solvents and changes in the pressure and temperature (Mukherjee, 2002). As the substance approaches its critical temperature, the properties of its gas and fluid phases join, bringing about just a single phase at the critical point: a homogeneous supercritical liquid. The heat of vaporization is zero at and past this critical point; thus, no distinction exists between the two stages. In the pressure–temperature diagram, the time when critical temperature and critical pressure meet is known as the critical point of the substance. Above the critical temperature, a fluid cannot be acquired by expanding the pressure, despite the fact that a solid might be formed under adequate pressure. The critical pressure is the vapor pressure at the critical temperature. In the region of the critical point, a small increment in pressure causes huge increments in the thickness of the supercritical phase.

Regarding this solubilizing impact, supercritical carbon dioxide looks like the nonpolar solvents hexane and benzene, and it has been especially well known for the extraction of unstable oils from plant material concerning numerous different substances, its dielectric steady may change with thickness, yet even at high densities, carbon dioxide has a constrained capacity to dissolve high-polarity compounds. However, it is possible to enhance its proclivity for more noteworthy compounds by expanding the liquid thickness (by changes in accordance with temperature and pressure) or addition of natural solvents (e.g., methanol, ethanol, and dichloromethane) or even water to the supercritical carbon dioxide. The addition of modifiers to carbon dioxide can enhance the extraction productivity by raising the solvency of the solutes. Two components have been proposed by Pereira and Meireles to clarify the impacts, as: solute–cosolvent collaborations, caused by an increase in solvent polarity and system swelling that encourages the contact of the solute by the solvent. These modifiers modify the extraction properties, but it should be recollected that the closeness of those compounds can change the fundamental temperature and pressure and can likewise need modifications to the systems for evacuating extraction liquid toward the end of the procedure (Mukherjee, 2002; Rostagno and Prado, 2013).

One disadvantage of SFE is that the mechanical assembly needed is more complex than for solvent extraction or steam distillation. The fundamental instrumentation contains a carbon dioxide supply (gas barrel), and a pump to attain the desired pressure and convey the controlled liquid to the extraction chamber, that is, in a very stove to change it to be held at the specified temperature (Houghton, 1998).

On–off qualities are required at the channel and outlets of the extraction chamber. The liquid leaving the sample chamber can be opened into an online investigative instrument (e.g., a gas chromatograph) or be gathered, depressurized, and the liquid permitted to vanish, leaving the separated material as a buildup. Solid samples for extraction should be finely divided, and fluid or wet examples might be blended with inactive adsorbents (e.g., celite) or drying specialists (e.g., magnesium sulfate) before placing in the chamber. SFE has been used for an extensive variety of chemical compounds. Mechanical cases of the utilization of SFE incorporate the decaffeination of espresso and separation of unpleasant standards from hops. Supercritical fluids may likewise be utilized as versatile stages in chromatography (supercritical liquid chromatography, SFC) (Mukherjee, 2002). Albeit supercritical solvents have higher diffusivity in the crude material grid than fluids, a decrease in the sample molecule measure by and large delivers an increase in the extraction yield acquired because of the increase in the contact surface among test and solvent, chiefly when diffusion is constrained by inside mass exchange protection.

6.3.12 Pressurized Liquid Extraction

In 1996, Richter et al. first described PLE. This method is now known by several names, namely, pressurized fluid extraction (PFE), accelerated fluid extraction, enhanced solvent extraction, and high-pressure solvent extraction. The concept of PLE is the application of high pressure to keep solvents liquid beyond their normal boiling points. High pressure facilitates the extraction process. Automation techniques are the main reason for the greater development of PLE-based techniques along with decreased extraction time and a reduced solvent requirement. The PLE technique requires a small amount of solvents because of the combination of high pressure and temperature, which provides faster extraction. The higher extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate and also decrease the viscosity and surface tension of the solvents, thus improving the extraction rate. Compared with traditional Soxhlet extraction, PLE was found to dramatically decrease the time required and the solvent use (Rostagno and Prado, 2013).

PLE can be performed in either static or dynamic mode. Static extraction mode is a batch process consisting of one or more extraction cycles with the addition of fresh solvent between each cycle. The extractor is pressurized through the solvent inlet while the outlet valve is kept closed. After the extraction, the valve is opened, releasing a mixture of solvent and extract to the collection. In the dynamic mode, the solvent is continuously pumped through the extractor containing the matrix, whereas the outlet valve is kept open during the extraction (Azmir et al., 2013) (Figs. 6.11 and 6.12).

6.3.12.1 *Illustrative Example*

Example 6.4

A technique has been created for the extraction of capsaicinoids from peppers by PLE; these compounds are dictated by reverse phase HPLC, with identification by fluorescence spectrophotometry and MS. The stability of capsaicin and dihydrocapsaicin was studied at different temperatures (50–200 °C), and a few extraction factors were tested: solvent (methanol, ethanol, and water), different percentages of water in the methanol (0%–20%) and in the ethanol (0%–20%), and the number of extraction cycles. The study assessed the repeatability (RSD < 7%) and the reproducibility (RSD < 7%) of the technique. Finally, the PLE technique was used to measure the capsaicinoids exhibited in three assortments of hot peppers grown in Spain, evaluating five capsaicinoids: nordihydrocapsaicin, capsaicin, dihydrocapsaicin, an isomer of dihydrocapsaicin, and homodihydrocapsaicin. Extractions were performed on a Dionex ASE 200 extractor (Dionex Corp., Sunnyvale, CA). The sample was blended with ocean sand (Panreac) and set in an 11-mL stainless-steel extraction cell.

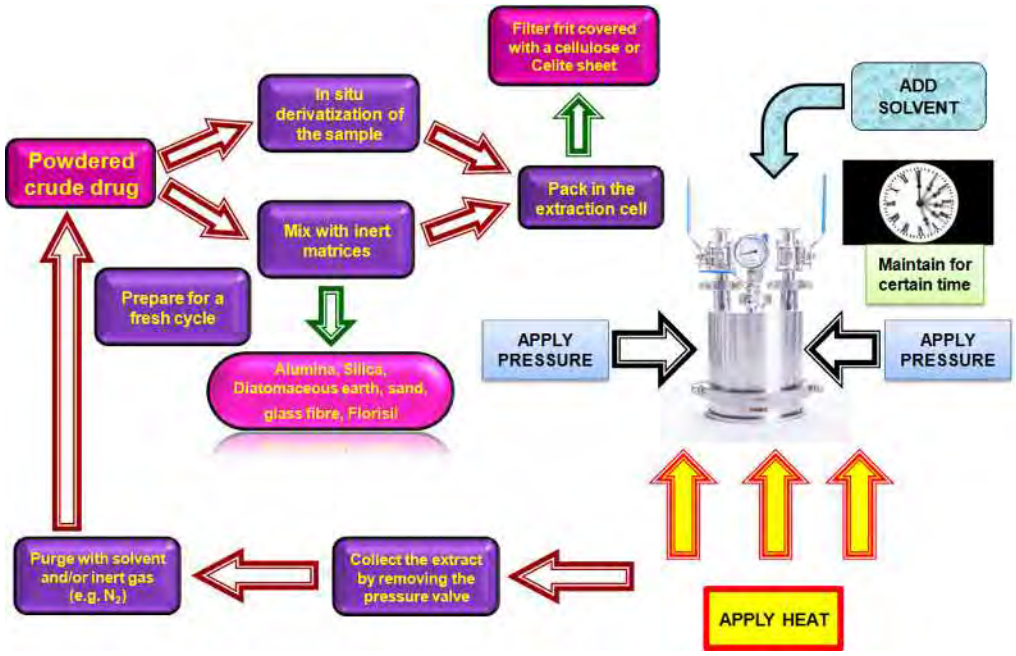

FIG. 6.11 Schematic diagram of static accelerated liquid extraction (pressurized liquid extraction).

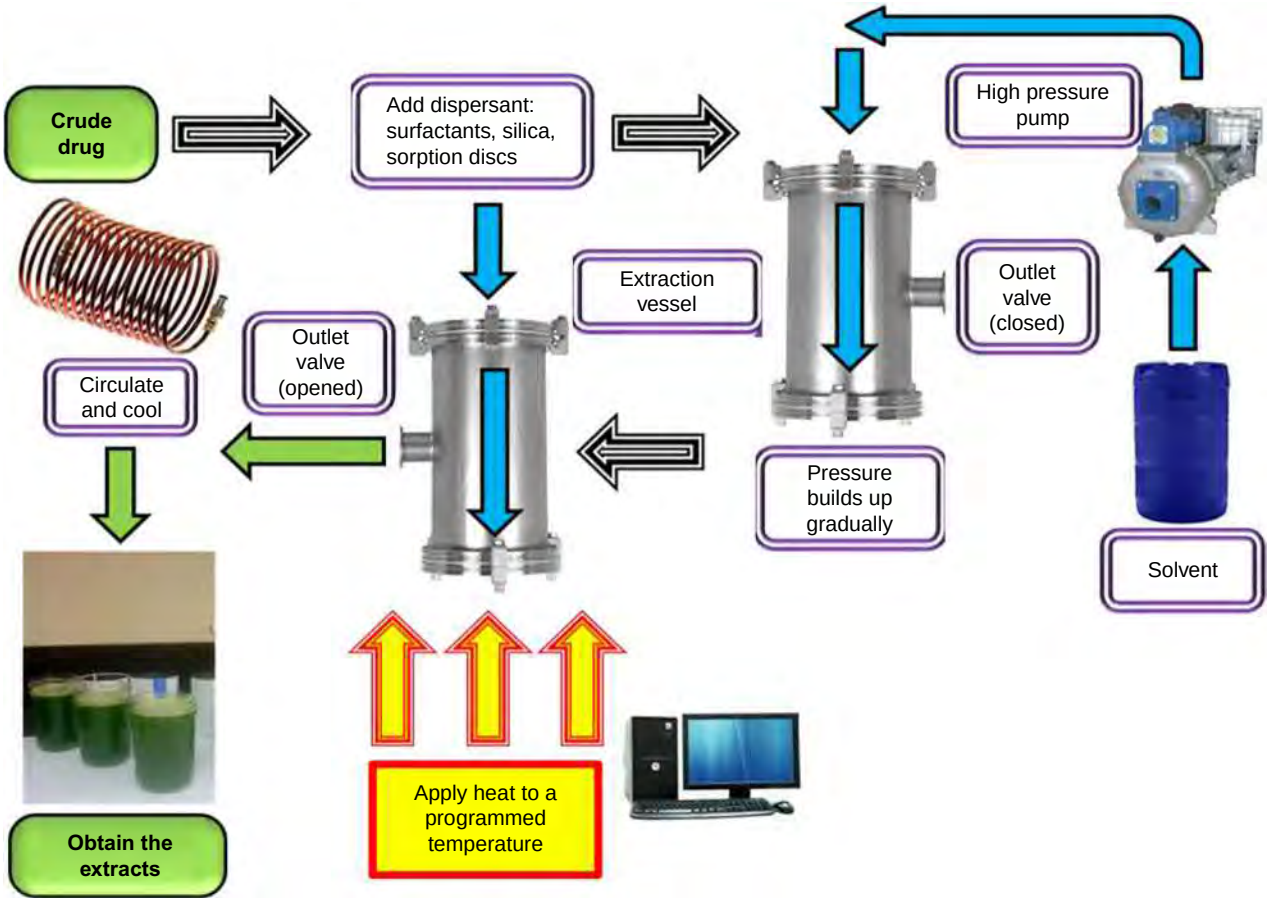

FIG. 6.12 Schematic diagram of a dynamic accelerated liquid extraction (pressurized liquid extraction).

A cellulose channel (Dionex Corp.) was set at the base of the extraction cell. The extraction chamber was loaded with the extraction solvent [water, ethanol, or methanol (0%–20% in water)], pressurized to 100 atm of pressure, and warmed to temperatures ranging from 50 to 200 °C. The extractions were finished with pepper test quantities of 0.7 g. The concentrates of peppers (volumes of <50 mL) were bested up to 50 mL, and these were the examples that were broken down by HPLC with fluorescence identification and MS (Barbero et al., 2006).

Example 6.5

PFE is a flexible system that permits the extraction of common bioactive compounds, for example, curcuminoids, aggravates that have medicinal properties and that give the yellow shade of turmeric. For the most part, utilized as a systematic apparatus, PFE utilizes elevated temperatures (313–473 K) and direct to high pressures (3.5–20 MPa) to encourage and improve the extraction procedure. Various properties, for example, the utilization of less solvent, lower extraction times, and no exposure of the mixtures to oxygen and light, are advantages over traditional procedures of solvent extraction. This study depicts the essentials and parameters affecting the procedure of PFE, investigating the most recent advancements and patterns in the extraction of bioactive compounds, for example, curcuminoids. It additionally discusses the likelihood of utilizing close-to-room temperature (313 K) and pressures in the range of 10–30 MPa as restricting to the utilization of high temperatures, in the extraction of colors from plant material rich in starch.

The PLE process performance is governed mainly by the choice of solvent, temperature, extraction time, and, to a lesser extent, by the pressure. However, the performance of the process depends on the matrix nature, the specific features of the target compounds and their localization inside the matrix. Therefore, it is necessary to know and establish the influence of these factors on the extraction process in order to obtain high yields and high-purity extracts.

6.3.12.2 Pressurized Hot Water Extraction (PHWE)

PHWE is an extraction technique that uses liquid water as an extractant (extraction solvent) at temperatures above the atmospheric boiling point of water (100 °C/273 K, 0.1 MPa), but below the critical point of water (374 °C/647 K, 22.1 MPa).

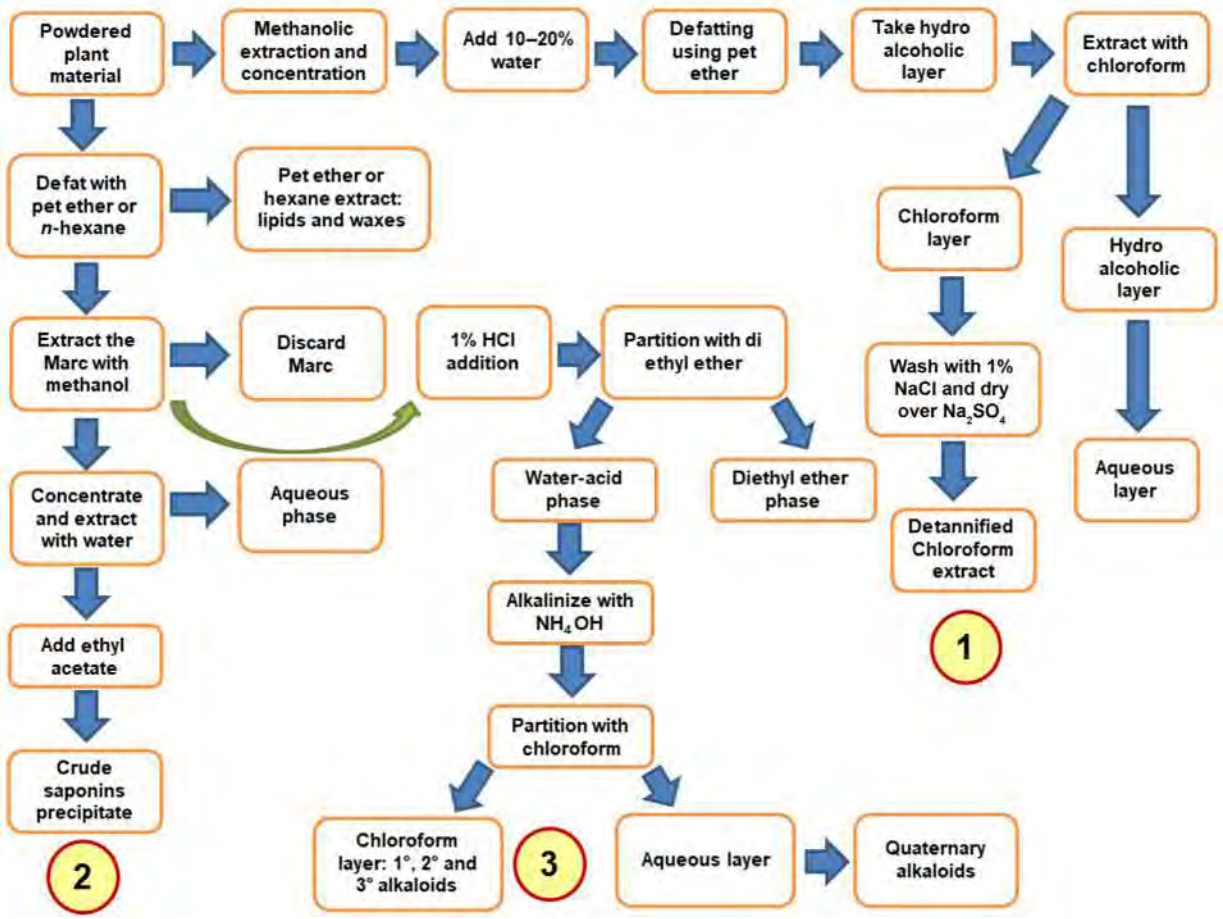

FIG. 6.13 General protocols for extraction of phytoconstituents (1—Preparation of a detannified extract; 2—fractionation method for obtaining saponin from crude drugs; and 3—general procedure to obtain an alkaloid extract from the alcoholic crude extracts of a plant).

The use of PHWE in analytical chemistry started with the work in environmental analysis by Hawthorne and colleagues in the mid-1990s and can also be referred to as SWE, superheated water extraction, and PLE or accelerated solvent extraction with water as a solvent. Detailed discussions are out of the scope of this book.

6.3.13 General Protocols for Extraction of Phytoconstituents

Some general protocols that may be useful for extracting phytoconstituents, such as detannified chloroform extract from plants, crude saponin from plants, and alkaloids from plants, are described in Fig. 6.13.

6.4 EFFECT OF SOLVENT, SOLVENT MIXTURES, AND SOLUTION ON EXTRACTION

The extraction agents or solvents utilized as a part of producing phytopharmaceuticals should have reasonable capability to dissolve the essential medicinal constituents and in this manner keep them uninflected from the raw substances containing the medications that are to be extracted. In pharmaceutical technology, the extraction agents or solvents are thought of as menstruum and the extract resolution isolated from the residual insoluble healthful formulation material is called miscella. Solvents that have a particular property for the extracted substances, for example, bitter principles, mucins, shades, tars, and so forth ought to (if in the slightest degree possible) not be diminished within the extraction of vegetable oils. As indicated by the quality definition in this technology, solvents are, under normal conditions, volatile, usually organic solvents acceptable at dissolving totally different volatile, liquid, or solid substances, while not themselves or the dissolved substance having chemicals modified. In pharmaceutical technology, this is not usually the case, as solvents, for example, oils, that are not volatile under typical conditions are likewise utilized. It is evidently expedient to rearrange varied solvents within the order of accelerating eluting strength.

The main inorganic solvents utilized as a part of a drug store are water and carbon dioxide, in spite of the actual fact that liquids, for example, alkali, SO_2, and HCN solutions are utilized for exceptional functions in pharmaceutical production. Water, pure organic liquids, and blends of organic liquids with water or with different organic liquids are utilized as extraction solvents. These organic liquids mainly concern hydrocarbons and their subsidiaries, for example, halogenated hydrocarbons, alcohol, esters, ketones, ethers, oils, and so on. Solvent selectivity, ease of use, cost, and environmental factors are the main considerations in the decision of an appropriate solvent or of a blend of a few solvents. The last two variables, specifically, are progressively increasing in importance because of the attention paid to them by administrators. This has turned out to be critical to the point that numerous producers are making genuine endeavors to formulate extraction forms so the least possible number of "safe" liquids, for example, water, bring down liquor and in uncommon territories, supercritical gases are utilized.

It should not go unmentioned here that for preparation of traditional formulations, extracts should fulfill the needs of the pharmacopeias. This is often possible simply once the predefined solvent is used. A level of call is obtainable by utilizing more cost-effective methanol instead of costly ethanol (on which obligations should be paid) if its polarity corresponds to that of the desired ethanol–water mix. As a dependable guideline, a 10th higher wood alcohol concentration typically relates to the desired alcohol fixation.

Generally, oleophilic formulation optional metabolites of assorted degrees of polarity are examined; still, a lot of water-solvent formulation constituents may well be of interest. The solvent(s) set for the extraction must cut the auxiliary metabolites under investigation, tough to evacuate, dormant, nontoxic, and not effectively combustible. Solvents should be refined or maybe twofold refined before use on the off chance that they are of low or unknown quality. A couple of plasticizers are typically found as contaminants in solvents, for example, dialkyl pthalate, tri-*n*-butyl acetyl citrate, and tri-butyl phosphate; these may well be consolidated amid solvent fabrication or on capability in plastic lined or plastic stoppered compartments. Fuel and chloroform typically contain dioctylphthalate [di-(2-ethylhexyl)phthalate or bis-(2-ethylhexyl)phthalate]. This substance moves into bioassays and taints formulation extracts and also changes are detected from them once the solvent is used as a region of mass and also the extract decreases in volume. Chloroform, dichloromethane, and fuel are usually the solvents of choice during a preparatory extraction of a formulation part. It has been incontestable that chloroform and its contaminants, CH_2Cl_2 and CH_2ClBr, might react with a couple of mixtures because of specific alkaloids (e.g., brucine, strychnine, and ephedrine), producing quaternary salts and other things. Basically, the closeness of hints of HCl might deliver deterioration, lack of association, or transition in numerous compounds (Britton, 1991). As a result of its nephro and toxic impacts, chloroform ought to be controlled in oxygenated regions using a metabolic process veil, or used below a smoke hood. Chloride is not the most toxic; however, it is rather a lot more volatile than chloroform. Methyl alcohol and 80% ethanol are solvents that are a lot more polar than the chlorinated hydrocarbons. It is believed that alcoholic solvents effectively enter cell layers, permitting the extraction of large quantities of endocellular segments. Conversely, chloroform, being of lower polarity, might wash out typically extracellular material. In this approach, alcohols break down basically polar metabolites along with medium- and low-polarity mixtures extracted by cosolubilization. It has been shown that a couple of artifacts could be created once utilizing methyl alcohol in the extraction procedure, example: trechonolide. An acquired from *Trechonaetes laciniata* was modified over into trechonolide B by methylation once warmed with methyl alcohol containing hints of acid. The alkyl group 12-cystisine acetic acid removed from *Euchresta japonica* was planned as rising from an esterification of relating acid during extraction. Water is from time to time utilized alone to induce a rough formulation extract; rather, a watery methanolic extract is parceled out between numerous solvents. Utilizing this technique, ethyl ethanoic acid derivation and diethyl ether are inclined to border emulsions with water, therefore, the growth of common salt to immersion might break the emulsion, in spite of the fact that on specific events it is vital to "break" the emulsion by action.

Extraction by steam refining is broadly used to acquire volatile terpenes, in spite of the fact that it has been seen that a reduction of up to a pair of pH units could happen on account of the break of vacuoles, which can produce unwanted responses in delicate mixtures (Charlwood and Charlwood, 1991). Diethyl ether was once used quite a bit for formulation extractions in spite of its unpredictability, combustibility, harmfulness, and its propensity to border touchy peroxides. Peroxides of divinyl ether are responsive and will deliver oxidization of unsaturated mixtures, for instance, carotenoids. Peroxides will be expelled from divinyl ether by shaking with $FeSO_4$. Thus, $(CH_3)_2CO$ could produce acetonides if 1,2-cis-diols are obtainable in an acidic medium. Extraction beneath acid or essential conditions often lead to explicit partitions; for example, anthocyanins are extracted by squashing new formulation material with wood spirit containing volume of w/v hydrochloric acid and alkaloids may be extracted in either acid or elementary media. It has been accounted for that the acid base treatment of extracts may produce artifacts as a result of revisions, for example, dihydroquercetin from *Pseudotsuga menziesii*, under basic/acid chemical action and heat (>100 °C) experiences epimerization at C2 and C3 ring constriction, producing alphitonin; scopine from *Hyoscyamus albus* produces oscine by adjustment of the epoxide (Matoasca and Walker, 1970) and also the decay and reaction of glycosides. It is important to understand the attributes of the formulation

secondary metabolites to be extracted (dissolvability, reactivity, and stability) keeping in mind the top goal to settle on the correct solvent for the extraction to avoid substance disintegration and also the development of artifacts. When the extraction is over, the solvent is removed by revolving evaporation at near to 30–40 °C because some unstable compounds may be degraded by higher temperatures. The remaining sticky or tarry buildup from the formulation extract (dry extract) is then weighed and ready by numerous techniques, as indicated by the target metabolites.

6.4.1 Solvents Used for Extractions

The number of solvents available for the extraction of prepared restorative formulation material and crisp therapeutic formulations is not large. Blends of organic solvents, for example, ether and ethanol, or blends of organic solvents, for example, liquor with water, are utilized to create certain impacts. Azeotropic blends are an exceptional sort of blend. These are blends of at least two liquids that boil at a constant temperature, that is, the vapor has the same composition as the liquid mixture (thus, cannot be separated by refining or amendment). In slow extraction by the Soxhlet procedure, in which the solvent is reused on to the extraction material by steady refining, solvent blends must be utilized on the off chance that they are azeotropic. Here it must be guaranteed that the structure of an azeotropically bubbling blend is pressure subordinate. As medication extractions are every now and again performed at subatmospheric pressures, the liquid blends utilized must be appropriate for this. Moreover, the organization of a blend viewed as azeotropic can be adjusted, for instance, by water being held amid soaking and resulting swelling of the medication material. The blend staying available for use never again boils azeotropically.

Out of the considerable number of solvents, water is the most vital of all extraction solvents. It is utilized either alone or blended with organic solvents, chiefly bring down liquor. The topic of the purity of water for the readiness of formulation extracts and what sort of sanitation ought to be utilized has regularly been discussed. Because medication formulations contain numerous mineral substances of shifting dissolvability and in significantly differing amounts, it is inappropriate to request the utilization of refined or demineralized water. It has been demonstrated that drinking water, which meets all the clean prerequisites, matches water of the quality required. Drinking water will be water that is reasonable for human use when it satisfies at any rate the following requirements:

- Drinking water and water utilized as a part of the foodstuffs business should dependably be free of sickness-causing pathogens and substances harmful to wellbeing.
- Water proposed for human use must be normally as free of microscopic organisms as may be expected under the circumstances and of acceptable taste.
- Drinking water must be free from contaminants, not colored, clear, cool, and free of any remote odor or taste.
- Water for local and mechanical use ought not to contain excessive salts, especially hardeners, iron, manganese, and organic substances (peat or humus).
- The following is likewise expressed in the World Health Organization Standards concerning the radiological consistency: "It is critical to keep the radioactivity in drinking water as low as would be prudent. Water with a higher substance of aggregate radioactivity can however still be flushed on the off chance that it contains less risky isotopes."
- Drinking water is for the most part the reason for alternate characteristics of water given in the pharmacopeias.

Water dissolvability of solvents is a noteworthy paradigm to consider for the extraction capability of a blend of solvents. Solvent-water blends rely upon the level of miscibility of the two parts. There exist genuinely extensive blend holes between the dissolvability of water in solvents and that of solvents in water. Contingent upon the miscibility with water the extracting idea of individual segments fluctuates a great deal in various ways. The miscibility criteria of various solvents with water and the other way around (at 20 °C) are shown in Table 6.2.

6.4.2 Extraction Enhancement by Emerging Extraction Solvents

6.4.2.1 Ionic Liquids

An ionic liquid is salt in liquid state in which the ion are poorly coordinated. They are highly solvating noncoordinating medium in which a variety of organic and inorganic solute are able to dissolve. Essentially, they are considered as milieu-accommodating substitutes for customary organic solvents due to their attractive physiochemical properties for extraction, which is chiefly affected by the size and trademark highlights of their cation and anion segments (Liu et al., 2005). Such remarkable properties incorporate simplicity of miscibility with different solvents, nonflammability, low substance reactivity, thermal stability, and insignificant vapor pressure. It is utilized widely in division science and pertinent in different extraction procedures, for example, liquid-stage miniaturized scale extraction, layer partition, and solid-state extraction, and for test

TABLE 6.2 Miscibility of Different Solvents With Water and Vice Versa (wt%)

Name of Solvent	Water Miscibility With Solvent	Solvent Miscibility With Water
Cyclohexane	0.01	0.01
n-Hexane	Nil	<0.01
Benzene	0.06	0.07
Toluene	0.05	0.06
Chloroform	0.2	0.8
Carbon tetrachloride	0.03	0.03
Methanol	∞	∞
Ethanol	∞	∞
n-Propanol	∞	∞
Isopropanol	∞	∞
n-Butanol	20.1	7.7
Isobutanol	15	10
Acetone	∞	∞
Methyl ethyl ketone	11.8	26.8
Acetic acid	∞	∞
Ethyl acetate	3.3	8.7
Diethyl ether	1.3	6.9

pretreatments (Zhang et al., 2014). As another approach, ILs can be consolidated into MAE systems as an extraction medium. This assertion depends on a few trials that affirm the prominent capacity of ILs to successfully absorb microwave light and scatter energy quickly through ionic conduction (Liu et al., 2012). Then, the expansion of microwave illumination gives quick heat exchange, expands yield, and reduces side residuals. Additionally, the rapid development of heat and pressure inside the material empowers target compounds to be liberated effectively from the natural network. Pan et al., (2016) described the microwave-assisted extraction (MAE) of lipid from three algal species (*C. sorokiniana, N. salina,* and *G. sulfuraria*) by utililizing 1-butyl-3-methylimidazolium hydrogen sulfate ([BMIM][HSO_4]), an imidazolium-based ionic liquid. This result demonstrated that, by interacting ionic liquid with MAE system, the extraction rate was increased by 10 times, comparing with solvent extraction system; the lipids were effectively extractable, because [BMIM][HSO_4] dexterously breaks down the algal biomass. In another exploratory examination, Liu et al. (2017) used bronsted-acidic ionic liquid amid MAE of pectin and naringin from pomelo peels, and their results showed that $[HO_3S(CH_2)_4mim]HSO_4$ was better than reference solvents, including water, HCl solution, and Na_2SO_4, with an unrivaled yield for pectin and aringin of 291.60 ± 7.25 mg/g and 8.38 ± 0.20 mg/g, respectively. In understanding, Liu et al. (2016) boosted the yield of fundamental oil, gallic acid, and ellagic acid from the lattice of eucalyptus leaves utilizing $[HO_3S(CH_2)_{4mim}]HSO_4$ as both an extraction solvent and hydrolysis catalyst in an MAE array. Turmeric, which is a perpetual formulation developed prevalently in tropical territories, is broadly recognized to have certain wellbeing regulating advantages, for example, mitigating diabetic, antitumor, hostile to microbial and cell reinforcement exercises. Because of its inborn bioactive constituents, that is, demethoxycurcumin, curcumin, and bisdemethoxycurcumin, all have a place with the class of polyphenols. To recoup these three parts from the formulation material, an MAE setup was effectively utilized with 0.3 mol/L 1-octyl-3-methylimidazolium bromide using an extraction time of just 8 min at a temperature of 55 °C, in contrast with the 90 min required for UAE (Liang et al., 2017). Not at all like other regular organic solvents, ILs are recyclable and cannot vanish into the environment or actuate the creation of contaminants. These investigations strengthen that the synergistic use of ionic liquid and microwaves gives energy savings and brings down time utilization; in this way the use of ILs in MAE methods is an effective and greener approach with broad extraction use.

6.4.2.2 Other Enhancements

Profound eutectic solvents, multiphase solvents, nonionic surfactants at vapor point temperature and so on are different enhancements in this field of extraction technology. Since their inception, MAE procedures have proven their practicality

for quick, powerful, and excellent recovery of nourishment segments. Its prevalence over regular strategies for extraction is primarily in light of its volumetric warming and high infiltration control, which fundamentally reduces extraction time and solvent use, in this way reducing the emission of carbon dioxide into the climate and presenting an ecofriendly approach (Perino et al., 2011). Moreover, MAE presents extra favorable circumstances, including lower preparation cost, high quality, and noteworthy energy savings contrasted with some conventional procedures that depend on heating sources by means of radiation, conduction, or convection (Chemat et al., 2017). Moreover, the introduction of crossover modes (MHG, UMAE, SFE, and so forth) as well as the joining of elective solvents can additionally expand general extraction effectiveness and guarantee greener use. For example, with reference to ecological effects, MHG can lessen the amount of carbon dioxide discharged into the environment (160 g carbon dioxide), when contrasted with customary hydrodistillation (2400 g carbon dioxide) (Vian et al., 2008). The solvent-free system can recover fundamental oils in focus states, without deposits, contaminants or indeed, even artifacts and decreases biomass waste by >20 times. Comparably, in UMAE, the synergistic utilization of microwaves and acoustic cavitation from ultrasonication, can rapidly deteriorate cell integrity and discharge regular cancer prevention agents, that is, phenolics, flavonoids, vitamin C from *Clinacanthus nutans* at an ideal time interim of 75 s, joined by bringing down solvent use, consequently limiting waste creation rather than run of the mill solvent extraction system. Then, extracting nourishment segments utilizing microwaves close by water as the extractant at subcritical or supercritical conditions displays a nontoxic and less destructive approach as opposed to acid-based strategies, particularly while recouping metals (Matusiewicz and Ślachciński, 2014). Over time, specific impediments might be brought about when these enhanced MAE procedures are used. For instance, high solvent volumes reachable at times, may diminish recovery and microwave adsorption by the material, in light of the fact that over the top vitality is consumed by the solvent, prompting diminish in mass exchange (Li et al., 2010). In addition, defeating proposals weaknesses could suggest utilizing rising solvents, for example, ionic liquids whose harmfulness levels are not completely settled. Likewise, uncommon thought to extra cost hardware and establishment should be inspected before appropriation. Other than that, the particular advantages proffered by consolidating MAE and other extraction techniques opens ways to new potential outcomes in expository science and sustenance fixings recuperation (Ekezie et al., 2017).

6.4.3 Properties of Solutions

The physical properties of substances might be delegated colligative, added substance, and constitutive. In the field of thermoactives, physical properties of frameworks are named broad properties, contingent upon the amount of the issue in the framework (e.g., mass and volume) and concentrated properties, which are free of the measure of the substances in the framework (e.g., temperature, pressure, thickness, surface strain, and consistency of an unadulterated liquid).

Colligative properties depend predominantly on the quantity of particles in a solution. The colligative properties of solutions are osmotic pressure, vapor pressure lowering, depression of freezing point, and elevation of the boiling point. The estimations of the colligative properties are roughly the same for amounts up to convergences of various nonelectrolytes in solution paying little heed to the species or substance nature of the constituents. In thinking about the colligative properties of strong in liquid solutions, it is expected that the solute is nonvolatile and that the pressure of the vapor over the solution is given completely by the solvent. Added substance properties rely upon the aggregate commitment of the particles in the atoms or on the total of the properties of the constituents in a solution. A case of an added substance property of a compound is the molecular weight, that is, the aggregate of the majority of the constituent particles. The majority of the segments of a solution are likewise added substance, the aggregate mass of the solution being the total of the majority of the individual segments. Constitutive properties rely upon the position and to a lesser degree on the number and sort of atoms inside a particle. These properties offer pieces of information to the constitution of individual compounds and gatherings of atoms in a system. Numerous physical properties might be halfway added substance and somewhat constitutive.

6.4.4 Types of Solutions

A solution might be characterized by the states in which the solute and solvent occur, and because three states (gas, liquid, and solid) exist; nine sorts of homogenous blends of solute and solvent are possible. In the development of solid solutions, the particles of the gas or liquid take up positions in the crystal lattice and carry on like iotas or atoms of solids. The solutes (regardless of whether gases, liquids, or solids) are separated into two primary classes: nonelectrolytes and electrolytes. Nonelectrolytes are substances that do not yield ions when dissolved in water and accordingly do not conduct electricity through the solution. Examples of nonelectrolytes are sucrose, glycerin, naphthalene, and urea. The colligative properties of solutions of nonelectrolytes are genuinely standard. A 0.1 M solution of a nonelectrolyte creates around an indistinguishable colligative impact from some other nonelectrolytic solution of equivalent focus. Electrolytes are substances that frame particles in solution, direct the electric current, and show obvious “peculiar”

colligative properties, that is, they deliver an impressively more prominent depression of the freezing point and boiling point rise than do nonelectrolytes of a similar concentration. Examples of electrolytes are hydrochloric acid, sodium sulfate, ephedrine, and phenobarbital. Electrolytes may be subdivided into strong electrolytes and weak electrolytes depending upon whether the substance is totally or just incompletely ionized in water. Hydrochloric acid and sodium sulfate are strong electrolytes, while ephedrine and phenobarbital are weak electrolytes. Binary and ternary azeotropic mixtures are exemplified in Table 6.3.

There are several physicochemical characteristics regarding the selection and properties of solvents, which are discussed below.

6.4.4.1 Azeotropic Mixtures

The mixture of various solvents with differing polarities regularly impacts the solubility properties of extractable materials. To use this phenomenon completely, the organization of the solvent should be picked so that a paired or ternary azeotropic blend is delivered. This has the preferred standpoint of endless supply of the extracts, the solvent boils always and the condensate, maybe after a little adjustment by substitution of parts specially held in the medication buildup, can be reused. These blends of solvents have potential use in the extraction of active phytochemical metabolites from unrefined medications, relying upon their different chemical nature as they can extract vast quantities of constituents in view of their temperament.

6.4.4.2 Solubility of Active Constituents

The dissolvability of a compound relies on the physical and chemical properties of the solute and the solvent, and in addition upon such factors as temperature, pressure, the pH of the solution, and to a lesser degree, the condition of subdivision of the solute. Of the nine possible sorts of blends, in light of the three states, only gases in liquids, liquids in liquids and solids in liquids are of specific pharmaceutical significance. A saturated solution is one in which the solute is in harmony with the strong stage (solute). Dissolvability is characterized in quantitative terms as the centralization of solute in an immersed solution at a specific temperature, and subjectively, it might be characterized as the unconstrained collaboration of at least two substances to shape a homogeneous solution.

An unsaturated or subsaturated solution is one containing the dissolved solute in a concentration below its equilibrium solubility at a distinct temperature. A supersaturated solution is one that contains a greater amount of the dissolved solute than it could normally contain at a particular temperature. A few salts, for example, sodium thiosulfate and sodium acetic acid derivation can be broken down in vast quantities at a raised temperature and after cooling, neglect to take shape from the solution. Such supersaturated solutions can be changed over to stable immersed solutions by seeding the solution with a *gem* of solute, by heated agitation, or by scratching the dividers of the holder. Supersaturation probably occurs when the little cores of the solute required for the start of crystallization are more solvent than bigger crystals, making it difficult for the cores to assemble and develop with resultant failure of crystallization.

6.4.4.3 The Phase Rule

Solubility may be described in a concise manner by the use of Gibb's phase rule.

$$F = C - P + 2$$

where F is the number of degrees of freedom, that is, the number of independent variables (usually temperature, pressure, and concentration) that must be fixed in order to completely determine the system, C is the smallest number of components that are adequate to describe the chemical composition of each phase, and P is the number of phases.

6.4.4.4 Solubility Expressions

The solubility of a drug may be expressed in a number of ways. The U.S. pharmacopeia lists the solubility of drugs as the number of milliliters of solvent in which 1 g of solute will dissolve. For example, the solubility of boric acid is given in the U.S. pharmacopeia as follows: 1 g of boric acid dissolves in 18 mL of water, in 18 mL of alcohol, and in 4 mL of glycerin. Solubility is also quantitatively expressed in terms of molality, molarity, and percentage. For substances whose solubilities are not definitely known, the values are described in pharmaceutical compendia by the use of certain general terms. Solubilities of drugs are found expressed in various units in the Merck Index. For exact solubilities of many substances, the reader is referred to the works of Seidell, Landolt-Bornstein, International Critical Tables, and Lange's Handbook of Chemistry.

TABLE 6.3 Binary and Ternary Azeotropic Mixtures

Solvent Mixture	Ratio	Boiling Point (°C)
Binary azeotropic mixtures		
n-Hexane:benzene	81:19	68.9
n-Hexane:chloroform	28:72	60.0
n-Hexane:ethanol	79:21	58.7
n-Hexane:*n*-propanol	96:4	65.7
n-Hexane:isopropanol	78:22	61.0
Chloroform:*n*-hexane	72:28 (R)	60.0
Chloroform:ethanol	93.07	58.4
Chloroform:methanol	88:12	53.5
Carbon tetrachloride:methanol	79:21	55.7
Methanol:*n*-hexane	27:73	50
Methanol:cyclohexane	37:63	54.2
Methanol:benzene	39:61	48.3
Methanol:carbon tetrachloride	21:79	55.7
Methanol:acetone	86:14	55.7
Methanol:ethyl acetate	19:81	54.0
Ethanol:water	95.57:4.43	78.15
Ethanol:ethyl acetate	30.6:69.4	71.8
Ethanol:methyl ethyl ketone	40:60	74.8
Isopropanol:ethyl acetate	23:77	74.8
Methyl ethyl ketone:methanol	86:14	55.9
Methyl ethyl ketone:water	89:11	73.6
Methyl ethyl ketone:benzene	37:63	78.3
Methyl ethyl ketone:carbon tetrachloride	29:71	73.8
Diethyl ether:ethanol	40:60	74.8
Ethyl acetate:water	95:5	34.2
Ternary azeotropic mixtures		
n-Propanol:cyclohexane:water	18:54.8:26.9	65.4
n-Propanol:carbon tetrachloride:water	8.9:62.8:29.3	68.5
n-Propanol:cyclohexane:water	10.3:60.3:29.4	66.5
Isopropanol:cyclohexane:water	19.2:54.3:23.5	62.1
Ethanol:carbon tetrachloride:water	23:57.6:19.4	61.8
Ethanol:ethyl acetate:water	12.4:60.1:27.5	70.3
Ethanol:benzene:water	22.8:53.9:23.3	64.9
Ethanol:cyclohexane:water	22.2:54.3:23.5	62.1
Isopropanol:benzene:water	19.2:54.8:26	64.2
n-Propanol:cyclohexane:water	10.3;60.3:29.4	66.5

6.4.4.5 Solvent–Solute Interactions

It is well known that water is a good solvent for salts, sugars, and similar compounds, whereas mineral oil and benzene are often solvents for substances that are normally only slightly soluble in water. These empiric findings are summarized in the statement, "like dissolves like." Such a maxim is satisfying to most of us.

6.4.5 Classification of the Solvents

6.4.5.1 Polar Solvents

The dissolvability of a medication is related in large measure to the polarity of the solvent, that is, to its dipole moment. Polar solvents disintegrate ionic solutes and other polar substances. Likewise, water blends in all extents with liquor and breaks down sugars and other polyhydroxy compounds. The capacity of the solute to build hydrogen bonds is a much more powerful factor than is the polarity, as reflected in a high dipole moment. In spite of the fact that nitrobenzene has a dipole moment of 4.2×10^{-18} esu cm and phenol an estimation of just 1.7×10^{-18} esu cm, nitrobenzene is a solvent just to the degree of 0.0155 mol/kg in water, while phenol is a solvent to the degree of 0.05 mol/kg at 20 °C.

Water breaks down phenols, alcohols, aldehydes, ketones, amines, and other oxygen- and nitrogen-containing intensifies that can frame hydrogen bonds with water. A distinction in acidic and essential character of the constituents in the Lewis electron contributor acceptor sense additionally adds to particular communications in solutions. The particles of water in ice are consolidated by hydrogen bonds to yield a tetrahedral structure. Albeit a portion of the hydrogen bonds are broken when ice dissolves, water still holds its ice-like structure in large measure at standard temperatures. This semicrystalline structure is separated when water is blended with another substance that is equipped for hydrogen bonding. At the point when ethyl liquor and water are blended, the hydrogen bonds among water and liquor particles. Notwithstanding the variables as of now listed, the dissolvability of a substance likewise relies upon basic features, for example, the proportion of the polar to nonpolar gatherings of the particle. As the length of a nonpolar chain of aliphatic liquor builds, the dissolvability of the compound in water diminishes. Straight-chain monohydroxy alcohols, aldehydes, ketones, and acids with more than four or five carbons cannot go into the hydrogen-fortified structure of water and subsequently are just marginally solvent. At the point at which extra polar gatherings are available in the particle, as found in propylene glycol, glycerin, and tartaric acid, water dissolvability increases enormously. Stretching of the carbon chain lessens the nonpolar impact and prompts increased water solvency.

Tertiary butyl ether is miscible with water, whereas n-butyl ether breaks up to the degree of around 8 g/100 cc of water at 20 °C. To sum up, polar solvents, such as water, go about as solvents as indicated by the concomitant systems:

(a) Owing to their high dielectric constant, to be specific around 80 for water, polar solvents decrease the power of attraction between oppositely charged particles in crystals, for example, sodium chloride. Chloroform has a dielectric constant of 5 and benzene one of around 2; henceforth, ionic mixtures are for all intents and purposes insoluble in these solvents.

(b) Polar solvents break valence bonds of possibly solid electrolytes by acid base responses because these solvents are amphiprotic. As an example, water realizes the ionization of HCl as takes after: Weak organic acids are not ionized significantly by water; their partial solvency is ascribed rather to the hydrogen bond development with water. Phenols and carboxylic acids, be that because it could, are promptly broken down in solutions of solid bases.

(c) Finally, polar solvents are fit for solvating particles and particles through dipole interactions, especially hydrogen bond development, which prompts the dissolvability of the compound. The solute must be polar in nature because it regularly should seek the obligations of the as of now related structure. The particle dipole connection between the sodium salt of oleic acid and water might be delineated all things considered.

6.4.5.2 Semipolar Solvents

Semipolar solvents, like ketones and alcohols may induce a definite degree of polarity in nonpolar solvent molecules. For example, benzene, that is instantly polarizable, becomes soluble in alcohol. In fact, semipolar compounds may act as intermediate solvents to impart miscibility of polar and nonpolar liquids. Accordingly, acetone increases the solubility of ether in water. Propylene glycol has been shown to increase the mutual solubility of water and peppermint oil and of water and benzyl benzoate.

6.4.5.3 Nonpolar Solvents

These solvents are at the extreme nonpolar end of the polarity chart. These include *n*-hexane, carbon tetrachloride, and petroleum ether. They are mainly used for defatting of a plant sample. This is done at the very beginning of an extraction process to remove the fats, oils, and waxes from a particular drug, generally the leaf or tender stems, sometimes the seeds.

6.4.6 Influence of Solvents

Weak electrolytes may act like strong electrolytes and like nonelectrolytes in solution. At the point at which the solution is of such a pH, to the point that the medication is altogether in the ionic frame, it carries on as an answer of a strong electrolyte and solvency does not constitute a major issue. In any case, when the pH is changed in accordance with an incentive at which unionized particles are delivered in adequate concentration to surpass the dissolvability of this shape, precipitation happens. A solute is more soluble in a blend of solvents than in one solute alone, which is known as cosolvency and the solvents in the mixture in the increasing order of dissolvability of the solute are called cosolvents. Around 1 g of phenobarbital is soluble in 1000 mL of water, in 10 mL of liquor, in 40 mL of chloroform, and in 15 mL of ether.

6.4.7 Combined Effect of pH and Solvents

- The solvent affects the solubility of a weak solution during a buffered solution in two ways.
- The addition of alcohol to a buffered solution of a weak solution will increase the solubility of the unionized species by adjusting the polarity of the solvent to an additional favorable extent.
- Being less polar than water, alcohol decreases the dissociation of a weak solution, and the solubility of the drug goes down because the dissociation constant is reduced (pK_a is increased) (Mukherjee, 2002).

6.4.8 Examples of Effects of Solvents on the Extraction

Tested solvents play an important role in the extraction of the total solid and phytochemical composition as well as the antioxidant capacity of *S. chinensis*. Acetone (50%, v/v) was found to be the optimal extraction solvent for extractable solids (12.2%), phenolic compounds (60 mg GAE/g DW), flavonoids (100 mg CE/g DW), proanthocyanidins (47.4 mg CE/g DW), and saponins (754 mg EE/g DW), as well as antioxidant capacity (ABTS 334mMTE/g DW, DPPH 470 mM TE/g DW, FRAP 347 mM TE/g DW, and CUPRAC 310 mM TE/g DW). The extract prepared from 50% acetone had high levels of bioactive compounds (TPC 555 mg GAE/g CRE, flavonoids 819 mg CE/g CRE, proanthocyanidins 392 mg CE/g CRE, and saponins 1880 mg EE/g CRE) as well as antioxidant capacity (ABTS 414 mM TE/g, DPPH 407 mM TE/g, FRAP 320 mg TE/g, and CUPRAC 623 mM TE/g), thus further confirming that 50% acetone is the solvent of choice. Therefore, 50% acetone is recommended for extraction of phenolic compounds, their secondary metabolites, saponins, and antioxidant capacity from the root of *S. chinensis* for further isolation and utilization (Ngo et al., 2017).

6.5 CHARACTERISTICS OF PHYTOCONSTITUENTS

6.5.1 Polarity

It is necessary to notice the connection between the strategy applied and the properties of the substances extracted. A general principle is like dissolves like. Thus nonpolar solvents can extract out nonpolar substances, and polar materials are extracted out by polar solvents.

6.5.2 pH

The ionizability of the compounds is important as the pH of the extracting solvent is often adjusted to make sure most extractions, such as alkaloids, are often extracted into polar liquid acid, as their basic nature ensures salt formation in acid. The salt dissociates into ions in aqueous solutions and also the substance dissolves because of hydration of the charged, protonated organic compound and also the anion. Solvents at basic pH might equally be used to extract acidic phytochemicals, for example, fatty acids and phenols. it's necessary to make sure that the compounds won't break down at the pH values utilized; for example, esters are liable to chemical reaction in alkali and many glycosides lose the sugar moiety in acid.

6.5.3 Thermostability

The solubility of compounds in solvents increases with increasing temperature and higher temperatures facilitate penetration of the solvent into the cellular structures of the organism to be extracted. However, any advantage gained here will clearly be lost if the compound is unstable at higher temperatures. The formation of artifacts, that is, new compounds not initially present in the organism under study, is a possibility with many extraction methods. It should be noted that when a traditional method of extraction is followed, these artifacts may actually be responsible for the biological effects observed (Mukherjee, 2002).

6.6 INTERFERING COMPOUNDS IN EXTRACTION OF DESIRED PHYTOCONSTITUENTS

It has been discovered that numerous normally occurring compounds may interfere with the separation and cleansing of a desired bioactive formulation constituent. A couple of general techniques are specified below that may help the reader to understand that contamination may have occurred amid extraction.

6.6.1 Lipids

While lipids are usually extracted from solvents of low polarity, they will coextract once polar solvents are used. These mixtures may be visualized by running a tending plate and using iodine vapor in a closed chamber to uncover dark colored spots. To expel the fats and associated waxes from an extract, the bottom formulation material may be mixed with oil ether or resolvent and dried before the full extraction procedure. The material in addition may be specifically extracted with the solvent of choice and defatted later. Phase action or vacuum-liquid chromatography utilizing oil ether or resolvent as eluents may likewise be used to clean out the nonpolar lipid compounds, allowing the ensuing fractionation of the extract (Coll and Bowden, 1986; Pelletier et al., 1986). Fats and waxes may be disposed of from a contaminated sample by filtration through an invert stage activity section, an ulterior within the lipids being control. An associated elective approach is to incorporate alcohol or methanol–water in adequate volume to break down the desired compound, using dissolution by sonication if essential and later on separation off the encourage.

Another strategy for the expulsion of fats and oils from watery or weak alcoholic extracts is an increase of inert fat that liquefies at a temperature below the brink of the extraction liquid. For this reason, exhausting paraffin or wax is to be further to the extract. The mix is to be warm till the purpose once all the wax liquefies and therefore the mix is mixed systematically. Heat is exhausted and permissible to cool down to temperature. The wax can set as a tough layer on the best purpose of the extract. Exactly pullulate the liquid beneath. The liquid need to be freed from fats that have all weakened within the wax.

6.6.2 Colors and Pigments

Undesirable colors, for example, chlorophyll and flavonoids might be available at high concentration levels depending upon the formulation part handled. They are not effortlessly dispensed with, but rather a portion of the accompanying techniques might be connected. Activated charcoal or actuated carbon is known to discolor solutions by a particular adsorption effect. The solution might be either transported across a reasonably short charcoal section, or the powder may be mixed with the liquid to be bleached, left to stay for a period of time, and sifted. The efficiency of the adsorption is increased by warming. Charcoal has the disadvantage that numerous active restorative mixtures will likewise be adsorbed, as on account of morphine, strychnine, and quinine (it is a result of this that charcoal has been used as a cure for alkaloid harming). Chlorophylls are usually disposed of in considerable amounts from the extracts by solvent parceling. Chlorophyll is more solvent in less polar solvents and, thus, can be expelled by solvent parceling. For this reason, the alcoholic extract is to be weakened to around 200th v/v alcohol with water and, at that point, extracted with diethyl ether or dichloromethane, and the chlorophyll can get into the less polar layer. Less polar constituents can likewise partition into the dichloromethane or ether layer, so an additional advance to fractionate this layer utilizing rejection gel is currently once again prudent. A trial with a little example and a succeeding TLC examination is usually projected keeping in mind the end goal to assess the impacts of decolorizing by these techniques. Extracts and parts may likewise be cleared up by separating them through Celite. An aqueous solution of 2%–5% lead (II) ethanoate (general precipitation reagent) has additionally been used for the evacuation of unsaturated fats, chlorophylls, and various colored materials. The sticky extract from the formulation is cleaved in 95% watery ethanol, with warming if necessary, and therefore the lead (II) ethanoate solution is then included, delivering an insoluble encourage that is separated off by suction through Celite or kieselguhr. Celite is a commercial brand of diatomaceous earth utilized as a filtration aid. Various purities of Celite can be acquired from numerous producers; Celite comprises roughly 90% SiO_2, and furthermore contains small amounts of CaO, Al_2O_3, and Fe_2O_3. This technique has been utilized broadly to acquire the predecontaminated portion containing sesquiterpene lactones, yet numerous different compounds might be coaccelerated, along these lines decreasing their general compound yields.

Utilization of avoidance gel is another method for separation of chlorophyll. This strategy isolates chlorophyll as it is an extensive atom and goes through the gel in the segment rapidly. Alternate segments in the extracts are held. The Sephadex LH-20 is suitable with a nonpolar solvent is permissible to swell for a few hours in the solvent in which the extract is broken down. The gel is packed into a section and presented by dissolving in as little a volume as could be expected under the circumstances, to the highest point of the segment. Elute with a similar solvent and gather aliquots of around 5 mL. A green band relating to the chlorophyll ought to be seen that moves rapidly through the gel. Other yellow groups (comparing to

the carotene) may likewise be seen. Proceed with elution until the point that every one of the colors have been expelled and gather no less than double the segment volume of consequent eluent.

Reverse phase chromatography is another strategy for expulsion of chlorophyll from alcoholic extract. This technique makes use of the nonpolar nature of chlorophyll. For this reason, the stationary stage is readied (RP-8 or RP-18 silica) by treating with methanol and pressed into a section. The segment is washed with a solvent in which the example is broken up. The extract is broken down in a small amount of water or methanol:water (1:1) and presented at the highest point of the section. It is to be eluted with 1:1 methanol:water taken after by methanol. The divisions are to be gathered as they will contain the vast majority of the parts other than chlorophyll. Elute the section with 100 mL acetone:methanol (1:1), which could elute some chlorophyll while rinsed with methylene chloride or ether to get the pigment.

6.6.3 Plasticizers

Plasticizers may contaminate solvents, filter papers, plastic mechanical assemblies, and chromatographic solid phases put away in plastic holders. Dioctylphthalate ester has usually been found to contaminate confines from formulations, and in unadulterated form, it is a yellow oil that shows a large cytotoxic action. When performing TLC on silica gel, it exhibits a pink-violet spot once showered with concentrated H_2SO_4 or concentrated sulfuric acid (4:1) and heated at 110 °C for 5 min. With $R_f = 0.4$ (oil ether-ethyl ethanoic acid derivation, 19:1). Plasticizers may be eliminated by refining the solvents, separating the sample through a reverse phase chromatographic phase, or by filtering the extract or test through porous alumina.

6.6.4 Water Soluble Polysaccharides and Other Carbohydrate Polymers

Sugar polymers, for example, starch, adhesive, gums and so on, are exceptionally basic parts with formulations, which can ruin extraction and fractionation because they frame thick solutions or gels with water. They occur in large amounts in marine green growth, in the sustenance stockpiling organs of the all the more profoundly advanced formulations and in specific creatures. The most well-known technique for expelling these substances from watery extracts is to encourage them by the expansion of the less polar water-solvent solvent, for example, ethanol or $(CH_3)_2CO$ and the subsequent accelerates would then be able to be evacuated by filtration or centrifugation.

6.7 EXTRACTION METHODS FOR SPECIFIC PHYTOCHEMICAL GROUPS

Solvent extraction is the most common strategy for extraction and it provides a general layout of the solvents that are suitable for extraction of the principle categories of compounds. The principle categories of compound to be considered are settled oils, fats and waxes, volatile or basic oils, carotenoids, alkaloids, glycosides, aglycones, synthetic resin compounds, polysaccharides, and proteins. Polarity and hydrogen ion concentration are two of the factors during the extraction. The techniques given during this area are general one in view of the regular properties of expansive categories of phytochemicals (Detailed methodology will be explained in Chapter 7).

6.7.1 Alkaloids

All alkaloids contain no less than one nitrogen atom and the compound is basic. This suggests salt development can happen within the sight of acid. This principal property of alkaloids is employed as a part of their extraction and additional tidy up. Two techniques may be used for alkaloid extraction. One is to alkalize the formulation material utilizing diethylamine or alkali and extract with an organic solvent. The soluble medium guarantees that the alkaloids are in their free base or unionized state. Most organic compound bases are of medium polarity and may be extracted utilizing chloroform, methylene chloride, or diethylether. A general solvent, for example, ethanol could likewise be used. The second technique is to treat the formulation material with watery acid. In this circumstance, the alkaloids form salts that are ionized and in this manner solvent in watery media. The organic compound will be recouped in free base form by basifying the watery extract (which deprotonates the alkaloid), followed by extraction into suitable organic solvent.

6.7.2 Carotenoids

The red, orange, and yellow colors observed in plant parts are due to carotenoids. They are generally tetraterpenoids in nature (containing around 40 carbon chain structures) and can be partitioned into hydrocarbons and oxygenated fractions, known as xanthophylls. The previous groups are relatively nonpolar and may be extracted into pet ether. Xanthophylls are of a more polar nature as they consist of ketone, aldehyde, acid, or epoxide groups, and can thus be extracted into ethanol or mixtures of ethanol and less polar solvents, for example, chloroform.

6.7.3 Fixed Oils, Fats, and Waxes

Fixed oils and fats differ only in their physical state at room temperature (liquid and solid, respectively), which forms a component of the level of unsaturation and chain length of the unsaturated fats. A heterogeneous blend of unsaturated fats, long-chain alcohol, paraffin, fixed oils, fats, and waxes is referred to as the fixed oils. These may be extracted with nonpolar solvents, for example, light oil or hexane. They may likewise break down in chloroform, ethanol, or methanol, yet these solvents will likewise extract out different kinds of phytochemicals. On a modern scale, these substances might be extracted by the procedure of articulation instead of solvent extraction. Where the proximity of fats and oils interferes with the extraction of different constituents, a defatting step, including solvent extraction of fats, is sometimes found to be quite useful.

6.7.4 Glycosides

Glycosides are naturally polar because of the availability of at least one sugar moiety in the molecular structure. Their isolation from plants is performed based on the nature and the aglycone moiety. Most glycosides can be isolated using polar solvents, for example, $(CH_3)_2CO$, C_2H_5OH, CH_3OH, water, or blends of any of these solvents. In the case of cardiac glycosides, the aglycone moiety (steroidal ring, cyclopentanoperhydro phenanthrene) is very large in size. These glycosides are freely soluble in chloroform, whereas their solubility in water is not appreciable. During aqueous extraction, the glycosides are decomposed into aglycone and sugar moieties. Aglycone and glycone moieties get hydrolyzed and partitioned. If the extraction of only the aglycone moiety is important, then, hydrolyzing the glycosidic fraction with dilute mineral acids, such as 10% H_2SO_4, liberates the aglycone moiety (this principle is employed in Borntrager's test, refer to Chapter 7). There are three types of glycosidic linkages, namely, O-glycosidic linkages, N-glycosidic linkages, and C-glycosidic linkages. In the case of C-linkages, the glycoside is resistant to acid hydrolysis. In this case, the separation of the aglycone moiety is performed by treatment of the glycoside with an $FeCl_3$ solution (such as Cascarosides—such hydrolysis is performed during the modified Borntrager's test). Such agents function as oxidizing agents, converting the monoanthrones to dianthrones.

6.7.5 Phenolic Compounds

Phenolic compounds exist in plant parts as free phenolic compounds or as glycosides. Phenolic compounds have many –OH groups, and so they are polar in nature and capable of forming hydrogen bonds in aqueous solutions. Due to their weakly acidic nature, they can form salts with alkali (NaOH, KOH, NaOEt, NaOMe etc.) very easily, forming phenolate salts. Polyphenol Oxidases are capable of polymerizing the phenols and forming high molecular weight polymers. In case of polymerization mediated by acids, the phenolics become darker in color.

6.7.6 Proteins

Amino acids, the monomeric units of protein structure, comprise both carboxylic ($–COO^-$) and ammonium (NH_3^+) groups, existing in the Zwitterionic form. Depending on the natures and proportions of the different amino acid groups, the protein remains either in an ionized or unionized state in different pH ranges. The pH, at which the protein remains in an unionized state, is known as the isoelectric point (pI), which depends on the amino acid composition of the protein. At a pH above the pI, the protein carries a net negative charge, whereas if the pH is below the pI, the protein carries a net positive charge. Extractions of most of the proteins require 70%–80% alcohol, which are generally water, dilute acid, or dilute base.

Preferential isolation of proteins may be performed by precipitating them using acetone, ethanol, or ammonium sulfate solution. Some of the proteins, such as globulins, are more soluble in salt solutions than in water. They may be extracted using 10% NaCl solution. Prolamines are better extracted using 70%–80% alcohol treatment, followed by dilution with water. After the precipitation of the proteins, purification is performed by resolubilization, ultrafiltration, gel filtration, affinity chromatography, or electrophoretic techniques.

6.7.7 Polysaccharides

Polysaccharide, such as starches and celluloses, are formed by polymerization of monosaccharides and oligosaccharides. Polysaccharides may be generally of three types, namely, completely water soluble polysaccharides (glycogen), hydrolysable by water and capable of gel formation (amylopectin), and those that are completely insoluble in water (cellulose). These variations in the solubility are attributed to the difference in the glucose linkages in the structures of the polysaccharides. Certain groups in the structure of glucose are also important in determining the solubility. Treatment with salts results in breakdown of the polyuronides. The presence of amino acids improves the solubility of the sugar in acids. Chitin is a polysaccharide forming the exoskeleton of the arthropods. Due to the presence of the acetamido group and establishment of the

hydrogen bonding renders it insoluble in water. Those polysaccharides which are broken down completely or partially in water may be extracted with cold or warm water. The polysaccharides may be filtered out using redissolution in water and dialysis, which removes the salts and other small atoms, such as monosaccharides. The filtered material is then dried in order to get the dried polymer. Isolation of polysaccharides may be achieved through gel filtration. Refinement of the polysaccharides is performed by treatment of the sample with solvents, which can break the matrix down and get the pure polysaccharide. Cellulose is obtained through treatment of wood with ethanol, sodium chlorite, sodium chloride, and finally water.

6.8 TREATMENT OF DRUG RESIDUE AFTER EXTRACTION–DOWNSTREAM PROCESSING

Every now and again the result of an extraction procedure cannot be utilized as a part of a viable application. Such items may contain undesirable aggravates that must be removed. Of specific significance is the removal of solvents utilized as a part of the extraction, if these substances cannot be allowed in the final product or because of the cost involved with the removal procedure. Furthermore, the physical properties (particle size and morphology, crystalline structure, and so on) are as vital as the chemical properties of the extract and can assume a determinant part on the natural movement and the possible common uses of the material. Besides, regular extracts generally are inclined to corrupted forms and should be figured with protective compounds. The detailing can likewise expand the functionalities of the extracts, permitting their use in pharmaceutical, restorative, or food applications with various goals.

6.8.1 Sanitization of Extracts and Elimination of Solvents

Remaining quantities of solvents are generally present in pharmaceuticals and food items because of the extraction systems connected in the generation forms. Increased awareness in the public arena with respect to both ecological and human wellbeing has stimulated the development of new innovative methodologies that permit the diminishment or (if possible) the end of solvents from particular items. Obviously, the best solution is to avoid the use of poisonous solvents in each case, yet in the situations in which this is not possible, solvent disposal strategies are required. Organic solvents may collect in lipid- and fat-rich cells of the human body, including the sensory system, brain, bone marrow, liver, and body fat, and can have various inconvenient impacts. Subsequently, leftover solvents have to be isolated, owing to their potential hazard to human health, as discussed in Chapter 4.

6.8.2 Removal of Solvents

Separation of solids from volatile solvents by vaporization is commonly known as concentration. This method requires the conversion of the solvent segment from a liquid state to the vapor state. Accordingly, concentration, as well as an increase in the temperature, can be utilized to accomplish this. A typical use of concentration is found in the generation of the last particles of some highly valued items, for example, vitamins and pharmaceuticals. On account of low creation rates a solitary stage is utilized; then again various stage evaporators working at various pressure (and in this way temperature) levels are utilized as a part of facilities intended for bigger generation limits, with a specific end goal to recuperate the idle heat substance of the solvent all the while. A pressure decrease must be utilized when the disintegration of the item with temperature is likely. Diverse techniques for energy supply offer ascent to new concentration forms. For instance, microwave drying depends on the use of electromagnetic energy; the microwave frequencies give a speedier and more homogeneous dissipation of solvents. It can be joined with vacuum, keeping in mind the end goal is to work at temperatures appropriate for thermolabile items.

Concentration may be carried out in several ways, such as by direct heating of the substance on a heating device, such as a water bath, or in a rotary vacuum evaporator. The rotary vacuum evaporator is the method of choice for a natural products chemist because in this method controlled heating helps to remove the solvent while retaining the thermolabile flavonoid compounds intact. The quantity of material to be processed per unit time and the characteristics of the solution being concentrated are important features to be considered in the choice of a vaporizer. Stable solutions are concentrated using a moderate apparatus, while heat-sensitive solutions are concentrated using techniques in which the exposure to heat is short, preferably under vacuum. The vaporization process is combined with circulation of the liquid, for example, with tube vaporizers with and without forced circulation.

6.8.2.1 Rotary Vacuum Evaporator

The Rotary evaporator is a common laboratory apparatus used for quick removal of solvents from reaction mixtures. This is done by heating mixtures gently under reduced pressures to accelerate the rate of evaporation. A vacuum evaporator used

to work by bringing down the pressure over a mass of fluid, thus reducing the boiling points. The rotary evaporator works by increasing the rate of evaporation of the solvent by:

- reducing the pressure to lower the solvent boiling point,
- rotating the sample to increase the effective surface area, and
- heating the solution.

The main components of a rotary evaporator are:

- A motor unit for rotating the evaporation flask or vial that contains the user's sample.
- A vapor duct serving as the axis for sample rotation, and a vacuum-tight conduit for the vapor to be drawn off the sample.
- A vacuum system for reducing the pressure substantially within the evaporator system.
- A heated fluid bath (generally water) to heat the sample.
- A condenser with either a coil passing coolant, or a "cold finger" into which coolant mixtures, such as dry ice and acetone, are placed.
- A condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it recondenses.
- A mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath.

The operation methodology of a Rotary vacuum evaporator is depicted in Fig. 6.14. There is a general 20/40/60 rule. It states that the temperature of the water bath should be 60 °C, causing a temperature of 40 °C of the sample and the vapor evolved is thereafter condensed at 20 °C. For lower temperature ranges, a 0/20/40 rule is applicable.

6.8.2.2 *Tube Vaporizers*

This is a conventional type of vaporizer. In this autocirculation type apparatus, a bundle of narrow vaporizer tubes are arranged concentrically around the white central "fall tube." The vaporizers are heated externally with steam. A liquid separator in the vapor chamber, placed above the tubes prevents the removal of droplets of the concentrated solution with steam. With its progress through the vapor tube, the crude extract gets separated into vapor and liquid. Another pump-circulation type modification of this apparatus has been introduced to combat the problem of low temperature difference and viscous solutions. This apparatus augments forced circulation of the liquid with higher heat transfer coefficient than an autocirculation vaporizer (List and Schmidt, 1989).

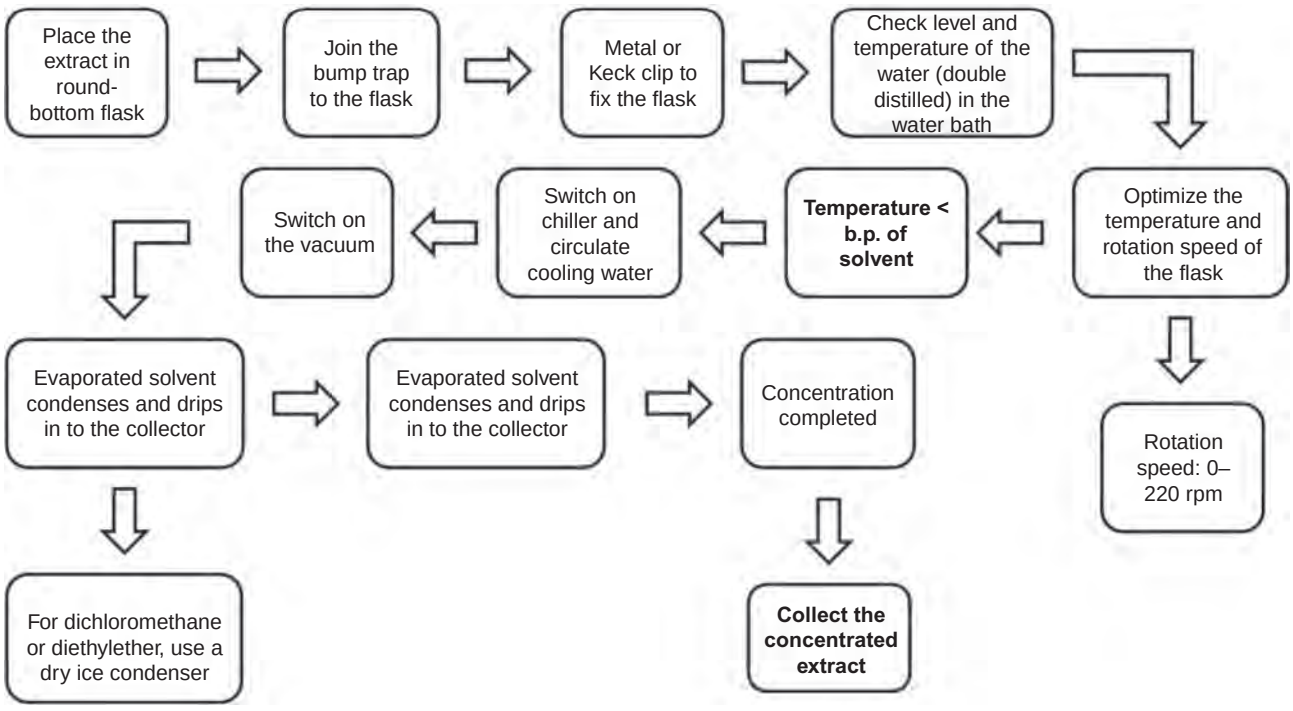

FIG. 6.14 Operation procedure of a Rotary vacuum evaporator.

6.8.2.3 Falling Film Vaporizer

To conserve sensitive material, this type of falling film vaporizer, also known as a spray film vaporizer has been introduced. Here, steam is released as spray film. In this mechanism, the sprayed material stays in the heated environment for a short duration of time and then goes out through the outlet. One disadvantage is that the solution needs to reach its boiling point prior to atomization and application on the film-forming surface (List and Schmidt, 1989).

6.8.3 Freeze Drying

Lyophilization may be defined as freeze drying and is a controllable method of dehydrating labile products by vacuum desiccation. It possesses the following features:

- ✓ Freezing of the liquid sample, followed by the conversion of water into ice and crystallization of crystallizable solutes as well as formation of an amorphous matrix comprising noncrystallizing solutes associated with unfrozen moisture.
- ✓ Sublimation of frozen ice under vacuum.
- ✓ Evaporation of moisture from the amorphous matrix.
- ✓ Desorption of chemisorbed water resident in the apparently dried cake (Jadhav and Moon, 2015).

Freeze drying is the evacuation of ice or other solidified solvents from a material through the procedure of sublimation and the removal of bound water through the procedure of desorption. Lyophilization and freeze drying are terms that are used interchangeably depending upon the situation in which the drying occurs. Controlled freeze drying keeps the temperature of the item sufficiently low amid the procedure to avoid changes in the appearance and attributes of the dried item. It is an amazing strategy for saving a wide assortment of heat-sensitive materials, for example, proteins, microorganisms, pharmaceuticals, tissues, and plasma. Here the process aims at sublimation of the ice directly to the vapor phase.

Sublimation occurs when a solid (ice) changes directly to a vapor without first going through a liquid (water) phase. Thoroughly understanding the concept of sublimation is a key building block to gaining knowledge of freeze drying. Sublimation is a phase change and heat energy must be added to the frozen product for it to occur. Sublimation in the freeze drying process can be described simply as:

FREEZE (The product is completely frozen) ⟹ VACUUM (The product is then placed in a deep vacuum, well below the triple point of water) ⟹ DRY (Heat energy is then added to the product causing the ice to sublime).

During lyophilization, the material is cooled to a temperature below its triple point and then a vacuum is applied to remove moisture from the material through sublimation. As we know, water has a total of three phases, solid, liquid, and vapor. The phase diagram of water must be consulted to determine when these three phases of water will be in an equilibrium condition with each other, or when any two phases will be in an equilibrium condition, or when the water phase will be changed completely from one phase to another phase, or at which pressure and temperature water will undergo a phase change. We can see the triple point in the above phase diagram of water. The phase diagram of water will locate one point in a curve at which all phases of water will coexist together. At the triple point, the pressure will be 4.58 mm of Hg and the respective temperature will be 0.0075 °C. Let us see one live example to understand the concept of the triple point or the concept of all three phases of water coexisting at a pressure of 4.58 mm of Hg and a respective temperature 0.0075 °C. The triple point is also called the nonvariant point because the degree of freedom at this point is zero. As the degree of freedom at the triple point is zero, none of the variables will be changed when all the phases exist. In simple terms, we can say that the three phases or the triple point will only be available at a particular pressure and temperature. Applications of freeze drying are many:

(a) Pharmaceutical and biotechnology

Pharmaceutical organizations regularly utilize freeze drying to increase the useful lifetime of items, for example, vaccines and different injectables. By expelling the water from the material and sealing the material in a vial, the material can be easily stored, shipped, and later reconstituted to its unique shape for infusion.

(b) Food industry

Freeze drying is used to preserve food and make it very lightweight. The process has been popularized in the form of freeze-dried ice cream, an example of astronaut food.

(c) Engineering industry

In chemical synthesis, items are regularly freeze dried to make them more stable, or easier to dissolve in water for ensuing use. In bioseparations, freeze drying can be used likewise as a late-stage purging method, because it can viably remove solvents. Moreover, it is able to concentrate substances with low molecular weights that are too small to possibly be removed by a filtration film.

(d) Other uses

Organizations, such as the Document Conservation Laboratory at the United States National Archives and Records Administration (NARA), have performed studies on freeze drying as a recovery method for water-damaged books and documents. In bacteriology freeze drying is used to conserve special strains.

(e) The advantages of lyophilization include

- Chemical decomposition is minimized.
- Removal of water without excessive heating.
- Enhanced product stability in a dry state.
- Ease of processing a liquid, simplifies aseptic handling.
- More compatible with sterile operations than dry powder filling.

There are essentially three categories of freeze dryers:

- *Manifold freeze dryer*: In manifold freeze dryers, a short, normally round tube is utilized to interface different compartments with the dried item to a condenser.
- *Rotary freeze dryer*: Rotating freeze dryers are generally utilized for drying pellets, 3D squares, and other pourable substances. The rotational dryers have a round and hollow supply that is pivoted amid drying to accomplish a more uniform drying all through the substance.
- *Tray style freeze dryer*: Tray style freeze dryers, as a rule, have a rectangular supply with racks on which items, for example, pharmaceutical solutions and tissue extracts, can be put in trays, vials, and different compartments.

Improved freeze-drying systems are being created to broaden the scope of items that can be freeze dried, to enhance the nature of the item, and to deliver the item faster and with less work. A lyophilizer comprises a vacuum chamber that contains item shelves equipped for cooling and warming containers and their contents. A vacuum pump, a refrigeration unit, and related controls are associated with the vacuum chamber. Chemicals are, for the most part, put in holders, for example, glass vials that are put on the shelves inside the vacuum chamber. Cooling components inside the shelves freeze the item. Once the item is frozen, the vacuum pump evacuates the chamber and the item is heated. Heat is exchanged by heat conduction from the shelves, through the vial, and eventually into the item (Nireesha et al., 2013).

6.8.4 Reverse Osmosis

In reverse osmosis a net stream of solvent diffuses from a concentrated solution to a dilute solution through a semipermeable membrane. Pressure is applied to the concentrated solution side with a specific endpoint to surpass the osmotic pressure and, therefore, solvent exchange through the layer exists from the concentrated solution to the dilute side. This procedure requires no phase change and can work at ambient temperature, which is favorable when managing particularly delicate materials. The separation component in the layer depends on the size, shape, charge, and interactions of the compounds with the films. Asymmetric cellulose acetate membranes 100 mm thick are usually utilized. To apply reverse osmosis, a molar mass of the solute >300 Da is typically required. This method is broadly connected to the food business in procedures, for example, juice processing. In pharmaceutical manufacturing, it is utilized to recover active compounds amid downstream treatment.

6.8.5 Particle Size Reduction

Much of the time, a fundamental postextraction step is the creation of particles of a particular size and morphology, either by PS reduction or by precipitation of the components in the extract. The essential goals of this progression are to reduce storage capacity volume prerequisites, and to encourage the product dosage, improving its bioavailability. Specifically, if the bioavailability is restricted by the dissolvability or dissolution rate of the compound (e.g., in drugs with low water dissolvability directed orally), the control of particle parameters, for example, PS and PSD, are of vital significance. The decrease of PS increases the dissolution rate of the solid because of the expansion of the proportion between surface area and the volume of the particles. In addition, the saturation solubility of the compound is likewise expanded when PS is reduced below a basic size of 1–2 mm. For instance, the solvency of $BaSO_4$ in water at 20 °C is 2.2 mg/L for particles over 5 mm, and achieves a value of 2.6 mg/L for particles of 100 nm. For pharmaceutical applications, the reduction of PS beneath 1–2 mm permits utilizing diverse administration routes (aspiratory, parenteral, and so on). Another parameter that impacts the dissolvability is the solid condition of the particles. It is notable that amorphous compounds generally indicate higher dissolvability in contrast with crystalline compounds. Besides, extraordinary polymorphic types of crystalline

compounds can demonstrate distinctive solubilities (e.g., caffeine can be delivered in two crystalline polymorphs with various solubilities in water). The control of polymorphism in solid forms is a key consideration in the long-term solidness of the compounds. Plus, the dissolvability and also the stability of specific medications can be enhanced by cocrystallization of the active substance with an excipient, as caffeine cosolidified with glutaric acid. Along these lines, to achieve the most noteworthy saturation solubility, the best rule is to use nanometer-sized particles and an amorphous state. It is essential for pharmaceutical items to maintain an amorphous state for the shelf life of the item. On the off chance that the item in the amorphous state is not sufficiently stable, the typical approach is to coprecipitate it with an amorphous polymer, As demonstrated previously, the control of PS is a main part of the procedure, and sizes below 1 mm are required when solvency issues exist. Ordinary methodologies, for example, crystallization or spray drying, normally deliver particles ranging in size from 10 to 100 mm (microparticles). For the generation of nanoparticles (particles below 1 mm in size, in pharmaceutical and related orders) there are two methodologies: bottom-up and top-down strategies. The top-down approach mechanically reduces the PS of micrometric powders acquired by conventional techniques to nanometric (nanonization). In the bottom-up approach, the item is acquired. Postextraction processes include improvement of functional attributes of extracts specifically in the nanometric measure from a clear solution or a colloidal solution of the item, after the evacuation of the solvent (Rostagno and Prado, 2013).

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Chapter 7

Bioactive Phytocomponents and Their Analysis

Chapter Outline

Quality Control and Evaluation of Herbal Drugs. https://doi.org/10.1016/B978-0-12-813374-3.00007-7

7.1 BIOACTIVE PHYTOCOMPONENTS AND THEIR CLASSIFICATION

The chemistry dealing with natural products is the oldest branch of organic chemistry. Natural products' chemistry probably started with curiosity about color, odor, taste, and folk medicinal cures. In the early days, natural product chemistry was focused on the isolation of natural products and the determination of their structure. In certain cases, this was followed by isolation and elucidation of the biosynthetic route. With rapid advances in isolation techniques, structural determination is becoming routine in most cases. The trend also shifted toward activity-monitored isolation and structure determination, particularly inclined toward bioassay-guided isolation. This involves the interaction between a ligand and its biopolymeric receptor. Understanding the mode of action of folk herbs and related products is even more complex because unfractionated or partly fractionated extracts are used (Albach et al., 2003).

Substances obtained from plants and animals, with or without purification, have been employed in medicine for a long time. Such crude drugs and galenicals prepared from them are still widely employed, although many of them have been replaced, to a greater or lesser extent by pure medicinal chemicals, which are either isolated from natural sources or prepared synthetically. The advantages in using a pure chemical compound in medicine are many and obvious, namely, that the physiological effects of such a compound are fixed and definite and not complicated by extraneous factors, such as variation in quality and the presence of variable amounts of other physiologically active galenicals. There is the fact that the nature of the active constituents of many crude drugs is still unknown. There is the economic question; for even when the active constituents have been isolated and identified, it may not be possible to produce the drug either from the plant or synthetically at a price that can compete with that of active preparation of the drug. There are many crude drugs produced from a natural mixture of medicinal substances that produce a therapeutic action that is so desirable and well established as to make it unnecessary to attempt the artificial preparation of a similar mixture (Mann et al., 1994).

The pharmacological actions of crude drugs are determined by the nature of their constituents. In many cases, it has been possible to isolate the constituents and determine their structure, and in some cases, it has even been possible to synthesize them. Where it is a commercial proposition to synthesize these chemical substances or to isolate and purify them on a large scale from nature, then the active constituents are often used in preference to the drug itself or a preparation of the drug (Ramstad, 1956; Kirtikar et al., 1975). This has certain advantages, such as:

(i) medically, it can be guaranteed to provide doses that are more exact;
(ii) pharmaceutically, it can lead to a more suitable and elegant preparation that is free from undesirable inert constituents; and
(iii) economically, it ensures a regular supply at fairly uniform prices.

The dried vegetable drug, although a dead plant or part of a plant, was, however, once a living organism in which many metabolic processes were taking place. In fact, it is precisely because of the chemistry involved in living processes that various constituents are present in the dead tissues of the drug. Of course, certain chemical processes that occur during the transformation from the living plant to the dead drug are also important and they very often determine the actual conditions in which the constituents occur in the drug. On a few occasions, however, the process that takes place during the drying of the drug or preparation of the drug for the market produces new constituents. These constituents were not formed as a result of metabolism while the plant was living. The majority of higher plants are autotrophic, that is, they contain chlorophyll and are able to synthesize all of the required substances from simple substances through photosynthesis. They can take in carbon dioxide and water and other nutrients and gas from the natural world as their starting material for the production of primary metabolites (simple carbohydrates). They must obtain this from other sources. The production of carbohydrates is a photochemical reaction. Because it is dependent on light, it can take place only during the day (WHO, 1998).

7.1.1 Classification of Bioactive Phytocomponents

Crude drugs are classified based upon the active constituents. The plants contain various constituents in them, such as alkaloids, glycosides, tannins, carbohydrates, and saponins. Irrespective of their morphological or taxonomical characters, drugs with similar chemical constituents are grouped together. The plant drugs chemically consist of a complex but organized mixture of organic and inorganic constituents. The organic constituents are pharmaceutically important groups and can be classified as follows:

(i) Carbohydrates

Crude drugs mostly consist of carbohydrates that are either simple or complex in their structure. The simple ones are the hexoses, pentoses, and the methyl pentoses. Slightly more complex are the di- and trisaccharides. These are soluble substances present in cell sap that are often associated with other constituents, for example, glycosides. The more complex carbohydrates are cellulose, starch, gums, and mucilage, found either as food reserves or as part of the plant skeleton, for example, the cell wall. The pharmaceutically important carbohydrates are polysaccharides and polyuronides.

(ii) Glycosides

These include a wide range of chemical subgroups containing a glycone (sugar) and an aglycone (nonsugar) moiety. On hydrolysis by water under the influence of enzymes or mineral acids, they yield a sugar together with some kinds of hydroxy organic compounds, such as alcohol, phenol, or a hydroxy acid. They are further classified into different groups as described later. The pharmaceutically important glycosides include saponin and anthracin derivatives.

(iii) Tannins

These include a class of exceedingly complex plant products, including pyrogallol tannin, flavotannins, pseudotannins, and others.

(iv) Proteins and amino acids

This constitutes a major class of natural products of different categories, including albumin, globulin, conjugated proteins, and others.

(v) Coloring matter

These are widely distributed in natural products. The majority consist of chlorophyll, anthoxanthins, and anthocyanins.

(vi) Fixed oils, fats, and waxes

They constitute a major class of compounds from plant and animal resources. They were among the earliest organic substances to be investigated and their general chemical nature was elucidated as long ago as 1811 by Chevreul. Almost all of them are the main ester derived from trihydric alcohol, glycerin.

(vii) Volatile oils

The medicinal action of many crude drugs is due to the presence of essential oils. They are usually secreted as such in plant tissues, but sometimes they are produced from more complex substances by chemical changes brought about after the collection of the plant. They consist mainly of a mixture of small molecules of hydrocarbons containing 10–15 carbon atoms.

(viii) Alkaloids

They constitute a major class of chemical groups present in plant drugs. Originally, this meant "alkali like" and was applied indiscriminately to all organic bases. Over the course of time, the term alkaloid has changed in significance and, presently, it includes mainly the cyclic nitrogenous bases, which occur in plants. Thus, alkaloids are naturally occurring organic substances having a cyclic nitrogenous nucleus, exhibiting basic properties, and having a pronounced physiological action (Wallis, 1985).

(ix) Carotenoids

Carotenoids are responsible for several of the red, orange, and yellow pigments found in the plant and animal kingdoms. They are typically tetraterpenoid derivatives (containing 40 carbon atoms) and may be divided into hydrocarbons and oxygenated forms, referred to as xanthophylls. Xanthophylls are very polar as they contain alcohol, ketone, aldehyde, acid, or epoxide groups, and thus may be extracted with ethyl alcohol or mixtures of ethyl alcohol and comparatively less polar solvents, such as chloroform (Houghton and Raman, 1998).

(x) Synthetic resin compounds

These exist as free phenols or as glycosides. Because of the multiplicity of chemical group functions, phenols tend to be comparatively polar and they dissolve in alcohol. As they are weak acids, they can even be extracted or partitioned with alkali as phenolate salts. An obstacle encountered with synthetic resin compounds is that they will bear intensive chemical process reactions by the action of polyphenol oxidases. This is the reason for the brown coloration in broken material once exposed to the air. The chemical process reaction is catalyzed by acid (Houghton and Raman, 1998).

(xi) Proteins

Most of the proteins are ionized at high or low pH due to the presence of free carboxylic, amino, and phenolic groups on the amino acid side chain. The isoelectrical point (pI) occurs when the pH carries no net charge and this may vary with every macromolecule depending on the constituent amino acids. At pH values higher than the pl, the macromolecule carries a net electric charge and, therefore, at pH values below the pl, a net positive charge is carried. As a result, most of the protein is extracted with water, buffers, dilute acid or base, or straightforward salt solutions. However, a lot of lipotropic proteins require the use of 70%–80% alcohol (Davin and Lewis, 2000; Tyler, 1982; Robinson, 1955).

Selective precipitation of groups of macromolecules in an exceedingly crude protein extract is achieved by gradual addition of solvent, ethyl alcohol, or ammonium sulfate. Conversely, for proteins with a larger solubility in salt solutions than water, for example, for globulins, a crude macromolecule mixture is extracted with a 10% sodium chloride solution and, therefore, the globulins are precipitated by the addition of water. For prolamines, extraction in 70%–80% alcohol is followed by precipitation by dilution with water. Resolubilization and more separation and ultrafiltration will follow the precipitation step, gel filtration, ion-exchange chromatography, or electrophoresis. Proteins vary in their stability to denaturing/coagulating agents like heat, pH, organic solvents, detergents, and air mass. These factors should be taken into consideration throughout the extraction process. Whenever the sample to be extracted contains high proportions of fat (e.g., some seeds), it is judicious to defat it by extraction with light petroleum before the extraction of proteins (Houghton and Raman, 1998).

(xii) Polysaccharides

Polysaccharides are polymers of sugar derivatives. Because of their polyhydroxylated nature, it might be expected that all polysaccharides would be soluble in water. However, this may not be the case, because the overall shape of the molecule and the strength of the intermolecular bonds must be overcome for hydration to occur, hence influencing the water solubility. Generally, there are three forms of sugar polymers, fully water soluble, partly soluble sugar polymers that swell to create gels, and lastly, water-insoluble sugar polymers. Examples of these three types are glycogen, amylopectin, and cellulose, which are all polymers of glucose. Solubility variations arise as a result of differences in molecular shape (globular, branched linear, and linear) because of variations in the type of linkage between glucose units. The presence of certain functional groups on sugar derivatives is also vital where solubility is concerned. As an example, polymers of sugar acids (polyuronides) can dissolve in alkali because of salt formation, whereas the presence of amino sugars enhances the solubility in acid. The acetyl amino group in chitin (a major component of the exoskeleton of arthropods) allows strong hydrogen bonding between chitin chains, making it very insoluble in water. Polysaccharides that completely or partially dissolve in water can be extracted using cold or warm water. Coeluting proteins can usually be removed by shaking the aqueous extract with 20% of its volume of chloroform followed by 20% of its volume of *n*-butanol or *n*-pentanol. Proteins usually coagulate at the interface of the organic and aqueous layers. Acidification to pH 4–5 assists this denaturation process. The crude polysaccharide mixture is precipitated by the addition of ethyl alcohol or acetone to the separated liquid layer. Divalent ions, such as calcium, can be used to precipitate out polyuronides, for example, calcium alginate. Purification of the polysaccharide fraction is achieved by redissolving in water and dialyzing to get rid of salts and smaller molecules, such as monosaccharides. This step is followed by freeze drying in order to obtain the dried polymer. Individual polysaccharides are obtained by gel filtration strategies (Houghton and Raman, 1998).

For water-insoluble polysaccharides, such as cellulose and chitin, "extraction" is achieved by treatment with solvents that dissolve out the impurities and leave the purified polysaccharide. To get cellulose, wood is treated in turn with ethyl alcohol (to remove resinous material), acidified sodium chlorite (to chlorinate the lignin, bleach), alkali (to rinse off chlorinated polymer and take away polyuronide pectins), and eventually water (to take away chlorinated lignin and remove polyuronide pectins). Cellulose can dissolve in strong mineral acid solutions, from which it is regenerated by the addition of water. However, these processes might result in degradation of the polysaccharide (Lee et al., 2015).

7.2 IMPORTANCE OF PHYTOCONSTITUENTS IN THERAPY

The therapeutic potentials of plant and animal origin have been used from ancient times by using simple processes, without the isolation of the pure compounds, that is, in the form of crude drugs or the galenicals prepared from them. The pharmacological action of a crude drug is determined by the nature of its constituents. Thus, the plant species may be considered as a biosynthetic laboratory, not only for the chemical compounds that are utilized as food by humans and animals (e.g., carbohydrates, proteins, and fats), but also for a multitude of compounds, including alkaloids, terpenoids, flavonoids, and glycosides, which exert definite physiological effects. These chemical compounds are responsible for the desired therapeutic properties. To obtain these pharmacological effects, the plant materials are used in their crude form or they may be extracted with suitable solvents to take out the desired components with the resulting principals being employed

as therapeutic agents. The phytochemistry of herbal drugs embraces a thorough consideration of these chemical entities, which are called constituents.

As the herbal drugs contain so many chemical compounds, it is essential to single out those responsible for the therapeutic effects, which are called active constituents. There are, of course, a number of examples in which the therapeutic effect of a crude drug, or a galenical preparation of the drug, differs to some extent from that of its active constituents after isolation. This may be due to the synergistic effect of several constituents of the crude drug, which can enhance or retard the desired action. It may also be due to the modifying effects by some constituents upon the physical properties of the others. For example, digitoxin is insoluble in water, but it can always be extracted by infusion with water, which is due to the presence of the saponin digitonin, which makes it soluble (Herbert, 2001).

In the context of the quality of the herbal medicine, the advantages of using the pure active constituents are obvious as these compounds have fixed and definite physiological effects without the presence of such extraneous factors as variations in quality and the presence of variable amounts of other physiologically active substances. There are, however, many obvious reasons for the continued use of crude drugs as such and the galenicals prepared from them. There are so many therapeutic potentials of herbal drugs for which the natures of the active constituents are still unknown. In this case, it is better to use the total plant extract of the desired part of the plant rather than to isolate the specific constituents.

Many crude drugs provide a natural mixture of medicinal substances, which produces a therapeutic action. One component may be responsible for the desired therapeutic effect, whereas the purpose of another may be to enhance the activity or to nullify the side effects. These therapeutic properties are so desirable and well established as to make it unnecessary to attempt an artificial preparation of similar mixture. Thus, from the pharmaceutical point of view, it is necessary to have a clear idea not only of the therapeutically active components, but also of the inert substances, such as cellulose, lignin, suberin, cutin, starch, albumin, coloring matter of plant origin, and several others of animal origin, such as keratin, chitin, muscle fiber, and connective tissue. Often the presence of inert substances modifies or prevents the absorbability or potency of the active constituents and makes it more difficult to isolate the main constituents. To eliminate or nullify the effects of these inert materials, the active moieties are extracted, crystallized, and purified for therapeutic use; these are named secondary substances (Bourgaud et al., 2006).

The secondary metabolites of plants that are of greater pharmaceutical importance represent either byproducts of anabolic or catabolic processes or they may be direct products of metabolism, having some significant biological functions (Forkmann and Heller, 1999). These constituents in plants are influenced by several factors in the plant cells, which play a key role in their modification. Ontogeny plays an active role in secondary metabolite production of varying concentrations. Production of secondary metabolites varies according to the stages of development of the plants. For example, the morphine content *of Papaver somniferum* is highest 2–3 weeks after flowering, while in earlier stages the related alkaloids, such as thebaine and codeine, predominate; if there is a delay, the morphine decomposes (Kutchan, 1998; Misra et al., 1999). Based on these concepts, various biosynthetic pathways leading to the formation of secondary metabolites have been derived, which is known as biogenesis or biosynthesis of drugs (Wallis, 1985; Dewies et al., 1964; Conn, 1981). A general understanding of the biogenetic pathways is shown in Fig. 7.1.

7.3 QUALITATIVE ANALYSIS OF CRUDE DRUG EXTRACTS AND ISOLATES

There are some characteristic general reagents that have been described for a few of the most common types of natural products found as plant secondary metabolites. It should be taken into consideration that none of these reactions is specific and a positive reaction allows only the presumption of the presence of a certain type of secondary metabolite because certain structural similarities with compounds of completely different types may result in false-positive reactions. A negative reaction does not exclude the presence of any compound by reason of the fact that such a compound may occur in too low a concentration for unambiguous detection (Raaman, 2006; List and Schmidt, 1989).

7.3.1 Tests for Alkaloids

(A) Dragendorff's test

If a few drops of Dragendorff's reagent (potassium bismuth iodide) are added to a small amount of drug solution, the formation of an orangish red color indicates the presence of alkaloids.

Dragendorff reagent

Dragendorff reagent is a solution of potassium bismuth iodide, which gives reddish-brown precipitates with alkaloids. Dissolve 8.0 g $Bi(NO_3)_3 \cdot H_2O$ in 30% (w/v) HNO_3 and 27.2 g KI in 50 mL water. Combine the solutions and let stand for 24 h, filtered, and made up with water to 100 mL. Use only in acid solutions in which an orange-brownish precipitate will

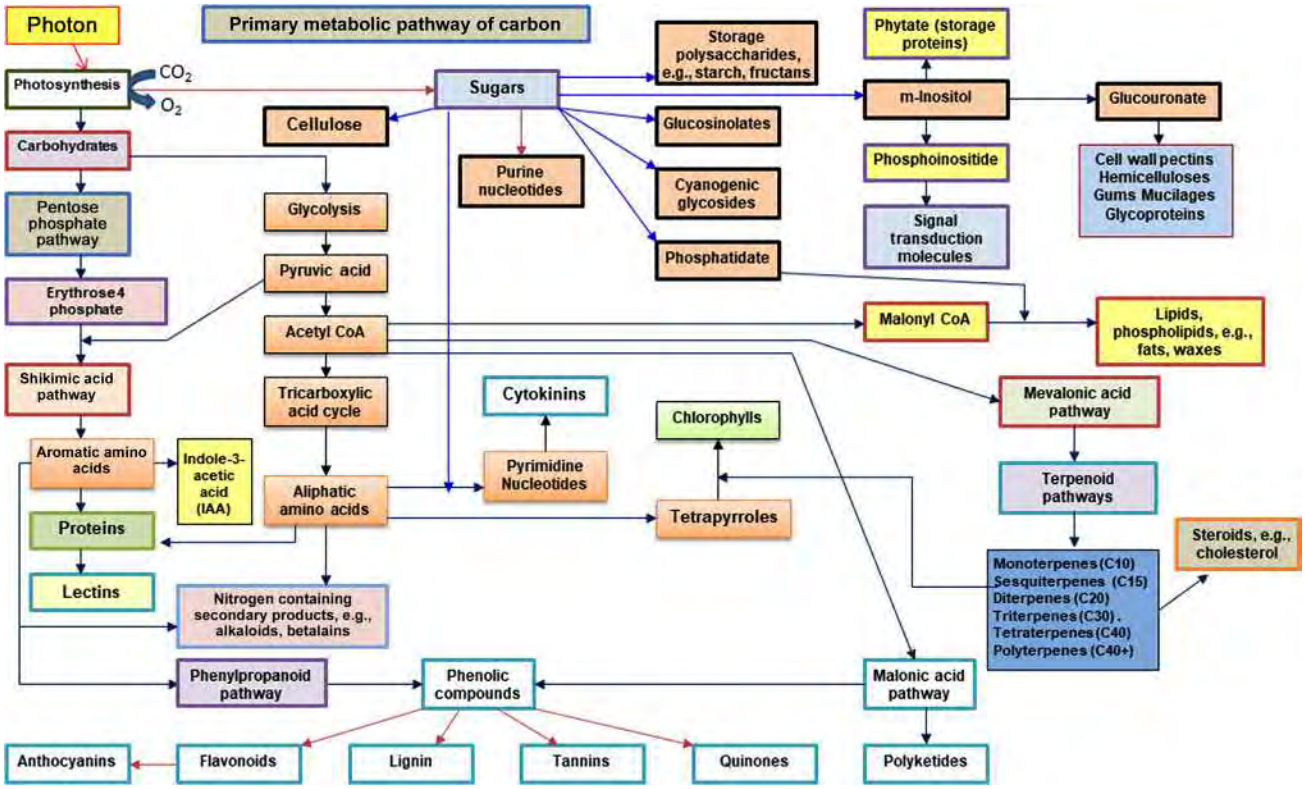


FIG. 7.1 General understanding of the biogenetic pathways for development of plant metabolites.

appear. The alkaloids may be recovered by treatment with sodium carbonate and subsequent extraction with ethyl ether. This reaction may also be performed on a filter paper or on a TLC plate by adding a drop of the reagent onto a spot of the sample.

(B) Mayer's test

Take a small amount of drug solution and add a few drops of Mayer's reagent (potassium mercuric iodide); the formation of a creamy-white precipitant indicates the presence of alkaloids (Bonner and Varner, 1965).

Mayer's reagent

Mayer's reagent is a solution of potassium mercuric iodide prepared by dissolving mercuric chloride and potassium iodide in water. Dissolve 1.36 g $HgCl_2$ in 60 mL water and 5 g KI in 10 mL water. Combine both solutions and make up with water to 100 mL. Add a few drops to an acidified solution (HCl or diluted H_2SO_4) containing the alkaloids and, if alkaloids are present, a creamy-white to yellowish precipitate will appear. Special care should be taken because the precipitate may be redissolved by ethanol in the solution or excess of reagent (Wallis, 1985).

(C) Hager's test

Take a small amount of drug solution and add a few drops of Hager's reagent (saturated aq. solution of picric acid); the formation of crystalline yellow precipitate indicates the presence of alkaloids.

Hager's reagent

Hager's reagent is a saturated solution of picric acid in water and gives yellow precipitates with alkaloids.

(D) Wagner's test

Take a small amount of drug solution and add a few drops of Wagner's reagent (dilute iodine solution); the formation of reddish-brown precipitate indicates the presence of alkaloids.

Wagner's reagent

Wagner's reagent is a solution of potassium triiodide in water. Dissolve 1.27 g I_2 (sublimed) and 2 g KI in 20 mL water, and make up with water to 100 mL. A brown precipitate in acidic solutions suggests the presence of alkaloids.

(E) Tannic acid test

Take a small amount of drug solution and add a few drops of tannic acid solution; the formation of buff-colored precipitate indicates the presence of alkaloids.

(F) Ammonia reineckate test

Take a small amount of drug solution, then add a slightly acidified (HCl) saturated solution of ammonia reineckate; the formation of pink flocculent precipitate indicates the presence of alkaloids.

Ammonium reineckate

Add 0.2 g hydroxylamine to a saturated solution of 4% ammonium reineckate, and acidify with dilute HCI. A pink precipitate will appear if alkaloids are present. The precipitate is soluble in 50% acetone, which may also be used to recrystallize it.

7.3.2 Tests for Tannins

(A) Ferric chloride test

Mix the extract with 1% ferric chloride solution; a blue, green, or brownish green color indicates the presence of tannins.

(B) Gelatin test

Treat the extracts with a 1% solution of gelatin containing 10% sodium chloride. If white precipitate appears, that indicates the presence of tannins.

(C) Goldbeater's skin test

Soak a small piece of Goldbeater's skin in 2% hydrochloric acid, rinse with distilled water, and place in the solution to be tested for 5 min. Wash with distilled water and transfer to a 1% solution of ferrous sulfate. A brown or black color on the skin indicates the presence of tannins. Goldbeater's skin is a membrane prepared from the intestine of the ox and behaves similarly to an untanned hide.

(D) Phenazone test

Take about 5 mL of an aqueous plant extract, then add 0.5 g of sodium acid phosphate; warm, cool, and filter. To the filtrate add a 2% solution of phenazone. All tannins are precipitated, the precipitate being bulky and often colored.

(E) Vanillin-hydrochloric acid test

Take plant extract and mix with a mixture of vanillin:alcohol:dilute HCl in the ratio 1:10:10. The reaction produces phloroglucinol, which along with vanillin gives a pink or red color.

7.3.3 Test for Glycosides

A single extract cannot be made to test for all types of glycosides. Glycosides vary widely in their chemical type, particularly in the nature of the aglycone. Their solubility properties correspondingly exhibit a wide range, and it is therefore necessary to use different extraction methods to test for different types of glycosides. The tests for the detection of the presence of glycosides in crude drugs are discussed below.

Detection of glycosides

For detection of glycosides, take about 100 mg of extract and hydrolyze with concentrated hydrochloric acid for 2 h on a water bath, filter it, then subject the hydrolysate to the following tests.

(i) Borntrager's test

Take 2 mL of hydrolysate filtrate, add 3 mL of ethyl acetate and shake well. The ethyl acetate layer will separate, then add 10% ammonia solution. The formation of a pink color indicates the presence of anthraquinone glycosides.

(ii) Legal's test

Take about 20 mg of the extract dissolved in pyridine. Then add sodium nitroprusside solution and make alkaline by using 10% sodium hydroxide solution. The presence of glycoside is indicated by a characteristic pink color.

(iii) Using NaOH reagent

Take a small amount of alcoholic extract and mix with 1 mL of water and add sodium hydroxide solution. A yellow color indicates the presence of glycosides.

(iv) Using Fehling's reagent

Hydrolyze the sample with 2 N HCl and conduct Fehling's test. Glycosides before hydrolysis show a weak reaction with Fehling's reagent and a strong reaction after hydrolysis.

7.3.3.1 Tests for Anthraquinone Glycosides

Borntrager's test

Take 1 g of plant extract and add 5–10 mL of dilute HCl, then boil on a water bath for 10 min and filter. Extract the filtrate with CCl_4/benzene and add an equal amount of ammonia solution to the filtrate and shake well. The formation of pink or red color in the ammoniacal layer indicates the presence of anthraquinone glycosides.

Modified Borntrager's test

Take 1 g of drug and add 5 mL dilute HCl followed by 5 mL ferric chloride (5%, w/v). Boil for 10 min on a water bath, then cool and filter, extract with carbon tetrachloride or benzene and add an equal volume of ammonia solution. The formation of a pink to red color indicates the presence of anthraquinone glycosides (Thompson, 1957).

7.3.3.2 Tests for Steroid Glycosides

Liebermann–Burchard test

Take alcoholic extract and extract with $CHCl_3$, then add a few drops of acetic anhydride followed by conc. H_2SO_4 from the side wall of a test tube to the $CHCl_3$ extract. The formation of a violet to blue-colored ring at the junction of the two liquids indicates the presence of steroid glycosides.

Salkowski test

Take alcoholic extract and extract with $CHCl_3$, add conc. H_2SO_s from the sidewall of a test tube to the $CHCl_3$ extract. The formation of a yellow-colored ring at the junction of two liquids, which turns red after 2 min, indicates the presence of steroid moiety.

Antimony trichloride test

Take alcoholic extract and extract with $CHCl_3$, add a saturated solution of $SbCl_3$ in $CHCl_3$ containing 20% acetic anhydride. The formation of a pink color on heating indicates the presence of steroids and triterpenoids.

Trichloro acetic acid test

Take alcoholic extract and mix with a saturated solution of trichloro acetic acid, which forms a colored precipitate.

7.3.3.3 Tests for Cardiac Glycosides

(i) Keller–Kiliani test

Take alcoholic extract and an equal volume of water and add 0.5 mL of strong lead acetate solution, shake, and filter. Extract the filtrate with an equal volume of chloroform. The chloroform extract is then evaporated to dryness and the residue dissolved in 3 mL of glacial acetic acid followed by the addition of a few drops of $FeCl_3$ solution. The resulting solution is transferred to a test tube containing 2 mL of conc. H_2SO_4. A reddish-brown layer is formed, which turns bluish green after standing due to the presence of digitoxose.

(ii) Legal test

Take the alcoholic extract and an equal volume of water and add 0.5 mL of strong lead acetate solution, shake, and filter. Extract the filtrate with an equal volume of chloroform and dry the chloroform extract. Dissolve the residue in 2 mL of pyridine and sodium nitroprusside, followed by the addition of NaOH solution to make alkaline. The formation of a pink color indicates the presence of glycosides or aglycon moiety.

(iii) Baljet test

The thick section of a leaf of digitalis or the part of the drug containing cardiac glycoside, when dipped in sodium picrate solution, forms a yellow to orange color in the presence of aglycones or glycosides.

(iv) 3,5-Dinitro benzoic acid test

To the alcoholic solution of the drug add a few drops of NaOH solution followed by a 2% solution of 3,5-dinitro benzoic acid. The formation of a pink color indicates the presence of cardiac glycosides.

7.3.3.4 Tests for Coumarin Glycosides

Ferric chloride test

Take a concentrated alcoholic extract of the drug, then add a few drops of alcoholic $FeCl_3$ solution. The formation of a deep green color, which turns to yellow on addition of conc. HNO_3, indicates the presence of coumarins.

Fluorescence test

Take an alcoholic extract of the drug and mix with 1 N NaOH solution (1 mL each). The development of a blue-green fluorescence indicates the presence of coumarins.

7.3.3.5 Tests for Cynophoric Glycoside

Sodium picrate test

Moisten the powdered drug with water in a conical flask and add a few drops of conc. sulfuric acid. Using the cork of the flask, fasten a piece of filter paper impregnated with sodium picrate solution followed by sodium carbonate solution. A brick red color, due to volatile HCN in the presence of cynophoric glycosides, then forms.

7.3.3.6 Tests for Flavonoid Glycoside

Ammonia test

A filter paper is dipped in an alcoholic solution of the plant sample material and exposed to ammonia vapor. The formation of a yellow spot on the filter paper indicates the presence of flavonoid glycosides.

Shinoda test

Take an alcoholic extract of the drug and add zinc powder and dilute HCl. The formation of a deep red to magenta color indicates the presence of dihydro flavonoids.

Vanillin HCl test

Take an alcoholic extract of the drug and add vanillin HCl solution. The formation of a pink color indicates the presence of flavonoids.

7.3.4 Test for Flavonoids

Many reagents have been described for the identification of flavonoids as discussed below, although they may give a false-positive reaction with other polyphenols.

(i) *Shinoda test*: Take an alcoholic extract solution of the sample, add magnesium powder and a few drops of concentrated HCl; orange, pink, and red to purple colors will appear when flavones, flavonols, the corresponding 2,3-dihydro derivatives, and/or xanthones are present. It is advisable to add *t*-butyl alcohol before adding the acid to avoid accidents from a violent reaction; the colored compounds will dissolve into the upper phase. By using zinc instead of magnesium, only flavanonols give a deep red to magenta color; flavanones and flavonols will give weak pink to magenta colors or no color at all.

(ii) *Sulfuric acid*: Flavones and flavonols dissolve into concentrated sulfuric acid to give a deep yellow solution. Chalcones and aurones produce a red or red-bluish solution. Flavanones give orange to red colors.

(iii) *Lead acetate test*: Add a few drops of lead acetate solution (10%) to the alcoholic solution; yellow precipitate is observed.

(iv) *Zinc-hydrochloric acid reduction test*: Take an alcoholic solution of the sample and treat it with a pinch of zinc dust and a few drops of concentrated hydrochloric acid; a magenta color is produced after a few minutes, which indicates the presence of flavonoids.

(v) *Ferric chloride test*: Add a few drops of neutral ferric chloride solution to a small quantity of alcoholic extract. A blackish green color is produced, which indicates the phenolic nucleus (Harborne, 1964).

7.3.5 Tests for Phytosterols

(i) *Liebermann–Burchard test*: Take 1 mL anhydrous acetic acid and 1 mL chloroform and cool. Add one drop of concentrated sulfuric acid. When the sample is added, either in the solid form or in solution in chloroform, blue, green, red, or orange colors that change with time will indicate the presence of phytosterols.

(ii) *Salkowski test*: Dissolve 1–2 mg of the sample in 1 mL chloroform and add 1 mL concentrated sulfuric acid, forming two phases. A red or yellow color indicates the presence of sterols and methylated sterols.

7.3.6 Tests for Triterpenes

(i) *Tschugajeu test*: An excess of acetyl chloride and a pinch of zinc chloride are added to a chloroform solution of the sample material, kept aside for the reaction to subside and warmed on a water bath; eosin red color is produced.

(ii) *Briekorn and Brinar test*: Take a chloroform solution and add a few drops of chlorosulfonic acid and glacial acetic acid (7:3). A red color is produced, which indicates the presence of triterpenes.

7.3.7 Test for Saponins

(i) *Foam test*: Because of their surface-active properties, they may be recognized by shaking an aqueous solution of the sample and observing the production of foam, which is stable for approximately 15 min.

(ii) *Hemolysis test*: Saponins can be also identified by their ability to hemolyze red blood cells. Take 2 mL of 1.8% sodium chloride solution in two test tubes; 2 mL distilled water is added to 1 and 2 mL of 1% extract to the other. Five drops of

blood are added to each tube and gently mixed with the contents. Hemolysis is then observed under the microscope in the tube containing the extract, which indicates the presence of saponin.

(iii) *Test for steroidal saponins*: The extract is hydrolyzed with sulfuric acid and extracted with chloroform. The chloroform layer is tested for steroids.

(iv) *Test for triterpenoidal saponins*: The extract is hydrolyzed with sulfuric acid and extracted with chloroform and this is tested for triterpenoids.

7.3.8 Other Polyphenols

Ferric chloride test: Take a small amount of extract solution and add a few drops of neutral ferric chloride (5%) dissolved in water or ethanol. A blackish green color is produced, indicating the presence of polyphenols.

7.3.9 Tests for Resins

(i) On trituration with water, resins produce a milky emulsion.

(ii) Take the sample material (0.5 g) and boil with hydrochloric acid (5 mL) for some time. Filter it and add ammonia to the filtrate. A blue fluorescence is obtained, which indicates the presence of resins.

(iii) To a fractured surface, add 50% nitric acid. A green color is produced.

(iv) To a fractured surface of the drug, add sulfuric acid (1 drop). A red color is obtained, which changes to violet on washing with water.

(v) Dissolve a small quantity of chloroform or ethanolic extract of the drug in 5–10 mL of acetic anhydride by means of gentle heat, then cool, and add 0.005 mL of sulfuric acid. A bright purplish red color, rapidly changing to violet, is produced, which indicates the presence of resins.

7.3.10 Tests for Fixed Oils and Fats

(i) *Iodine value*: The iodine value is the mass of iodine in grams that is consumed by 100 g of fats or oil. An iodine solution is violet in color and any chemical group in the substance that reacts with iodine will make the color disappear at a particular concentration. The amount of iodine solution required to keep the solution violet is a measure of the number of iodine-sensitive reactive groups. It is a measure of the amount of unsaturation and, the higher the iodine value, the greater is the chance for rancidity.

(ii) *Saponification value*: The saponification value is the number of milligrams of potassium hydroxide required to saponify 1 g of fat. It is a measure of the average molecular weight of all the fatty acids present.

(iii) *Hydroxyl value*: The hydroxyl value is the number of milligram of potassium hydroxide (KOH) required to neutralize acetic acid combined with hydroxyl groups, when 1 g of a sample is acetylated.

(iv) *Ester value*: The ester value is the number of milligram of potassium hydroxide (KOH) required to saponify the ester contained in 1 g of a sample.

(v) *Unsaponifiable matter*: The principle is the saponification of the fat or oil by boiling under reflux with an ethanolic potassium hydroxide solution. Unsaponifiable matter is then extracted from the soap solution by diethyl ether. The solvent is evaporated and then the residue is dried and weighed.

(vi) *Acid value*: It is the amount of free acid present in fat as measured by the number of milligrams of potassium hydroxide needed to neutralize it. As the glycerides in fat slowly decompose, the acid value increases.

(vii) *Peroxide value*: One of the most widely used tests for oxidative rancidity, the peroxide value is a measure of the concentration of peroxides and hydroperoxides formed in the initial stages of lipid oxidation. Milliequivalents of peroxide per kilogram of fat are measured by titration with iodide ions. Peroxide values are not static and care must be taken in handling and testing samples. It is difficult to provide specific guidelines relating peroxide value to rancidity. High peroxide values are a definite indication of a rancid fat, but moderate values may be the result of a depletion of peroxides after reaching high concentrations.

7.3.11 Tests for Carbohydrates

7.3.11.1 Reagents

(a) *Iodine solution*: Add a few crystals of iodine to a 2% potassium iodide solution until the color becomes deep yellow.

(b) *Fehling's reagent A*: Dissolve 34.65 g copper sulfate in distilled water and make up to 500 mL.

(c) *Fehling's reagent B*: Dissolve 125 g potassium hydroxide and 173 g Rochelle salt (potassium sodium tartrate) in distilled water and make up to 500 mL.
(d) *Benedict's qualitative reagent*: Dissolve 173 g sodium citrate and 100 g sodium carbonate in 800 mL water. Heat to dissolve the salts and filter if required. Dissolve 17.3 g copper sulfate in about 100 mL water, add it to the above solution with stirring, and make up the volume to 1000 mL with water.
(e) *Barfoed's reagent*: Dissolve 24 g copper acetate in 450 mL boiling water. Immediately add 25 mL of 8.5% lactic acid to the hot solution. Mix well, cool, and dilute to 500 mL.
(f) *Seliwanoff's reagent*: Dissolve 0.05 g resorcinol in 100 mL of dilute (1:2) hydrochloric acid.
(g) *Bial's reagent*: Dissolve 1.5 g orcinol in 500 mL of concentrated HCl and add 20–30 drops of 10% ferric chloride.

7.3.11.2 Identification of Carbohydrates

The following phytochemical tests can be useful for the identification of carbohydrates in samples:

(i) *Molisch reagent*: Solution I—1% α-naphthol in 80% ethanol; solution II—concentrated H_2SO_4. Add 2–3 drops of solution I to a sample solution and acid, without mixing, to form an upper phase. A purple ring will appear at the interface as a result of the reaction between α-naphthol and furfural and 5-hydroxymethyl furfural aldehydes produced by dehydration of saccharides. A red color will appear if α-naphthol is replaced by 5% thymol. This reagent may be used with crude aqueous extracts, as well as pure compounds.
(ii) *Aniline acetate reaction*: Take the solid sample and heat it over a flame. Allow the vapors to react with aniline acetate impregnated on a filter paper placed over the vapor. A red color will appear in the presence of heterocyclic aldehydes produced from carbohydrate dehydration.

7.3.11.3 Some Distinctive Qualitative Chemical Analyses of Monosaccharides

Different carbohydrates, mostly monosaccharides, can be distinguished by the following tests:

- While considering the chemical nature for characterization, all carbohydrates give a positive result with Molisch's test and give a purplish color if treated with alcoholic α-naphthol in the presence of sulfuric acid.
- All the carbohydrates reduce Fehling's solution.
- When warmed with an alkali solution, they caramelize to give a yellowish brown solution.
- They all react with phenyl hydrazine to give osazones having a characteristic shape.
- Glucose and fructose can be distinguished from each other by Pinoff's test and Seliwanoff's test as follows:
  - In Pinoff's test, the fructose gives a red color after boiling for a very short time.
  - In Seliwanoff's test, the fructose gives a red color after boiling for a few minutes, whereas with glucose the color takes much longer to develop.
- The naturally occurring pentoses have an aldose structure and common pentoses include ribose, arabinose, and xylose. They can be detected in the following ways:
  - When treated with concentrated hydrochloric acid, they are converted to furfuraldehyde, which reacts with aniline to give a red color.
  - When treated with a phloroglucinol solution, they produce a red color.
- A number of delicate color reactions have been developed for use in paper chromatography of sugars, which can be utilized for distinguishing hexoses, pentoses, and methyl pentoses as follows:
  - When treated with aniline hydrogen oxalate, aldoses are colored brown, pentoses red, methyl pentoses brown, and ketoses are not colored at all.
  - When treated with naphthol resorcinols, hexoses are colored gray, pentoses blue, and methyl pentoses green.

7.3.11.4 Some Distinctive Qualitative Chemical Analyses of Disaccharides

The disaccharides are the condensation products of two monosaccharides, with the commonest being formed from two hexose units. The reducing disaccharides of pharmaceutical importance are maltose, cellobiose, lactose, gentiobiose, and rutinose. The commonest nonreducing disaccharide is sucrose. They have the following distinctive features.

- They do not reduce Fehling's solution.
- They give a yellowish brown color with alkali.
- They do not form osazones.

- Disaccharides can be hydrolyzed to the constituent hexoses by warming with dilute acid (sucrose ⟶ glucose + fructose); this will then reduce Fehling's solution, if made alkaline.
- Some drugs contain free sugars that can be extracted with water. Both licorice and gentian contain small quantities of reducing sugar, which can provide a test for the reducing sugar.
- Digitalis leaves contain reducing sugars, released from glycosides during drying and storage. An aqueous extract must be decolorized before the test can be applied.

7.3.11.5 Some Distinctive Qualitative Chemical Analyses of Polysaccharides

Polysaccharides are substances formed from four or more monosaccharides, although a majority of them have a large number of constituent units. Those formed from hexoses are known as hexosans and those from pentoses are known as pentosans.

Starch

Starch is the most abundant polysaccharide and occurs in most organs of the higher plants as well-defined granules with characteristic features, such as shape, size, hilum, and striations, which are useful aids for the identification of the drugs themselves. The commercial starches useful in pharmaceutical preparations are:

- Potato starch from the tuber of *Solanum tuberosum*.
- Wheat starch from the seeds of *Triticum aestivum*.
- Maize starch from the seeds of *Zea mays*.
- Rice starch from the seeds of *Oryza sativa*.
- Maranta arrowroot from the rhizomes of *Maranta arundinacea*.

Chemically, starch consists of amylose and amylopectin in varying proportions, based on the nature of the starch itself, while both are polysaccharides made up of a large number of D-glucose molecules joined through alpha-type linkages. Both amylose and amylopectins are insoluble in water, but in hot water amylose dissolves to form a nonviscous solution, while amylopectin is insoluble but swells to form a viscous gel. In hot water, starch granules swell up, allowing the water to penetrate until it is able to reach the hilum zone. Here the amylose that is present goes into solution, exerting considerable pressure on the rest of the granules, which have already changed in character because of the effect of the water, causing them to break up. Thus, the mucilage of starch is a colloidal gel of amylopectin dispersed in a colloidal solution of amylose. Besides this, the other distinguishing characters of starch are:

- Starch reacts with iodine at cold temperatures to give a blue starch iodide complex. The color disappears on warming to a temperature to 95°C and reappears when the mucilage is cooled again.
- As starch (amylose and amylopectin) contains no free reducing groups, it does not reduce Fehling's solution.
- Starch can be hydrolyzed to the constituent sugar by treatment with dilute acids, which will reduce Fehling's solution.
- By controlled hydrolysis of starch with dilute acids, it is possible to attain a series of hydrolytic products that differ in their behavior in water and their reaction with iodine solution and Fehling's solution.

7.3.12 Test for the Presence of Carotenoids

Five hundred milligram of the sample should be warmed with 30 mL of chloroform in a test tube. It should be filtered and divided into two equal portions and evaporated to dryness in evaporating dishes. Each extract should be reconstituted in 0.5 mL of chloroform. To the first sample, 0.5 mL concentrated sulfuric acid should be added. A deep blue to indigo color should be formed, which indicates the possible presence of carotenoids. To the second sample, 0.5 mL of a 2% (w/v) solution of antimony trichloride should be added in chloroform. A permanent or transient blue color should be formed, which indicates the possible presence of carotenoids (Mukherjee, 2002).

7.4 ALKALOIDS AND THEIR ANALYSIS

The alkaloids are one type of secondary metabolite found in different parts of the plants of different families. Alkaloids generally have at least one nitrogen atom in their structure, which is usually found to be part of a heterocyclic ring, though there remain a few exceptions to this rule. The protoalkaloids, such as ephedrine or mescaline, have nitrogen as part of the side chain, that is, they are alkaloidal amines. The alkaloids are generally crystalline solids. A few alkaloids, which do not contain oxygen in their structure, are volatile liquid in nature, such as nicotine, coniine, and sparteine. There are some nonvolatile liquid alkaloids also, such as pilocarpine, which also contains an oxygen atom as part of a furan ring in its structure.

Invariably, the alkaloids contain an N-atom in the tertiary-amine form (R_3N), for example, morphine and reserpine; less frequently in the secondary-amine form (R_2NH), for example, ephedrine; and very rarely in the primary-amine form (RNH_2), for example, norpseudoephedrine. Furthermore, whenever an N-atom occurs in either the tertiary or secondary form, it essentially constitutes an integral part of the ring system, namely, the heterocyclic ring system.

In most of the alkaloids containing a tertiary nitrogen atom in their structure, there remains a methyl substituent on the nitrogen atom, such as morphine, cocaine, colchicine, dextromethorphan, codeine, physostigmine, vinblastine, and vindesine. Other than the tertiary nitrogen atoms, the alkaloids may also contain a quaternary nitrogen atom, such as in D-tubocurarine. They possess properties a little bit different from the true alkaloids, such as the absence of H– in the N-atom.

Alkaloids, generally, are basic in nature, by virtue of the basic nitrogen atom present in the ring. Hence, they are prone to the formation of their respective salts with various acids. The degree of basicity of the alkaloids mostly depends upon the prevailing influence caused by the electrostatic status of the N-atom present in the alkaloid molecule, for instance, the number of N-atoms present in the alkaloid, whether the N-atom is located in the ring or in the side chain, and the presence of an alkyl group (e.g., methyl) to the N-atom. Another vital factor, which establishes the degree of basicity of an alkaloid, is the presence of a 1, 2, 3, or 4 degree N-atom, or atoms, in it. In fact, such apparent differences in the degree of basicity arising from the various structural features are eventually reflected in the different dissociation constant values (pK_a values) of various alkaloids. The alkaloidal bases may be water soluble or the alkaloidal salts may be soluble in organic solvents. There are also some remarkable differences in the solubilities between different salts of a particular alkaloid, such as:

- *Alkaloidal base-water soluble*: Caffeine, ephedrine, colchicine, pilocarpine, berberine, pilocarpine.
- *Alkaloidal salts-organic solvent soluble*: Lobeline hydrochloride and apoatropine hydrochloride.
- *Alkaloidal bases lacking solubility in organic solvents*: Narceine, pilocarpine (insoluble), and morphine (sparingly soluble, solubility in ether 1:5000).
- *Varying solubilities of different salts of a particular alkaloid*: Quinine hydrochloride is slightly soluble in water (1:1000), but quinine sulfate is highly soluble in water (1:1).

The solubility of the alkaloids is an important parameter from the perspective of extraction, separation, isolation, and characterization by chromatographic and spectroscopic techniques. If the difference in the solubilities of the alkaloid in two solvents is appreciably high, then we may use it for fractional crystallization of the alkaloid. The order of the solubility of most of the alkaloids is: chloroform > acetone > ethanol > methanol > ethyl acetate > ether > *n*-hexane.

All alkaloids contain a minimum of one nitrogen atom and, in a majority of cases, the compound is basic. This suggests that salt formation can occur in the presence of acid. This elementary property of alkaloids is employed in their extraction and further clean-up. Two strategies may be used for alkaloid extraction. One of them is to use diethylamine or ammonia to basify the plant material. The alkaline medium ensures that the alkaloids are in their free base or unionized state. Most alkaloidal bases, being of medium polarity are usually separated using chloroform, methylene chloride, or diethylether, or with a general solvent, such as ethanol. The second method involves treating the material with aqueous acid. The alkaloids form salts that are ionized in this situation and are therefore soluble in aqueous media. By basifying the aqueous extract (which deprotonates the alkaloid), the alkaloid can be recovered in free base form, followed by extraction into a suitable organic solvent (Houghton and Raman, 1998). Knowledge of the solubility of alkaloids and their salts is of considerable pharmaceutical importance. Not only are alkaloidal substances often administered in solution, but the difference in solubility between alkaloids and their salts also provides methods for the isolation of alkaloids from the plant and their separation from the nonalkaloidal substances present. While the solubilities of different alkaloids and salts show considerable variation, as might be expected from their extremely varied structure, it is true to say that free bases are frequently sparingly soluble in water but soluble in organic solvents. With salts, the reverse is often the case, they being usually soluble in water but sparingly soluble in organic solvents. For example, in water, strychnine hydrochloride is much more soluble than the strychnine base.

The isolation procedure of alkaloids is depicted schematically in Fig. 7.2. The alkaloids generally exist in the plant body as salts of weak organic acids, such as oxalic acid or tannic acid. Alkaloids, existing in the crude drug as salts of weak acids, should be liberated from the extract/fraction only by the use of mild alkalis instead of strong alkalis, such as NaOH or KOH. This helps to avoid hydrolysis (cocaine, hyoscyamine), solubilization (phenolic alkaloids—morphine, codeine, cephaline, etc.), or emulsification of fatty substances (a disturbing problem) during isolation. Ammonium hydroxide is used for isolation.

The free alkaloid base may be extracted with organic solvents—water miscible (alcohols) or water immiscible (chloroform, ether, or benzene). Alternatively, the crude drug can be directly extracted with acidified water, to convert the alkaloidal salts in hydrochloride or, preferably sulfate salts. They are then basified to the free bases by ammonium hydroxide and thereafter extracted with organic solvent, preferably chloroform. In case of tannate salts of alkaloids existing in the

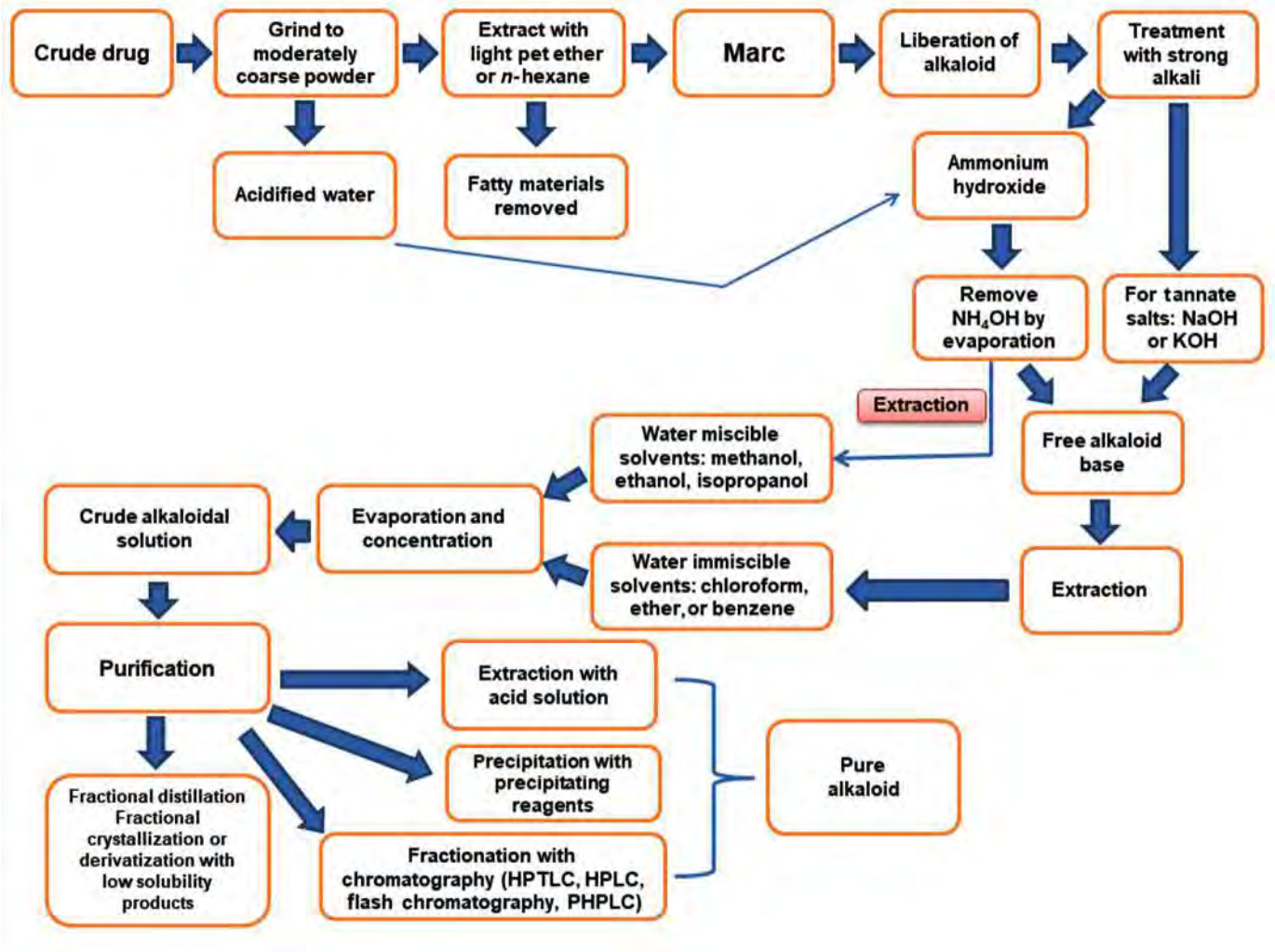


FIG. 7.2 Isolation and purification of alkaloids from crude plant material.

crude drug, instead of ammonium hydroxide, the crude drug is treated with sodium or potassium hydroxide to liberate the alkaloid. If liberation is not possible, then the drug is first treated with HCl or H_2SO_4 to convert the salts to hydrochloride or hydrobromide salts and then treated with sodium or potassium hydroxide to liberate the bases. Because of the high polarity and water solubility of the *N*-oxides of alkaloids, they are discarded by the normal alkaloid extraction procedures. Before performing these treatments, first the nonalkaloidal fatty portions of the herb are removed by extraction with petroleum ether or *n*-hexane in a Soxhlet apparatus. After that, again, for confirmation of the absence of alkaloids in the pet ether extract, we have to perform qualitative tests for alkaloids in an aliquot of the pet ether extract, acidified with a dilute mineral acid.

Extraction of the alkaloids from the crude drug may be carried out by one of the three processes of Soxhlet extraction, the Stas-Otto process, or the Kippenburger process. The Soxhlet extraction process involves soaking the crude powdered drug with dilute acid, packing it in a thimble and extracting with organic solvents. The extract is then concentrated to get an aqueous residue. In the Stas-Otto process, tartaric acid is used to moisten the drug prior to hydroalcoholic extraction. The concentrated extract is washed with pet ether to remove fatty matter. Then, the pet ether extract is again washed with water, to remove any dissolved alkaloid. The two aqueous extracts are combined thereafter. They are finally extracted with alcohol to get the alkaloid. In the Kippenburger process, the crude drug is first moistened with tannin and glycerol. Then, it is heated at 50°C to coagulate the proteins. This material is extracted with pet ether and the residual pet ether is removed by heating with an infrared lamp. The fat-free crude plant extract is subsequently acidified and shaken with chloroform, successively to remove the bulk of the alkaloids, namely, atropine, codeine, colchicine, narcotine, nicotine, papaverine, spartenine, and thebaine. In the case of opium alkaloids, passing CO_2 though the aqueous extract helps in the isolation of narceine and morphine.

7.4.1 Importance of Alkaloids in Phytotherapy

Alkaloids constitute a major class of the chemical groups present in plant drugs. Originally, alkaloid meant "alkali like" and was applied indiscriminately to all organic bases. Alkaloids may be described as naturally occurring organic substances, having a cyclic nitrogenous nucleus exhibiting basic properties and having a pronounced physiological action.

Among the other alkaloids present in a plant, several have been found to be antimicrobially active, the most active being vindoline and apparicine. Recently it was found that strictosidine in combination with strictosidine glucosidase has activity against some fungi and Gram-positive bacteria. As this alkaloid is found to be stored at high levels in the vacuoles of cells in young tissues, which also contain a high level of the specific glucosidase, it was postulated that this alkaloid plays a role as a phytoanticipin in plant defense. Several antimicrobially active alkaloids have been isolated from plants of the genus *Strychnos* (Loganiaceae). Dimeric tertiary toxiferine-type alkaloids were identified as the active compounds in some African chewing sticks. These alkaloids have activity against both Gram-positive and Gram-negative bacteria and, in particular, against some *Streptococcus* species connected with caries. *N*-oxides of these alkaloids, also found in the plant, were less active. The antidiarrheal activity of bisnordihydrotoxifeine has been reported and the activity seems to be related to an antagonistic effect on the stimulant activity of endogenous compounds on the gastrointestinal smooth muscle.

The harmane-type alkaloids are known to be phototoxic to bacteria and insects. Similarly, brevicolline, an *N*-methylpyrrolidine substituted harmane derivative, is phototoxic for microorganisms. The mechanism of the phototoxicity of *E. coli* has been studied. Harmalol was found to be active in this test system, whereas harmaline, harmine, harmane, and nonharmane were inactive. Although oxygen was required for the phototoxicity, no clear correlation was found between photoproduction of singlet oxygen or hydrogen peroxide by the alkaloids in the cells and phototoxicity. Strong antimicrobial activity was found for cathinone and derivatives and the activity of these alkaloids is enhanced by light. Yuechukene, from a *Murraya* species (Rutaceae), is an antimicrobially active dimeric prenylindole alkaloid. Murrayanine, a carbazole alkaloid having an indole nucleus, was found to exhibit antimicrobial activity. Clavine alkaloids, which are produced by some fungi as well as some plants of the Convolvulaceae, and a series of semisynthetic derivatives, were shown to have activity against various human pathogenic bacteria and *Candida albicans*. The polypyrroloindolinic alkaloid quadrigemine B isolated from *Psychotria* species has both cytotoxic and bactericidal activities. The isoquinoline alkaloid group is second in size to the indole alkaloids. Of the various classes of isoquinoline alkaloids, several include compounds with strong antimicrobial activity. One of the few alkaloids that have been used as an antibiotic is cepharanthine, which was employed in Japan as a prophylactic drug against tuberculosis during World War II. It was also used against leprosy. Several thalictrum alkaloids were reported to have activity against Gram-positive bacteria, in particular *Mycobacterium smegmatis*. Several aporphine alkaloids having an oxoaporphine skeleton have potential therapeutic activity. Liriodenine and dehydroglaucine are active against a broad spectrum of organisms, with MIC values similar to those of known antibiotics, such as streptomycin. Their methiodides have similar, but weaker, activities, except against yeasts, against which they are more active.

Lysicamine, which has two *O*-methyl groups instead of the methylenedioxy group in liriodenine, was inactive against *C. albicans*. Conversion into the quaternary nitrogen derivative resulted in an active compound, though not as active as the liriodenine metho compound (Mukherjee, 2002).

The antimicrobial activity of chelidonine and sanguinarine has long been known, particularly their tuberculostatic properties. Sanguinarine was more active, although the tuberculostatic concentration was difficult to determine because of the instability of this alkaloid. These two alkaloids and chelerythrine have antifungal activity, and the inhibitory effect is antagonized by ergosterol. The antimicrobial activity of sanguinarine, chelerythrine, and their tertiary pseudoalcoholate derivatives against some Gram-positive and Gram-negative bacteria and *Candida* species has been reported. The pseudoalcoholates were more active than the corresponding quaternary alkaloids, probably because they are able to pass through cell membranes and act as pro drugs, which in the cells are converted into active quaternary alkaloids. Solanum alkaloids have been reported to have antifungal properties. Solacongestidine, verzine, and solafloridine were much more active than solasodine and tomatidine. These alkaloids were found to inhibit ergosterol and cholesterol biosynthesis from lanosterol. It has been reported that the fungitoxic activity of α-tomatine and some of its hydrolysis products was closely correlated with the ability to complex with sterols, and not with the surfactant activity.

7.4.2 Classification of Alkaloids

The classification of alkaloids may be done in different ways, such as biosynthetic classification, chemical classification, pharmacological classification, and taxonomic classification.

7.4.2.1 Chemical Classification

Chemically, the alkaloids are a diverse set of structures. According to the basic skeleton of the alkaloids, they may be

(i) True alkaloids.
(ii) Proto alkaloids (alkaloidal amines).
(iii) Pseudoalkaloids (diterpenes).

(i) *True alkaloids*: They are heterocyclic nitrogen-containing compounds. They are potent pharmacological agents. Generally, they are crystalline white or colorless solids, with a few exceptions, such as nicotine, sparteine, pilocarpine, and coniine. These alkaloids occur in a limited number of species and families and are those compounds in which decarboxylated amino acids are condensed with a nonnitrogenous structural moiety. The primary precursors of true alkaloids are such amino acids as L-ornithine, L-lysine, L-phenylalanine/L-tyrosine, L-tryptophan, and L-histidine. Examples of true alkaloids include biologically active alkaloids, such as cocaine, quinine, dopamine, morphine, and usambarensine. True alkaloids are able to form water-soluble salts with mineral as well as organic acids. They may occur in plants in a free state, as salts or as *N*-oxides (Aniszewski, 2007).

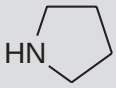
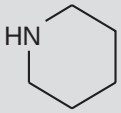
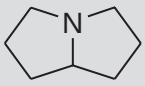
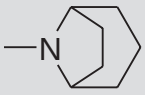
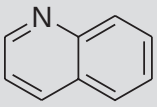
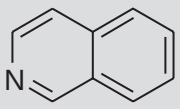
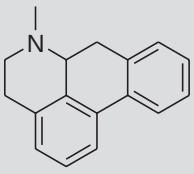
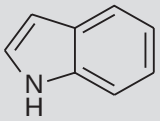
(ii) *Protoalkaloids*: They are derived from L-tyrosine and L-tryptophan and have nitrogen in their formula, which is not part of the heterocyclic ring. The nitrogen atom generally remains in the side chain. Examples include hordenine, mescaline, yohimbine, stachydrine, and 4-hydroxystachydrine. The last two alkaloids have a pyrroline nucleus and are basic alkaloids in the genus Boscia and Capparidacea family. *Boscia angustifolia* is used for the treatment of mental illness, and occasionally to combat pain and neuralgia.

(iii) *Pseudoalkaloids*: They are derived from nonamino acid precursors. The pseudoalkaloids result from amination or transamination reactions of the different pathways related to amino acids. Pseudoalkaloids can be derived from acetate and phenylalanine. They may be terpenoid or steroid in nature, in which the nitrogen atom has been inserted in a later stage. Examples of pseudoalkaloids include such compounds as coniine, capsaicin, ephedrine, solanidine, caffeine, theobromine, and pinidine (Table 7.1).

7.4.2.2 Distribution of Alkaloids in Plant Kingdom

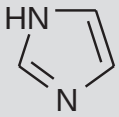
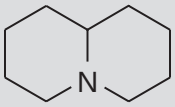
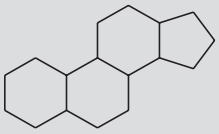
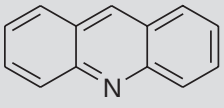
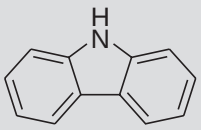
Distribution of the alkaloids in the plant kingdom and also, within a particular plant species, in different plant parts needs special attention. The latter topic is very interesting. Alkaloids get biosynthesized in a particular plant organ, and then get translocated to some other organ. Examples include the translocation of the alkaloid nicotine from the root to the leaves of the plant. Isoquinoline alkaloids are found predominantly in the latex of the opium plant, in contrast to the seeds. The quinoline alkaloids are found exclusively in the bark of the plant *Cinchona* sp. On the other hand, in case of *Colchicum*,

TABLE 7.1 Chemical Classes of Alkaloids

Name of the Class	Structure of the Basic Skeleton	Name of the Compound
Pyrrole and pyrrolidine alkaloids	 **Pyrrolidine**	Hygrine
Pyridine and piperidine	 **Pyridine**  **Piperidine**	Arecoline, anabasine, coniine, lobeline, pelletierine, trigonelline
Pyrrolizidine	 **Pyrrolizidine**	Echimidine, symphitine, senecionine, seneciphylline, necine, platiphylline, senecionine, heliotrine, senkirkine
Tropane	 **Tropane**	Cocaine, hyoscyamine, hyoscine, atropine
Quinoline	 **Quinoline**	Quinine, quinidine, cinchonine, cinchonidine, cupreine, camptothecin
Isoquinoline	 **Isoquinoline**	Morphine, codeine, thebaine, heroin, narcotine, papaverine, berberine, emetine, sanguinarine
Aporphine	 **Aporphine**	Boldine, glaucine, xanthoplanine, laurotetanine, laurifoline
Indole	 **Indole**	Ergot alkaloids, vinca alkaloids, physostigma alkaloids, strychnine, brucine

Continued

TABLE 7.1 Chemical Classes of Alkaloids—cont'd

Name of the Class	Structure of the Basic Skeleton	Name of the Compound
Imidazole	 **Imidazole**	Pilocarpine, isopilocarpine, pilosine
Quinolizidine	 **Quinolizidine**	Cytisine, luburnine, lupanine, sparteine
Steroidal alkaloids	 Steroid skeleton	Protoveratrine, solanidine, conessine, furtremine
Acridine and acridone alkaloids	 Acridine	Glycobismine-D, glycobismine-E, glycocitrine-III, acrifoline, arborinine, acronycine
Carbazole alkaloids	 **Carbazole**	Mahanine, mahanimbine

the alkaloid colchicine is found both in the seeds and the corm. The distribution of alkaloids in the plant kingdom is also of importance. Among the angiosperms, Apocynaceae, Papaveraceae, Ranunculaceae, Rubiaceae, Solanaceae, and Berberidaceae are the most important alkaloid-bearing plant families. Among the monocotyledons, the Amaryllidaceae and Liliaceae are the alkaloid-bearing plant families (Table 7.2). The yield of the alkaloids in the alkaloid-bearing plants varies, for example, nicotine—8% of the dry weight of tobacco leaves and morphine—14% of high-grade opium (Reddy et al., 2005).

The following is a brief account of some alkaloids found in different plant families:

- Chelidonine is found in *Chelidonium majus*.
- Lycopodine from *Lycopodium clavatum*.
- Intermedine from *Commiphora abyssinica*.

The *N*-oxides of the alkaloids have better pharmacokinetic and pharmacodynamic properties. This is possibly due to a belief that such compounds represented artifacts arising during the extraction and work up of tertiary alkaloids. Second, it may be attributed to either parallel or convergent evolution of certain complex biochemical pathways. Specific alkaloids are ordinarily confined to specific plant families (hyoscyamine in Solanaceae, colchicine in Liliaceae). Nicotine, which is found in a number of widely scattered plant families, is an exception to this rule. The occurrence of ergot alkaloids in the fungus *Claviceps purpurea* and certain *Ipomoea* species (Convolvulaceae) is also an exception (Mukherjee, 2002).

TABLE 7.2 Pharmacological Activities of Alkaloids in the Plant Kingdom

Family	Name of Alkaloid	Name of Plant Species	Pharmacological Activity
Agaricaceae	Bufotenin	*Amanita muscaria* (L.) Lam. (Mushroom)	Hallucinogen
	Muscarine	*A. muscaria* (L.) Lam. (Mushroom)	Acetylcholine-like
	Psilocybin Psilocin	*Psilocybe quebecensis* (Mushroom)	Hallucinogen
Amaryllidceae	Gallanthamine	*Galanthus nivalis* L. *Galanthus alexandri* Porcius *Galanthus imperati* Bertol. (Bulb) *Narcissus pseudonarcissus* L. (bulb)	Alzheimer disease
Apocynaceae	Alstonine	*Alstonia scholaris* (L.) R.Br. (Bark)	Antimalarial
	Aspidospermine	*Aspidosperma album* Mart. & Zucc. (Bark) *Aspidosperma araracanga* (Mart. & Zucc.) (Bark)	Respiratory stimulant
	Yohimbine	*Pausinystalia yohimbe* (K. Schum.) Pierre ex Beille (Bark)	Aphrodisiac
	Conessine	*Holarrhena antidysenterica* (bark)	Antidysenteric
	Ellipticine	*Ochrosia fatuhivensis* Fosberg & Sachet (bark)	Anticancer
	Akuammigine	*Picralima nitida* (Stapf) T. Durand & H. Durand *Picralima klaineana* Pierre (seeds)	Antimalarial
	Reserpine	*Rauvolfia serpentina* (L.) Benth. ex Kurz (Roots)	Tranquilizer
	Serpentine	*R. serpentina* (L.) Benth. ex Kurz (Roots)	
	Vincristine	*Catharanthus roseus* (L.) G.Don (Whole plant)	Anticancer
	Vinblastine	*C. roseus* (L.) G.Don (Whole plant)	Anticancer
Aristolochiaceae	Aristolochic acid	*Aristolochia eriantha* Mart. (Rhizome)	Tumor-inducing
Berberidaceae	Berberine	*Berberis aristata* (root bark)	Antibacterial
		Mahonia aquifolium (Bark)	Antimalarial
Boraginaceae	Indicine *N*-oxide	*Heliotropium arborescens* L. *Heliotropium corymbosum* Ruiz & Pav. *Heliotropium peruvianum* L. (leaves)	Anticancer
Cactaceae	Mescaline	*Lophophora williamsii* (Lem.) J.M.Coult. (leaves)	Hallucinogen
Celastraceae	Maytansine	*Maytenus nemorosa* Marais	Anticancer
Erythroxylaceae	Cocaine	*Erythroxylum coca* Lam. (leaves)	Local anesthetic
Fabaceae	Trigonelline, Choline	*Trigonella foenum-graecum* Seeds	Antihyperglycemic

Continued

TABLE 7.2 Pharmacological Activities of Alkaloids in the Plant Kingdom—cont'd

Family	Name of Alkaloid	Name of Plant Species	Pharmacological Activity
Palmaceae	Arecoline	*Areca catechu* L.	Anthelmintic
Solanaceae	Capsaicin	*Capsicum annuum* L. (fruits)	Used as counterirritant
	Hyoscine	*Atropa belladonna* L. (herb)	Travel sickness, amnesia
	Hyoscyamine	*Datura stramonium* L. (herb) *Hyoscyamus niger* L. (herb) *Duboisia myoporoides* R.Br. (herb) *Mandragora officinarum* L. (roots)	Antagonist of acetylcholine (Anticholinergic). Preoperative treatment.
	Tigloidine	*D. myoporoides* R.Br. (herb)	Parkinson disease
	Solanidine	*Solanum tuberosum* (tubers)	Toxic
	Solasodine	*S. tuberosum*, *Solanum khasianum*	
	Nicotine	*Nicotiana tabacum* L.	Insecticidal, smoking acquittal
	Solanine	*S. tuberosum* and *Lycopersicon esculentum* (Kakhia)	Highly toxic
	Tomatidine	*L. esculentum* Linn.	Precursor for many alkaloids
Sapindaceae	Caffeine	*Paullinia cupana Kunth*	CNS stimulant
Taxaceae	Paclitaxel	*Taxus brevifolia* Nutt.	Anticancer
	Baccatin	*T. brevifolia* Nutt.	Anticancer.
Theaceae	Caffeine	*Camellia sinensis* (L.) Kuntze (leaves)	CNS stimulant
	Theophylline	*C. sinensis* (L.) Kuntze (leaves)	Antiasthmatic

7.4.2.3 Biosynthetic Classification of Alkaloids

The chemical structure of alkaloids and the biosynthetic precursors of those alkaloids are closely related. So, classification based on the biosynthetic precursor is going to be quite useful. Anthranilic acid, histidine, lysine, ornithine, phenylalanine, and tryptophan are the principal amino acids from which most of the alkaloids are produced (Trease and Evans, 1983; Tyler, 1999). An account of the different biosynthetic-origin-based classifications of the alkaloids is shown in Table 7.3.

(i) Indole alkaloids derived from tryptophan.
(ii) Piperidine alkaloids derived from lysine.
(iii) Pyrrolidine alkaloids derived from ornithine.
(iv) Phenylethylamine alkaloids derived from tyrosine.
(v) Imidazole alkaloids derived from histidine.

The alkaloids are classified according to the nature of the basic nucleus present in the molecule. Various drugs containing crude alkaloids are listed in Table 7.4.

TABLE 7.3 Classification of the Alkaloids Based on Biosynthetic Origin

Amino Acid	Alkaloid	Family	Example Structure
Lysine	Quinolizidine alkaloids	Fabaceae	Lupanine, sparteine, cytosine
	Lycopodium alkaloids	Lycopodiaceae	Lycopodine
	Piperidine alkaloids	Punicaceae, Crassulaceae	Pelletierine, sedamine
Ornithine	Tropane alkaloids	Solanaceae, Erythroxylaceae	Hyoscyamine, cocaine
	Pyrrolizidine alkaloids	Asteraceae, Boraginaceae, Fabaceae	Senecionine, heliotrine
	Nicotiana alkaloids	Solanaceae	Nicotine, anabasine
Tryptophan	Monoterpene indole alkaloids	Apocynaceae	Strychnine, vincamine, yohimbine, ajmalicine, etc.
	Simple indole alkaloids	Fabaceae	Physostigmine
	Quinoline alkaloids	Rubiaceae, Cornaceae	Quinine, cinchonine, camptothecin
	Ergot alkaloids	Claviceps, Convolvulaceae	Ergotamine, lysergic acid
	β-Carboline alkaloids	Loganiaceae, Zygophyllaceae	Harman, harmaline
Phenylalanine/Tyrosine	Ephedra alkaloids	Ephedraceae	Ephedrine
	Tetrahydroisoquinoline alkaloids	Rubiaceae	Emetine
	Benzylisoquinoline alkaloids	Papaveraceae, Berberidaceae	Papaverine
	Benzophenanthridine alkaloids	Papaveraceae	Sanguinarine
	Protoberberine alkaloids	Berberidaceae, Papaveraceae	Berberine
	Morphinan alkaloids	Papaveraceae	Morphine, codeine
	Aporphine alkaloids	Monimiaceae	Boldine
	Phenylethylisoquinoline alkaloids	Colchicaceae	Colchicine
	Aristolochia alkaloids	Aristolochiaceae	Aristolochic acid
Anthranilic acid	Ruta alkaloids	Rutaceae	Skimmianine, dictamine

TABLE 7.4 Brief Account of the Alkaloid-Containing Medicinal Plants and Their Main Constituents

Class of Alkaloid	Drug	Botanical Source	Main Alkaloids
Pyridine derivatives	Areca nuts	*Areca catechu*	Arecoline, arecaine, guvacine
	Black pepper	*Piper nigrum*	Piperine (5%–8%)
	Fenugreek seeds	*Trigonella foenum-graecum*	Trigonelline, choline
	Hemlock fruit	*Conium maculatum*	Coniine (0.5%–1.5%), *N*-methyl coniine, conhydrine, pseudoconhydrine, and γ-coniceine
	Pomegranate bark	*Punica granatum*	Pelletierine, pseudopelletierine, *N*-methyl isopelletierine

Continued

TABLE 7.4 Brief Account of the Alkaloid-Containing Medicinal Plants and Their Main Constituents—cont'd

Class of Alkaloid	Drug	Botanical Source	Main Alkaloids
Tropane derivatives	Belladonna leave and root	*Atropa belladonna*	Hyoscyamine (0.1%–0.7%)
	Henbane leaves and seeds	*Hyoscyamus niger*	Hyoscyamine (0.05%–0.10%), thyoscine (traces)
	Stramonium leaves and seeds	*Datura stramonium*	Hyoscyamine (0.2%–0.5%)
	Datura leaves and seeds	*Datura fastuosa*, var. *alba* and *Datura metel*	Hyoscine (0.2%–0.5%)
	Coca leaves	*Erythroxylum coca*, *Erythroxylum truxillense*, and varieties	Cocaine, cinnamyl-cocaine, α- and β-truxillines, tropacocaine. Total alkaloids, 0.7%–2.5%
Quinoline derivatives	Red cinchona bark	*Cinchona succirubra*	Quinine, cinchonidine, cinchonine, quinidine. Total alkaloids, 5%–6%
	Nux vomica seeds	*Strychnos nux-vomica*	Strychnine, brucine. Total alkaloids, 2%–3%
	Ignatius beans	*Strychnos ignatii*	Strychnine, brucine. Total alkaloids, 2%–3%
Isoquinoline	Berberis stem	*Berberis aristata*	Berberine
	Blood root	*Sanguinaria canadensis*	Sanguinarine, chelerythrine, protopine
	Calumba root	*Jateorhiza columba*	Palmatine, jateorhizine
	Hydrastis rhizome	*Hydrastis canadensis*	Hydrastine (1.5%–3.0%), berberine (3%), canadine
	Opium	*Papaver somniferum*	Morphine (7%–16%), narcotine, codeine, thebaine, narceine papaverine
Glyoxaline derivatives	Jaborandi leaves	Species of *Pilocarpus*	Pilocarpine, isopilocarpine, pilosine (in *P. microphyllus*), pilocarpine (in *P. jaborandi*)
Purine derivatives	Cocoa seeds	*Theobroma cacao*	Theobromine (1.2%)
	Coffee seeds	*Coffea arabica*	Caffeine (1%–1.5%)
	Guarana	*Paullinia cupana*	Caffeine (2.5%–5.0%)
	Kola nuts	*Cola vera*	Caffeine (2.5%–3.5%)
	Tea leaves	*Camellia thea*	Caffeine (1.5%), theobromine (traces)
Alkaloids of unknown or doubtful constitution	Alstonia bark	*Alstonia scholaris* and *A. constricta*	Ditamine, echitenine, ditamine (in *A. scholaris*). Alstonine, porphyrine (in *A. constricta*)
	Aconite root	*Aconitum napellus*, *Cytisus scoparius*	Aconitine (0.3%–0.6%), picraconitine, aconine. Total alkaloids (0.5%–1.5%)
	Broom tops	*C. scoparius*	Sparteine
	Calabar beans	*Physostigma venenosum*	Physostigmine (eserine). Total alkaloids (0.1%–0.3%)
	Colchicum corn and seeds	*Colchicum autumnale*	Colchicine (0.2%–0.8%)
	Ergot	*Claviceps purpurea*	Gelsemine, gelsemimine, gelsemoidine
	Ipecacuanha root	*Psychotria ipecacuanha*	Emetine, cephaline, psychotrine. Total alkaloids, 2%–3%
	Lobelia herb	*Lobelia inflata*	Lobeline, lobelidine
	Pellitory root	*Anacyclus pyrethrum*	Pyrethrine (pellitorine)
	Stavesacre seeds	*Delphinium staphisagria*	Delphinine, delphisine, delphinoidine
	White hellebore rhizome	*Veratrum album*	Protoveratrine, jervine, rubijervine. Total alkaloids 0.5%–2.0%
	Yew leaves, shoots, and fruits	*Taxus baccata*	Taxine

7.4.3 Pyridine–Piperidine Alkaloids

On reduction, the tertiary base, pyridine, is converted into the secondary base, piperidine. These two nuclei form the basis of this group, which is sometimes divided into three subgroups:

- Derivatives of piperidine, including lobeline from lobelia.
- Derivatives of nicotinic acid, including arecoline from Areca.
- Derivatives of both pyridine and pyrrolidine, including nicotine from tobacco.

Alkaloids in this group include:

Conine—from Hemlock fruit
Pelletaerine—from Pomegranate root bark
Arecoline—from Areca seed
Lobelin and related alkaloids—from Lobelia
Ricinine—a toxic alkaloid from castor seed

7.4.3.1 Areca

Areca, areca nut, or betel nut is the dried, ripe seed of *Areca catechu* Linn. (Fam. Arecaceae). Areca is the Spanish and Portuguese term for the betel nut. Catechu is the East Indian name for an astringent extract or juice. Areca contains several alkaloids that are reduced pyridine derivatives, for example, arecoline (arecaidine methyl ester), arecaidine (*N*-methyl guvacine), guvacine (tetrahydronicotinic acid), and guvacoline (guvacine methyl ester). The total alkaloid content can reach 0.45%. Arecoline, the most abundant and physiologically most active alkaloid, is a liquid occurring to the extent of about 0.2%. Areca also contains tannin (about 15%), lipids, volatile oils, and gum. Areca is classified as an anthelmintic in veterinary practice and is employed as a vermicide and taeniafuge.

7.4.3.2 Lobelia

Lobelia or Indian tobacco consists of the dried leaves and tops of *Lobelia inflata* Linn. (Fam. Lobeliaceae). The drug contains 14 alkaloids, of which lobeline is the major and most important one. Lobeline, (−) lobeline, or alpha lobeline (to distinguish it from a mixture of the lobelia alkaloids formerly designated as lobeline) occurs as colorless crystals that are slightly soluble in water but readily soluble in hot alcohol. Lobeline produces similar, but weaker pharmacological effects than nicotine on the peripheral circulation, neuromuscular junctions, and the central nervous system. For this reason, lobeline sulfate was formerly incorporated in tablets or lozenges that were intended to aid in breaking the tobacco habit (smoking deterrents). The majority of controlled studies showed that lobeline had only a placebo effect on decreasing the physical craving for cigarettes, so these products were removed from the market.

7.4.3.3 Nicotine

Nicotine is a pyridine alkaloid obtained from the dried leaves of the tobacco plant *Nicotiana tabacum* (Fam. Solanaceae). The plant is a tall annual herb indigenous to tropical America, and the leaves contain from 0.6% to 9.0% nicotine and a lesser amount of nornicotine. Nicotine is a colorless to pale yellow, very hygroscopic, oily, volatile liquid with an unpleasant, pungent odor, and a sharp, burning, persistent taste. Nicotine is a ganglionic cholinergic receptor agonist with complex pharmacological actions that include effects mediated by binding to receptors in autonomic ganglia, the adrenal medulla, the neuromuscular junction, and the brain. Chronic use of nicotine may result in psychological and physical dependence. As a temporary aid for the cessation of cigarette smoking, the drug is available in transdermal systems, and it is also available bound to an ion-exchange resin in a chewing gum base. These alternative sources of nicotine help reduce the withdrawal symptoms associated with nicotine addiction (Baird, 1994).

7.4.4 Tropane Alkaloids

Tropane is a dicyclic compound formed by the condensation of a pyrrolidine precursor (ornithine) with three acetate-derived carbon atoms. Both pyrrolidine and piperidine ring systems can be discerned in the molecule. The 3-hydroxy derivative of tropane is known as tropine. Its esterification with (−) tropic acid yields hyoscyamine (tropine tropate), which may be racemized to form atropine. Some of the therapeutically important alkaloids of this group are described

in the next section. Two important groups of alkaloids belonging to this group are Solanaceous alkaloids and Cocoa alkaloids.

The Solanaceae drugs hyoscymus, stramonium, and belladonna herbs and roots contain hyoscyamine, which can be extracted quite easily as sulfate in aqueous solution. They also contain the related alkaloid hyoscine. This alkaloid and the optically inactive isomer atropine can be detected by the Vitali–Morin test.

Vitali–Morin test

The tropane alkaloid is treated with fuming nitric acid, followed by evaporation to dryness and addition of methanolic potassium hydroxide solution to an acetone solution of nitrated residue. Violet coloration takes place due to a tropane derivative. On addition of silver nitrate solution to a solution of hyoscine hydrobromide, yellowish-white precipitate is formed, which is insoluble in nitric acid, but soluble in dilute ammonia. These are based upon the ecgonine molecule in which the acid grouping is converted to a methyl derivative and the secondary alcoholic group is esterified.

7.4.4.1 Belladonna

Belladonna leaf, belladonna herb, or deadly nightshade leaf consists of the dried leaf and flowering or fruiting top of *Atropa belladonna* Linn. or of its variety *acuminata* (Fam. Solanaceae). Belladonna leaf yields not less than 0.35% of alkaloids. Atropa is from Atropos, meaning inflexible, the name of the Greek Fate who cuts the thread of life, and probably alludes to the poisonous character of the drug. Belladonna is from the Italian "bella," meaning beautiful, and "donna" meaning lady. The juice of the berry, when placed in the eyes, causes dilation of the pupils, thus giving a striking appearance. The plant is a perennial herb that grows to a meter in height. It is indigenous to central and southern Europe and to Asia. It is cultivated in sunny locations in England, Germany, India, and the United States. At present, the chief source of supply is the Balkans (BeMiller, 1992).

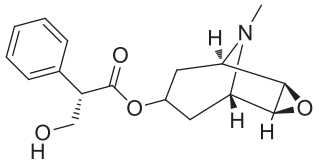
The poisonous character of the plant has been known for many years, particularly in its indigenous localities. It was the subject of many treatises during the 18th century. Its mydriatic properties were first recorded in 1802, but its analgesic properties were not recognized until 1860. The leaves were used earlier than the root, whose use did not occur until about 1860. The leaf yields alkaloids in concentrations ranging up to more than 1%. About three-fourths of the isolated alkaloid mixture is (−) hyoscyamine; the remainder is atropine. The latter compound exists, at most, only in traces in fresh plant material. Atropine is formed by racemization during the extraction process. Small but varying amounts of other bases are found in the root but not in the leaf. These include apoatropine, belladonnine, cuscohygrine, and scopolamine. The yield of alkaloids averages as follows: roots, 0.6%; stems, 0.05%; leaves, 0.4%; unripe berries, 1.19%; ripe berries, 0.21%; and seeds, 0.33%.

Belladonna acts as an antimuscarinic agent, which accounts for its use as a spasmolytic drug. It is used as adjunctive therapy in the treatment of peptic ulcers, functional digestive disorders, including spastic, mucus, and ulcerative colitis, diarrhea, diverticulitis, and pancreatitis. It possesses anticholinergic properties and is used to control excess motor activity of the gastrointestinal tract and spasm of the urinary tract.

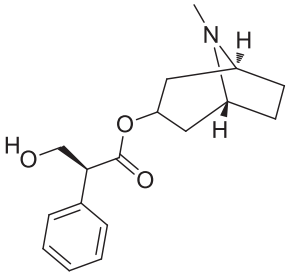
7.4.4.2 Solanaceous Alkaloids

The principal alkaloids of this group are (−) hyoscyamine, atropine [(±)-hyoscyamine], and scopolamine (also known as hyoscine). Atropine is an antidote in cases of poisoning caused by cholinesterase inhibitors, such as physostigmine and organophosphate insecticides. Scopolamine has a depressant activity on the central nervous system and is used to treat motion sickness. It is also employed for preanesthetic sedation and for obstetric amnesia in conjunction with analgesics, and to calm delirium. Toxicity symptoms that can occur during the therapeutic use of atropine, scopolamine, and belladonna tincture are skin rash, skin flushing, mouth dryness, and difficulty in urination, eye pain, blurred vision, and light sensitivity.

Hyoscyamine, the tropine ester of (−)-tropic acid, is asymmetric and accounts for the natural occurrence of the optical isomer. When (−)-hyoscyamine is extracted from the plants in which it occurs, it is usually racemized during the process and thus converted into the (±) compound, which is atropine. Hyoscyamine sulfate is extremely poisonous and occurs as white, odorless crystals, or as a crystalline powder; it is deliquescent and is affected by light. Hyoscyamine sulfate is an anticholinergic. It is used to aid in the control of gastric secretion, visceral spasm, hypermotility in spastic colitis, and pylorospasm and associated abdominal cramps. In Parkinsonism, it is used to reduce rigidity and tremors and to control associated diarrhea and hyperhidrosis. Hyoscyamine is the tropyl ester of tropic acid and scopolamine is the scopyl ester of tropic acid.



Scopolamine



Hyoscyamine

Atropine sulfate occurs as colorless crystals or as a white, crystalline powder. It is extremely poisonous. It effloresces in dry air and is slowly affected by light. Atropine sulfate is an anticholinergic. Used in surgery as an antisialogogue to control bronchial, nasal, pharyngeal, and salivary secretions, it is usually injected intramuscularly before induction of anesthesia. During surgery, the drug is given intravenously when reduction in pulse rate and cessation of cardiac action is attributable to increased vagal activity. Scopolamine or hyoscine is an alkaloid that is particularly abundant in *Datura fastuosa* var. *alba* and in *Datura metel*. It is an ester that, upon hydrolysis, yields tropic acid and scopoline, a base resembling tropine. It occurs as an almost colorless, syrupy liquid from its chloroformic solution and as colorless crystals from its ester solution. It is levo rotatory. Scopolamine hydrobromide or hyoscine hydrobromide occurs as colorless or white crystals or as a white, granular powder that is odorless and slightly efflorescent in dry air. It is extremely poisonous. Scopolamine hydrobromide is classified as an anticholinergic. At usual therapeutic doses, scopolamine is a central nervous depressant, whereas atropine is a stimulant. For this reason, scopolamine hydrobromide is used for preanesthetic sedation and for obstetric sedation in conjunction with analgesics; it is also employed for calming delirium. It is administered subcutaneously or intramuscularly in a single dose.

(i) Hyoscyamus

Hyoscyamus or henbane is the dried leaf, with or without the stem and flowering or fruiting top of the *Hyoscyamus niger* (Solanaceae) and contains not less than 0.04% of the alkaloids of hyoscyamus. *Hyoscyamus* is the ancient Greek and Latin name formed from two Greek words, meaning hog and bean. The plant is poisonous to swine. The alkaloids, hyoscyamine, and scopolamine, 0.05%–0.15%, of which three-fourths is hyoscyamine, are the active principles.

(ii) Stramonium

Stramonium, jimson weed, or Jamestown weed consists of the dried leaf and flowering or fruiting tops with branches of *Datura stramonium* Linn. or of its variety *tatula*. Stramonium seed is the ripe seed of *D. stramonium*. The ripening capsules are gathered and dried until the seeds shake out. The seeds are reniform, flattened, 3–4 mm in length, bluish black, and minutely reticulate.

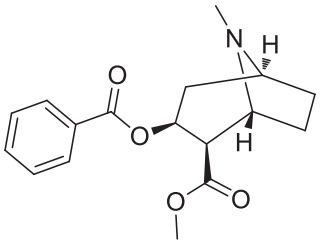
Stramonium seeds contain about 0.4% alkaloids, principally hyoscyamine with a small proportion of scopolamine and traces of atropine. Stramonium is generally regarded as a noxious weed and has frequently caused poisoning in children when ingested. The chief toxic symptoms are those of atropine poisoning; dilated pupils, impaired vision, dryness of the skin, secretions, extreme thirst, hallucinations, and loss of consciousness. Because of the potential for psychotropic effects, stramonium seeds have also been agents of abuse.

7.4.4.3 Coca Alkaloids

(i) Cocaine

Coca or coca leaves have been described as the dried leaves of *Erythroxylum coca*, known commercially as Huanuco coca, or of *Erythroxylum truxillense* Rusby, known commercially as *Truxillo coca* (Fam. Erythroxylaceae). Coca leaves contain three basic types of alkaloids: derivatives of ecgonine (cocaine, cinnamylocaine, α- and β-truxilline), tropine (tropacocaine, valerine), and hygrine (hygroline, cuscohygrine). Huanuco coca contains 0.5%–1% of ester alkaloids, derivatives

of tropine and ecgonine, of which cocaine constitutes the major part. Cuscohygrine is the principal nonester alkaloid in the leaf. *T. coca* has a somewhat lower content of ester alkaloids, but a much higher percentage (up to 75%) of this quantity is cocaine.

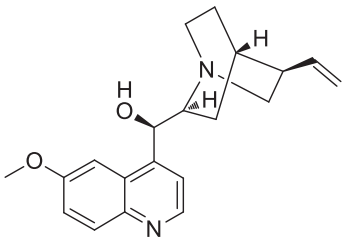


Cocaine

Cocaine is an alkaloid obtained from the leaves of *E. coca* and *E. truxillense* and their varieties. As explained subsequently, much of the alkaloid is actually prepared by semisynthesis from plant-derived ecgonine. Cocaine hydrochloride is the hydrochloride of the alkaloid cocaine. It occurs as colorless crystals or as a white, crystalline powder. Cocaine hydrochloride is an ingredient in Brompton's cocktail, which is widely used to control severe pain associated with terminal cancer. Because of its CNS stimulant properties, cocaine counteracts the narcotic induced sedation and respiratory depression associated with the narcotic analgesic ingredient (morphine or methadone) used in the cocktail. It also potentiates the analgesic effect.

7.4.5 Quinoline Alkaloids

Alkaloids containing quinoline as their basic nucleus include those obtained from cinchona (quinine, quinidine, cinchonine, and cinchonidine). *Cinchona* and its alkaloids are the only members of this group that are therapeutically important at present. Cinchonine, which is isomeric with cinchonidine, is the parent alkaloid of the quinine series. Quinine and its isomer quinidine represent 6-methoxycinchonine. The main alkaloids in this group are cinchona alkaloids. Cinchona bark contains about 30 alkaloids, of which the most important are quinine, quinidine, cinchonine, and cinchonidine. They occur in the bark as cinchotannates, which must be liberated from the acid by a mild alkali before they can be extracted with a suitable organic solvent.



Quinine

The Thalleoquin test: It consists of adding saturated bromine water to a solution of the alkaloid and then making alkaline with ammonia. Wagg's modification is an attempt to standardize conditions for the test to give it a quantitative value.

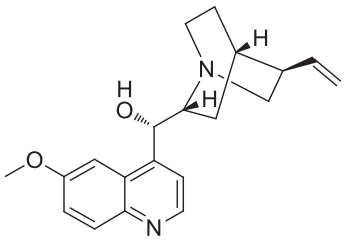
7.4.5.1 Cinchona Alkaloids

Cinchona, cinchona bark, or Peruvian bark is the dried bark of the stem or root of *Cinchona succirubra*, or its hybrids, known in commerce as red cinchona; or of *Cinchona ledgeriana*, *Cinchona calisaya*, or hybrids of them with other species of *Cinchona*, known in commerce as calisaya bark or yellow Cinchona (Fam. Rubiaceae).

Quinidine is a stereo isomer of quinine and is present in cinchona bark to the extent of 0.25%–1.25%. Quinidine sulfate is the sulfate salt of an alkaloid obtained from various species of *Cinchona* and their hybrids and from *Remijia pedunculata*, or prepared from quinine. It is odorless, has a bitter taste, and darkens when exposed to light. It is readily soluble in water, alcohol, methanol, and chloroform.

Quinine is the diastereo isomer of quinidine. It occurs as white, odorless, bulky, bitter crystals, or as a crystalline powder. It darkens when exposed to light and effloresces in dry air. It is freely soluble in alcohol, ether, and chloroform but

slightly soluble in water. Quinidine is used to treat various cardiac arrhythmias, such as premature atrial AV junctional, and ventricular contractions, such as atrial and ventricular tachycardia, atrial flutter, and atrial fibrillation. Its antiarrhythmic mechanism is through membrane stabilization. Quinine sulfate is the sulfate salt of an alkaloid obtained from the bark of the *Cinchona* species. It occurs as white, odorless, bitter, fine, needlelike crystals that are usually lusterless. It becomes brownish when exposed to light. It is not readily soluble in water, alcohol, chloroform, or ether.



Quinidine

Qualitative test for Cinchona

(i) *Thalleioquin test*: Take extract of cinchona powder and add one drop of dilute sulfuric acid and 1 mL of water. Add bromine water dropwise until the solution acquires a permanent yellow color and add 1 mL of dilute ammonia solution; an emerald-green color is produced, which indicates the presence of cinchona.

(ii) Take the powdered drug and heat with glacial acetic acid in a dry test tube. The presence of cinchona is indicated if red fumes evolve and condense in the top portion of the tube.

(iii) Take Cinchona bark, moisten with sulfuric acid and observe it under ultraviolet light. It shows a blue fluorescence due to the methoxy group of quinine and quinidine.

(iv) The powdered drug (0.5 g) is mixed with HCl (20 mL) and water (5 mL) and filtered. Potassium chloride (0.01) is added to the filtrate (2 mL). If emetine is present, a yellow color develops, which, on standing for 1 h, gradually changes to red.

7.4.6 Isoquinoline Alkaloids

The isoquinoline structure occurs in a considerable number of alkaloids in widely separated plant families. They represent the largest single group of plant alkaloids, and there is great variation in their chemical structures. Some of the important isoquinoline subgroups are the benzyl isoquinolines represented by papaverine and tubocurarine, the benzophenanthridines represented by sanguinarine, the pthalideisoquinolines that contain a ϒ-lactone ring and are represented by hydrastine, the morphinans represented by the opium alkaloids (codeine, morphine, thebaine), the protoberberines represented by berberine, and those with the mertine skeleton. In addition, with the isoquinoline group of alkaloids, the nitrogen is frequently in the quaternary form as in the case of berberine, sanguinarine, and tubocurarine, which greatly influences their solubility properties. Thus, this group is divided into three subgroups.

- Benzyl isoquinoline
- Bisbenzyl isoquinoline
- D-Tubocurarine

All of these occur, along with about 25 other alkaloids, in opium. In this class, the first subgroup includes morphine and codeine, and the second subgroup includes papaverine and narcotine. Morphine salts can be extracted quite easily with water. The alkaloids in opium occur partly as meconates, meconic acid being specific to opium. It can be characterized by the following chemical test.

Radulescu's test

In addition to the usual precipitation reactions, morphine gives a blue color with ferric chloride. When a dilute solution of morphine is treated with nitrous acid and the solution then made alkaline with a solution of ammonia, an orange-brown color is produced. This is Radulescu's test and is the basis of the colorimetric determination of small quantities of morphine.

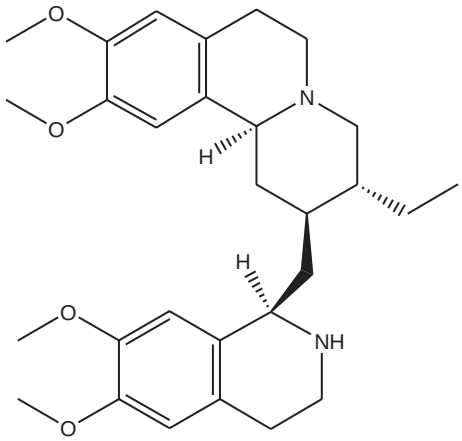
The last subgroup under this group includes D-tubocurarine from *Tube curare* and from *Pareira brava* root and the alkaloids occurring in Ipecacuanha root. Ipecacuanha contains the phenolic alkaloids cephaeline and psychotrine, and the nonphenolic alkaloids methyl psychotrine and emetine. The latter is the most important and can be extracted with difficulty with acids and may be oxidized to give an orange-red-colored compound.

7.4.6.1 Ipecac

Ipecac consists of the dried rhizome and roots of *Cephaelis ipecacuanha*, known in commerce as Rio or Brazilian ipecac, or of *Cephaelis acuminata*, known in commerce as Cartagena, Nitrogua, or Panama ipecac (Fam. Rubiaceae). Ipecac yields not less than 2% of the ether-soluble alkaloids of ipecac. *Cephaelis* is from two Greek words, meaning head and to collect or roll up and refers to the inflorescence; ipecacuanha is Portuguese from the Brazilian Indian ipekaaguene, meaning a creeping plant that causes vomiting; acuminata refers to the acute apex of the leaf.

In Rio (Brazilian) ipecac, the total alkaloid content reaches slightly over 2%, about one-third cephaeline and two-thirds emetine. In Cartagena (Colombia) ipecac and in Panama ipecac, the total alkaloid content reaches 2.2%. Ipecac, in the form of syrup, is used in the treatment of drug overdoses and in certain poisonings. It produces emesis through a local irritant effect on the gastrointestinal mucosa and a central medullary effect by stimulation of the chemoreceptor trigger zone. The usual dose in adults and children older than 1 year of age is 15 mL, followed by one or two glasses of water and may be repeated once in 20 min if emesis does not occur.

Emetine or methylcephaeline is an alkaloid obtained from ipecac or prepared synthetically by methylation of cephaeline. It was discovered by Pelletier and Magendie in 1817. Emetine hydrochloride is hydrate hydrochloride of emetine. It occurs as a white, odorless, crystalline powder that becomes yellowish when exposed to light. It is freely soluble in water and alcohol.

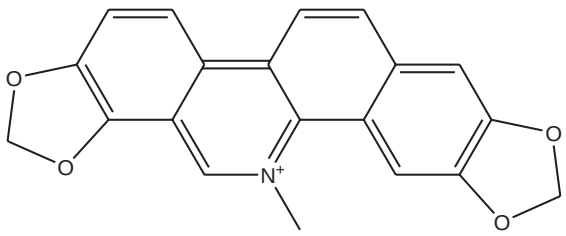


Emetine

Emetine hydrochloride is an antiamoebic and acts primarily in the intestinal wall and the liver. It inhibits polypeptide chain elongation, thereby blocking protein synthesis. The drug is not administered orally because it produces nausea and vomiting. Emetine hydrochloride has been used extensively as an antiprotozoan, particularly in the treatment of amebic dysentery, pyorrhea alveolaris, and other amebic diseases; however, it is no longer approved for these uses in the United States because it can accumulate in the body, producing potentially lethal toxic effects. It possesses expectorant and emetic properties.

7.4.6.2 Sanguinaria

Sanguinaria or bloodroot is the dried rhizome of *Sanguinaria canadensis* Linn. (Fam. Papaveraceae). Sanguinaria contains alkaloids, including sanguinarine (about 1%), chelerythrine, protopine, and allocryptopine. These alkaloids are colorless but tend to from colored salts.

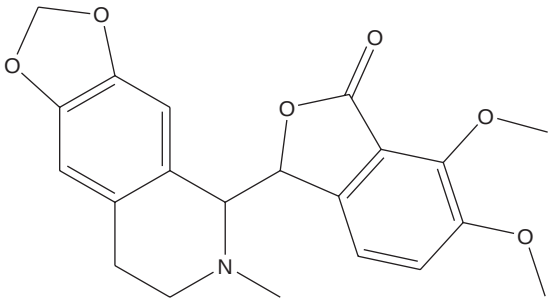


Sanguinarine

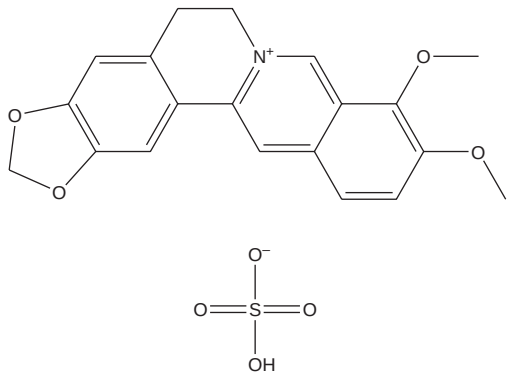
Sanguinarine yields reddish salts with nitric or sulfuric acids; yellowish salts are formed with chelerythrine. Sanguinarine is a benzophenanthridine type of isoquinoline alkaloid. All alkaloids of sanguinaria are found in other members of the Papaveraceae. Sanguinaria has stimulating, expectorant, and emetic properties. In addition, sanguinaria extract, representing a mixture of the total alkaloids, has been incorporated into toothpaste and mouthwash for the prevention of the development of dental plaque and subsequent periodontal disease.

7.4.6.3 Hydrastis

Hydrastis or goldenseal consists of the dried rhizome and roots of *Hydrastis canadensis* Linn. (Fam. Ranunculaceae). Three alkaloids have been isolated from hydrastis; hydrastine, berberine, and canadine. Of these, hydrastine (1.5%–4%) is the most important. Hydrastis yields not less than 2.5% of anhydrous ether-soluble alkaloids.



(–)β-Hydrastine



Berberine

Hydrastine is a phthalideisoquinoline and is readily soluble in chloroform, alcohol, and ether, but almost insoluble in water. Berberine is readily soluble in water but almost insoluble in ether. The salts of berberine form yellow crystals.

7.4.6.4 Tubocurarine Chloride

Curare or South American arrow poison is a crude dried extract from the bark and stems of *Strychnos castelnaei*, *Strychnos toxifera*, *Strychnos crevauxii* (Fam. Loganiaceae), and from *Chondrodendron tomentosum* (Fam. Menispermaceae). Tubocurarine chloride is standardized by the "head-drop" crossover test in rabbits in which groups of animals for testing and for control are used on alternate days (crossover). The standard "head-drop" dose is the least amount of the drug capable of producing muscle relaxation so that the head of the animal drops in a characteristic manner. Tubocurarine chloride is a nondepolarizing neuromuscular blocking agent and is employed intramuscularly or intravenously as a skeletal muscle relaxant to secure muscle relaxation in surgical procedures without deep anesthesia. It is also used to control convulsions of strychnine poisoning and of tetanus; it is an adjunct to shock therapy in neuropsychiatry and a diagnostic aid in myasthenia gravis.

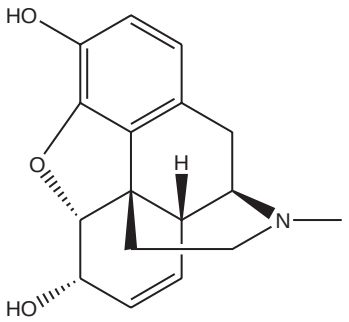
7.4.6.5 Opium

Opium or gum opium is the air-dried milky exudate obtained by incising the unripe capsules of *P. somniferum* Linn. or its variety *album* (Fam. Papaveraceae). Morphine is the most important of the opium alkaloids. Morphine and the related alkaloids

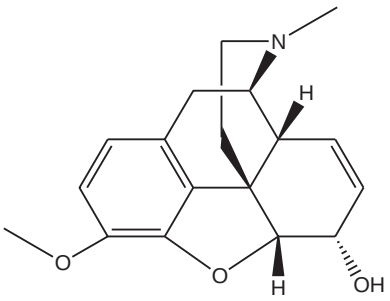
are morphinan isoquinoline derivatives. The molecule contains a phenolic and an alcoholic hydroxyl group. The alkaloid and its salts occur as white silky crystals, sometimes in cubic masses, or as a fine crystalline powder. It is stable in air, odorless, and bitter tasting. Centrally acting analgesics, in most cases, have certain structural features in common, which are:

- a central carbon atom with no hydrogen substitution (quaternary),
- a phenyl group or isostere attached to this carbon atom,
- a tertiary nitrogen atom, and
- a two-carbon bridge separating the tertiary nitrogen and the central carbon atom.

Codeine is the most widely used opium alkaloid. It may be either obtained from opium (0.2%–0.7%) or prepared from morphine by methylation or from thebaine by appropriate reduction and demethylation. Codeine is methylmorphine in which the methyl group replaces the hydrogen of the phenolic hydroxyl group. Codeine and its salts occur as fine needles or as white crystalline powders that effloresce in air.



Morphine



Codeine

Codeine and its salts are narcotic analgesics and antitussives; they are used as sedatives, especially in allaying coughs. Although its action is similar to that of morphine, codeine is considerably less toxic and involves much less danger of habit formation. Diacetylmorphine or heroin is formed by the acetylation of morphine; the hydrogen atoms of both the phenolic and alcoholic hydroxyl groups are replaced by acetyl groups. The action of heroin is similar, but more pronounced, than that of morphine. Because of its potency and the danger of habit formation, its use in medicine has been discontinued.

Qualitative test for opium

(i) An aqueous extract of opium with $FeCl_3$ solution gives a deep reddish-purple color, which persists on addition of HCl. This indicates the presence of meconic acid.

(ii) Morphine gives a dark violet color with conc. H_2SO_4 and formaldehyde.

7.4.7 Indole Alkaloids

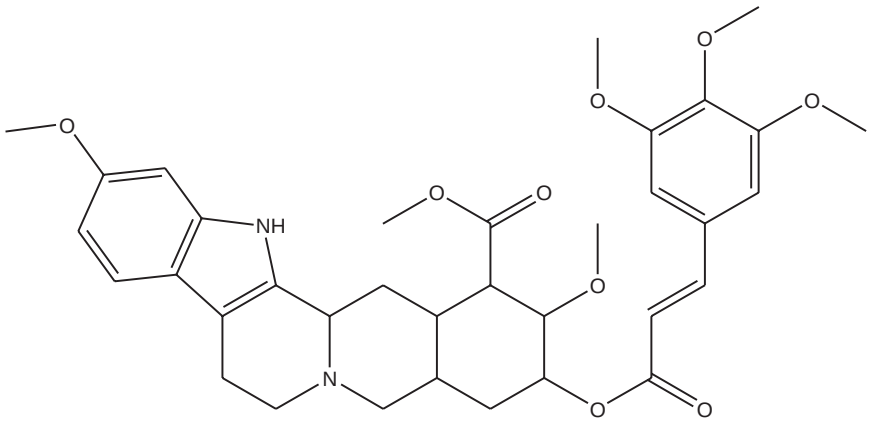
A number of important alkaloids possess an indole ring as part of their structure. Strychnine and brucine (dimethoxystrychnine) from nux vomica and physostigmine from physostigma belong to this group. However, strychnine and brucine also contain a quinoline nucleus and some authors classify them in the quinoline group. The important drugs and their alkaloids of the indole group are rauwolfia, reserpine, catharanthus (vinca), vinblastine, vincristine, nux vomica, strychnine, brucine, physostigma, physostigmine, ergot, ergotamine, ergonovine, and yohimbine (Stöckigt and Ruppert, 1999; Hibino and Choshi, 1961).

7.4.7.1 Rauwolfia serpentina

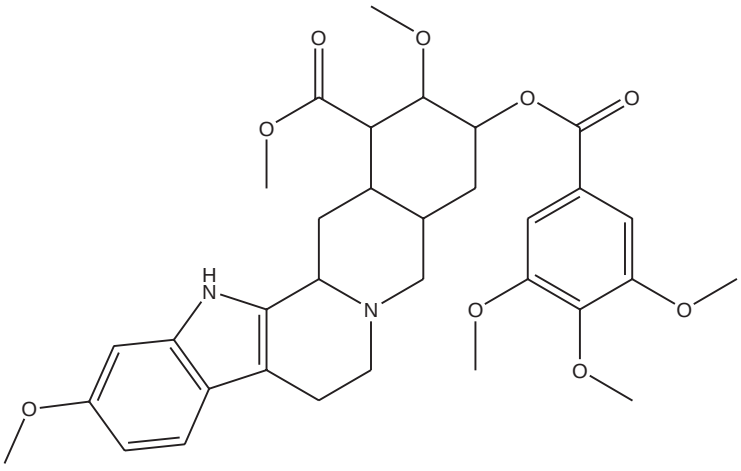
R. serpentina is the dried root of *R. serpentina* (Fam. Apocynaceae). Powdered rauwolfia is a fine or very fine powder that is adjusted, if necessary to confirm to the official requirements for reserpine–rescinnamine group alkaloids by admixture with lactose or starch or with a powdered *R. serpentina* containing a higher or lower content of these alkaloids. It contains not less than 0.15% and not more than 0.2% of reserpine–rescinnamine group alkaloids, calculated as reserpine. Reserpine is the chief alkaloid and has strong hypotensive and sedative activity. A total alkaloidal determination is not indicative of activity unless the proportion of alkaloids is known.

Because at least 50 alkaloids have been isolated, it is easy to understand the claim that the whole root exhibits a medicinal action that is different from that of reserpine. A definite lowering of blood pressure in hypertensive states, a slowing of the pulse, and a general sense of euphoria follow administration. It is used in mild essential hypertension and also as adjunctive

therapy with other agents in more severe forms of hypertension. It is also indicated to relieve the symptoms in agitated psychotic states, such as schizophrenia, in patients unable to tolerate other antipsychotic agents. Reserpine is a white or pale buff to slightly yellow, odorless, crystalline powder that darkens slowly when exposed to light and rapidly when in solution. Reserpine is an antihypertensive and antipsychotic agent. Rescinnamine is an alkaloid that occurs in several species of *Rauwolfia*.



Rescinnamine

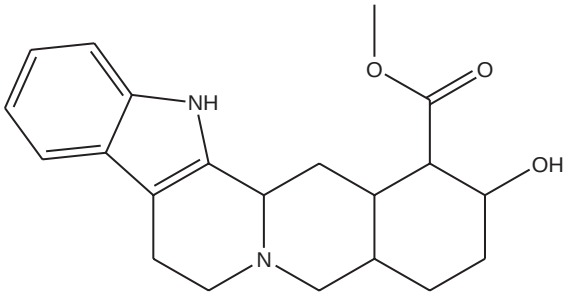


Reserpine

Its appearance, properties, and solubility are somewhat similar to those of reserpine. Chemically, it is the methyl reserpate ester of 3,4,5-trimethoxycinnamic acid. It is used to treat mild essential hypertension. Deserpidine (canescine, recanescine) is an alkaloid obtained from the root of *Rauvolfia canescens* L. Chemically, it is 11-demethoxyreserpine. It has the same uses as *R. serpentina* and reserpine and is claimed to have fewer side effects. In mild or moderate hypertension, rauwolfia or its derivatives may be the sole therapy, but in more severe hypertension, rauwolfia acts synergistically with more potent hypotensive agents. Products are available that use combinations of rauwolfia, deserpidine, or reserpine with thiazide diuretics and/or other antihypertensive agents.

7.4.7.2 Yohimbine

Yohimbine is an indole alkaloid of the corynane type obtained from the bark of the West African tree *Pausinystalia yohimbe* (Fam. Rubiaceae). The bark contains about 6% of a mixture of alkaloids, the principal one of which is yohimbine. Yohimbine has successfully treated impotence in patients with vascular or diabetic problems. Its peripheral autonomic nervous system effect is to increase cholinergic and to decrease adrenergic activity. In male sexual performance, erection is linked to increased cholinergic activity, which results in increased penile blood inflow, decreased penile blood outflow, or both, causing erectile stimulation.



Yohimbine

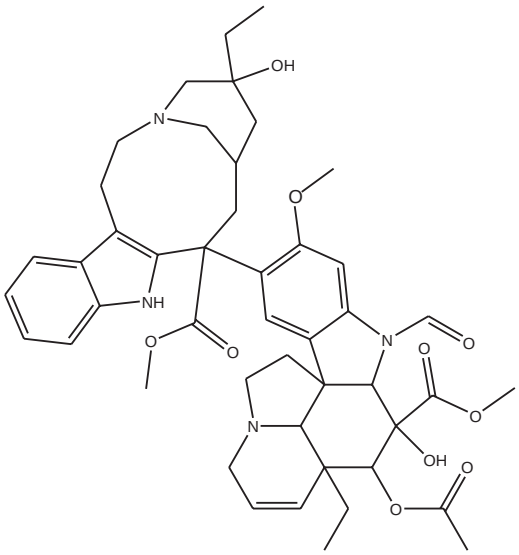
7.4.7.3 Catharanthus Alkaloids

Catharanthus or vinca is the dried whole plant of *Catharanthus roseus* (Fam. Apocynaceae). During the course of a modern scientific investigation prompted by the folklore reputation of this plant as an oral hypoglycemic agent, the ability of certain fractions to produce peripheral granulocytopenia and bone marrow depression in rats was observed by the Canadian group of Noble, Beer, and Cutts. Continued study led to the isolation of an alkaloid, vinblastine, which produced severe leukopenia in rats.

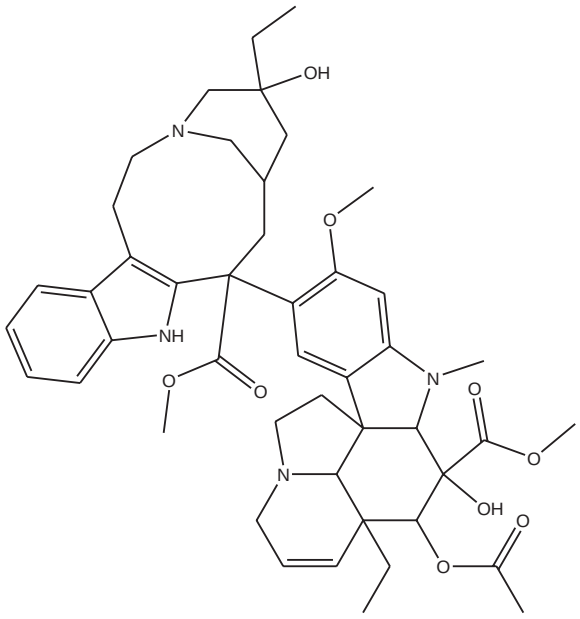
Recognizing the anticancer potential of this plant, G.H. Svoboda and coworkers at Eli Lilly and Co. isolated an extremely large number of alkaloids from the plant. Of these, four bisindole compounds, vinblastine, vinleurosine, vinrosidine, and vincristine, possess demonstrable oncolytic activity. Because these active alkaloids exist in the crude drug in relatively small amounts, enormous quantities are required for commercial production. Nearly 500 kg of cathranthus is utilized to produce 1 g of vincristine. To satisfy the demand, the plant is collected from both natural and cultivated sources in Madagascar, Australia, South America, the West Indies, Europe, India, and the southern United States.

More than 90 different alkaloids have been isolated from *Catharanthus*. They are generally indole and dihydroindole derivatives, some of which occur in other members of the Apocynaceae. These include ajmalicine, tetrahydroalstonine, serpentine, and lochnerine. The alkaloids with antineoplastic activity belong to a new class of bisindole derivatives. These alkaloids are composed of an indole and a dihydroindole unit, catharanthine, and vindoline, respectively. The former belongs to the ibogane type and the latter to the aspidospermane type of monoterpenoid indole alkaloids.

The most characteristic effect of these drugs is the arrest of cell division at metaphase, in a manner resembling the effect of colchicine. Both vinblastine and vincristine bind tightly to tubulin and interfere with the functioning of the microtubule system, which is a component of the mitotic spindle. Recent findings indicate that the alkaloids actually inhibit the polymerization of tubulin into microtubules.



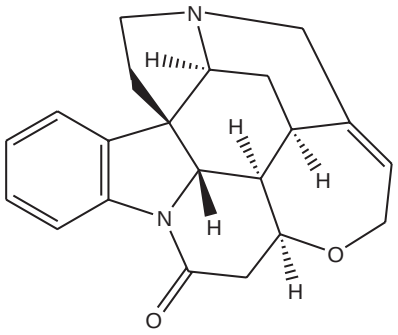
Vincristine



Vinblastine

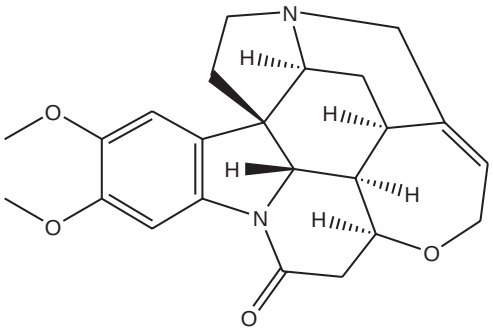
7.4.7.4 Nux vomica

Nux vomica is the dried, ripe seed of *Strychnos nux-vomica* Linn. (Fam. Loganiaceae). Strychnos is the Greek name for a number of poisonous plants; nux-vomica is from two Latin words and means a nut that causes vomiting. Nux vomica contains alkaloids, 1.5%–5%, consisting chiefly of strychnine and brucine, the former comprising from one-third to one-half of the total amount. Strychnine and brucine (dimethoxystrychnine) are modified corynane-type monoterpenoid indole alkaloids and are obtained from nux vomica or ignatia by extraction with dilute sulfuric acid. The solution is concentrated. The alkaloids are precipitated with lime, separated by means of solvent, and purified by recrystallization.



HCl

Strychnine



Brucine

Strychnine is interesting pharmacologically and is a valuable tool in physiologic and neuroanatomic research. It is extremely toxic, functioning as a central stimulant.

Qualitative test for Nux vomica

(i) *Strychnine test*: To a section of endosperm, add ammonium vanadate and sulfuric acid. Strychnine in the middle portion of the endosperm is stained purple.
(ii) *Potassium dichromate test*: Strychnine gives a violet color with potassium dichromate and conc. sulfuric acid.
(iii) *Brucine test*: To a thick section, add concentrated nitric acid. The outer part of the endosperm is stained yellow to orange due to the presence of brucine.
(iv) *Hemicellulose test*: To a thick section, add iodine and sulfuric acid. The cell walls are stained blue, which indicates the presence of Nux vomica.

7.4.7.5 Physostigmine

Physostigma, calabar bean, or ordeal bean is the dried, ripe seed of *Physostigma venenosum* (Fam. Fabaceae), yielding not less than 0.15% of the alkaloids of physostigma. Calabar bean contains several alkaloids, such as physostigmine (eserine), eseramine, geneserine, and physovennie. Physostigmine is the major alkaloid and is present in the cotyledons to the extent of 0.04%–0.3%. Physostigmine or eserine is an alkaloid usually obtained from the dried, ripe seed of *P. venenoum*. It occurs as a white, odorless, microcrystalline powder that may acquire a red tint when exposed to heat, light, air, or contact with traces of metal.

7.4.7.6 Ergot

Ergot, rye ergot, or secale cornutum was formerly defined in the official compendia as the dried sclerotium of *C. purpurea* (Fam. Clavicipitaceae) developed on plants of rye, *Secale cereale* Linn. (Fam. Gramineae). Ergot was required to yield not less than 0.5% of the total alkaloids of ergot calculated as ergotoxine and water-soluble alkaloids equivalent to not less than 0.01% of ergonovine. Ergot contains or produces a large number of alkaloids, the most important of which are ergonovine, ergotamine, and a mixture of ergocristine, ergokryptine, and ergocornine, which has been marketed for many years under the name of ergotoxine.

The alkaloids are often separated into two groups based on their solubility in water. Ergonovine is the principal component of the water-soluble fraction. Ergotamine and the ergotoxine group are insoluble in water and are often referred to as peptide alkaloids. Significant semisynthetic alkaloids include methylergonovine, dihydroergotamine, ergoloid mesylates, methysergide, and LSD. Ergot contains a number of alkaloids closely related in chemical structure, based on either lysergic or isolysergic acid, which can easily be identified based on their solubility. These alkaloids may be extracted quite easily from de-fatted ergot with ether, after being made alkaline with ammonia. They can then be transferred from the ether to an acid solution, tartaric acid being preferred. With a solution of *p*-dimethyl-aminobenzaldehyde in sulfuric acid containing a trace of ferric chloride, they give a blue color, which reaches a maximum intensity after 5 min.

Qualitative test for ergot

(i) Ergot shows a red-colored fluorescence under UV light.

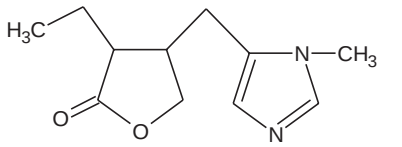
(ii) Take extracted ergot powder and mix with a mixture of $CHCl_3$ and sodium carbonate. The $CHCl_3$ layer will be separated, then add a mixture of *p*-dimethylaminobenzaldehyde (0.1 g), H_2SO_4 (35% (v/v), 100 mL), and 5% ferric chloride (1.5 mL). A deep blue color will be produced, which indicates the presence of ergot alkaloids.

7.4.8 Imidazole Alkaloids

The imidazole (glyoxaline) ring is the principal nucleus in pilocarpine from *Pilocarpus*. Pilocarpine is a monoacidic tertiary base containing a lactone group, as well as the imidazole nucleus. Obvious structural similarities suggest that this alkaloid is probably formed from histidine or a metabolic equivalent, but experimental confirmation of such a biosynthetic origin is lacking. Pilocarpus and pilocarpine are the important drugs of this group.

7.4.8.1 Pilocarpine

Pilocarpus or jaborandi consists of the leaflets of *Pilocarpus jaborandi* (*Pernambuco jaborandi*), or of *P. pinnatifolius* (*Paraguay jaborandi* Fam. Rutaceae). The plants are shrubs indigenous to Brazil. All of the commercial kinds of pilocarpus, when freshly dried, yield from 0.5% to 1% of the alkaloids pilocarpine, isopilocarpine, pilocarpidine, and pilicarpine. Isopilocarpine, pilocarpodine, and pilosine are also present in some of the species.



Pilocarpine

Pilocarpine directly stimulates the muscarinic receptors in the eye, causing constriction of the pupil and contraction of the ciliary muscle. In narrow-angle glaucoma, miosis opens the anterior chamber angle to improve the outflow of aqueous humor. In chronic open-angle glaucoma, the increase in outflow is independent of the miotic effect. Contraction of the ciliary muscle enhances the outflow of aqueous humor via indirect effects on the trabecular system.

7.4.9 Steroidal Alkaloids

The steroidal alkaloids are derived biosynthetically from six isoprene units and could be classified as triterpenoids or steroids; however, they also contain nitrogen, giving them basic properties. The nitrogen may be part of a ring system, usually incorporated at a late stage in biosynthesis, or it may be as an *N*-methyl substituted amino group. Steroidal alkaloids have been found in the plant families Apocynaceae, Buxaceae, Liliaceae, and Solanaceae.

7.4.9.1 Veratrum viride

American or green hellebore consists of the dried rhizome and roots of *V. viride* (Fam. Liliaceae). It contains a large number of alkaloids, customarily classified into three groups on the basis of their chemical constitution. Group I, consisting of esters of the steroidal bases (alkamines) with organic acids, includes cevadine, germidine, germitrine, neogermitrine, neoprotoveratrine, protoveratrine, and veratridine. Group II includes pseudojervine and veratrosine, which are glucosides of the alkamines. The alkamines themselves—germine, jervine, rubijervine, veratramine, and germitrine—are probably the most important therapeutically. The complexity and relative instability of these constituents account for the problems encountered in the biologic standardization of this drug. *V. viride* possesses hypotensive, cardiac depressant, and sedative properties. It has been used in the treatment of hypertension. Small doses principally affect blood pressure without notably changing the respiratory or cardiac rate. The drug has its most uniform effects in small doses. White hellebore or European hellebore is the dried rhizome of *Veratrum album* Linn. (Fam. Liliaceae). It is similar to *V. viride* but is indigenous to central and southern Europe. White hellebore is similar in appearance and structure to green hellebore, but the external color is much lighter. White hellebore possesses hypotensive properties, but the crude drug is not used therapeutically. Both white and green hellebores are also employed as insecticides.

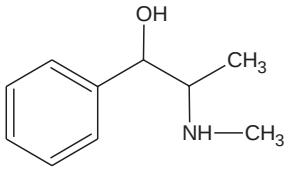
7.4.10 Alkaloidal Amines

The alkaloids in the group do not contain heterocyclic nitrogen atoms. Many are simple derivatives of phenylethylamine and, as such, are derived from the common amino acids, phenylalanine or tyrosine. Some of the alkaloids in this category,

whose biosynthesis has been studied utilizing labeled precursors, include mescaline in the peyote cactus (*Lophophora williamsii*), ephedrine in *Ephedra distachya*, cathionone in the khat plant (*Catha edulis*), and colchicine in the autumn crocus (*Colchicum autumnale*).

7.4.10.1 Ephedrine

Ephedra or Ma Huang is the entire plant of the over ground portion of *Ephedra sinica* Stapf (Fam. Gnetaceae). Ephedrine occurs as white, rosette, or needle crystals, or as an unctuous mass. It is soluble in water, alcohol, chloroform, ether, and in liquid petrolatum. The latter solution is turbid if the ephedrine is not dry.



(–)-Ephedrine

In large doses, ephedrine may cause hypertension, headache, dizziness, palpitations, vomiting, and because it is a central nervous system stimulant, nervousness, and insomnia.

7.4.10.2 Colchicine

Colchicum seed is the dried, ripe seed of *C. autumnale* Linn. (Fam. Liliaceae). Colchicum corn is the dried corn of the same species. Colchicum contains the alkaloid colchicine, up to 0.8% in the seed and 0.6% in the corn. Colchicine has antimitotic activity. In its presence, the chromosomes of the cell will continue to divide without the formation of spindle fibers and subsequent cell division to form daughter cells. Any numeric change in chromosome number in a cell entails a mutation that becomes evident in several of the characteristics of the experimental plant. New varieties of plants of economic and pharmacognostic value may result from further research. The interrelationship between the action of colchicine and mitosis is being investigated in animals; preliminary experiments show that injections of colchicine can affect the dispersal of tumors; thus, it has been employed experimentally in the treatment of various neoplastic diseases.

Khat or Abyssinian tea consists of the fresh leaves of *C. edulis* (Fam. Celastraceae). Khat contains a potent phenylalkylamine alkaloid called (−) cathinone. It has pharmacological properties analogous to those of (+) amphetamine and is of similar potency with a similar mechanism of action, namely, the induction of catecholamine release from storage sites. The young, fresh leaves that come from the tips of the branches contain the optimum amount of cathinone. In older leaves, it is converted to the weakly active compounds (+) norpseudoephedrine (80%) and (−) norephedrine (20%). This conversion also occurs rapidly during the drying of young leaves.

7.4.11 Purine Bases

The purines are derivatives of a heterocyclic nucleus consisting of the six-membered pyrimidine ring fused to the five-membered imidazole ring. Purine itself does not occur in nature, but numerous derivatives are biologically significant. The pharmaceutically important bases of this group are all methylated derivatives of 2,6-dioxypurine (xanthine). Caffeine is 1, 3,7-trimethylxanthine, theophylline is 1,3-dimethylxanthine, and theobromine is 3,7-dimethylxanthine. Caffeine is synthesized from the same precursors in *Coffea arabica* as are the purine bases in all other biologic systems that have been investigated. The purines can be identified by the Murexide test, giving a purple color when the residue, obtained after heating with an oxidizing agent, is exposed to the vapors of ammonia. Caffeine readily sublimes as prismatic crystals.

7.4.11.1 Caffeine-Containing Drugs

Kola, cola, or kolanuts is the dried cotyledon of *Coca nitida*, or of other species of Cola (Fam. Sterculiaceae). It yields not less than 1% of anhydrous caffeine.

Kolanuts contain caffeine, up to 3.5%, and theobromine, less that 1%. In the fresh nuts, these purine derivatives are bound to the tannin kolacatechin. During the drying process, the complex is split, yielding free caffeine and theobromine and converting the colorless kolacatechin to the red-brown kola red. Kola possesses the central stimulating action of caffeine. It is an ingredient in several carbonated beverages.

(i) Caffeine

Caffeine or 1,3,7-trimethylxanthine occurs in coffee, tea, cocoa, guarana, kola, and mate. Although caffeine can be produced synthetically, it is usually prepared from tea, tea dust, or tea sweepings, or recovered from coffee roasters. Caffeine is

anhydrous or contains one molecule of water of hydration. Caffeine occurs as a white powder or as white, glistening needles matted together in fleecy masses. It has a bitter taste. Caffeine may be sublimed without decomposition when heated.

Qualitative test for caffeine

(i) Caffeine and other purine alkaloids give a murexide color reaction. Take caffeine in a petri dish to which hydrochloric acid and potassium chlorate crystals are added and heated to dryness. A purple color is obtained by exposing the residue to vapors of dilute ammonia. With the addition of fixed alkali, the purple color disappears.

(ii) Caffeine also produces a white precipitate with tannic acid solution.

7.4.11.2 Theophylline

Thea or tea consists of the prepared leaves and leaf buds of *Camellia sinensis* (Fam. Theaceae) (Fernández-Cáceres et al., 2001), a shrub or tree with alternate, evergreen leaves. The tea tree is indigenous to eastern Asia and is now extensively cultivated in China, Japan, India, and Indonesia. The generic name is Greek and means goddess; sinensis refers to its Chinese origin. Green tea is prepared in China and Japan by rapidly drying the freshly picked leaves in copper pans over a mild artificial heat. The leaves are often rolled in the palm of the hand as they dry. Black tea is prepared in Sri Lanka and India by heaping the fresh leaves until fermentation has begun. They are then rapidly dried artificially with heat. Theophylline, or 1,3-dimethylxanthine, is isomeric with theobromine and was first isolated from tea in 1885. It is prepared synthetically from caffeine or by other means. Theophylline occurs as a white, odorless, bitter crystalline powder that is soluble in about 120 parts of water. It is rendered more soluble when combined with basic compounds.

Theophylline and related compounds are utilized principally as smooth muscle relaxants for the symptomatic relief or prevention of bronchial asthma and for the treatment of reversible bronchospasm associated with chronic bronchitis and emphysema. In addition, theophylline possesses diuretic properties.

7.4.11.3 Theobromine

Theobromine or 3,7-dimethylxanthine is a compound prepared from the dried, ripe seed of *Theobroma cacao* (Fam. Sterculiaceae) or is made synthetically. It occurs as a white, crystalline powder with a bitter taste and sublimes at about 260°C. The base is slightly soluble in cold water or in alcohol but is readily soluble when mixed with salts that from basic solutions, such as calcium salicylate, sodium acetate, or sodium salicylate. Theobromine is a diuretic and a smooth muscle relaxant. It has little stimulant action on the central nervous system.

7.5 VOLATILE OILS, SPICES, AND THEIR ANALYSIS

The medicinal action of many crude drugs is due to the presence of essential oils. They are usually secreted as such in plant tissues, but sometimes they are produced from substances that are more complex through chemical changes brought about after the collection of the plant. They consist mainly of a mixture of small molecules of hydrocarbons containing 10–15 carbon atoms. Volatile oils contain mainly mono- and sesquiterpenes and phenolic compounds. They are comparatively nonpolar and may be extracted with light petroleum. However, solvents, such as chloroform and dichloromethane, are notably more applicable whenever the coextraction of fats and waxes is to be avoided. Steam distillation is a popular method for the selective extraction of volatile oils. Methods, such as the advanced phytonics technique, supercritical fluid extraction, and extractions into fixed oils or waxes, are also used (Purseglove et al., 1981).

Volatile oils are the odorous principals found in various plant parts. Because they evaporate when exposed to the air at ordinary temperatures, they are called volatile oils, ethereal oils, or essential oils. The last term is applied because volatile oils represent the "essences" or odoriferous constituents of the plant. Spices are dried, fragrant, aromatic, or pungent plant parts that contain a volatile oil and are used primarily in food as a seasoning rather than for nutrition. A very large number of plants contain substances that are entirely, or almost entirely, volatile without decomposition. While most of them are heterogeneous liquids, a few are solids or semisolids at room temperature. The volatile oils may occur in flowers, leaves, fruits, seeds, barks, and woods, but invariably exist in specialized secretary cells, for example, the vittae in the umbelliferous fruits. Volatile oils and spices of pharmaceutical importance are shown in Table 7.5.

Depending on the plant family, volatile oils may occur in specialized secretory structures, such as glandular hairs (Lamiaceae), modified parenchymal cells (Piperaceae), oil-tubes called vittae (Apiaceae), or in lysigenous or schizogenous passages (Pinaceae, Rutaceae). They may be formed directly by the protoplasm, by decomposition of the resinogenous layer of the cell wall, or by the hydrolysis of certain glycosides. In the conifers, volatile oils may occur in all tissues; in the rose, they appear in appreciable quantities only in the petals; in cinnamon, only in the bark and the leaves; in the apiaceous fruits, only in the pericarp; in the mints, only in the glandular hairs on the stems and leaves; and in the orange, one kind of

TABLE 7.5 Pharmaceutically Important Volatile Oils and Spices

Name	Part of Plant	Biological Source	Active Constituents	Use
Anise	Dried fruit	*Pimpinella anisum* (Apiaceae)	1%–3% volatile oil containing 80%–90% anethole	Flavoring agents
Basil	Fresh leaves	*Ocimum sanctum* (Labiatae)	Volatile oil, cineol	Flavoring agent, expectorant
Black cumin	Dried fruits	*Nigella sativa* (Ranunculaceae)	45%–60% carvone, limonene and cymene	Carminative, stimulant, diuretic
Cajuput	Fresh leaves and twigs	*Melaleuca leucadendron* (Myrtaceae)	50%–60% cineol, terpineol	Flavoring agent
Caraway	Dried fruit	*Carum carvi* (Apiaceae)	3%–6% volatile oil containing 50%–85% carvone, 40%–50% limonene	Flavoring agent, carminative
Chenopodium	Fresh flowering and fruiting plant except the roots	*Chenopodium ambrosioides* and *C. anthelmintica* (Chenopodiaceae)	1%–4% volatile oil containing 70% ascaridole	Anthelmintic
Cinnamon	Bark	*Cinnamomum zeylanicum* (Lauraceae)	60%–70% cinnamic aldehyde, phenols, hydrocarbons	Flavoring agent, astringent, germicide
Citronella oil	Dried leaves	*Cymbopogon winterianus*, *C. nardus* (Poaceae)	5%–55% citronellol, 25%–40% geraniol	Perfume insect repellant
Clove	Unexpanded flower buds	*Eugenia caryophyllus* (Myrtaceae)	14%–21% volatile oil containing eugenol, sesquiterpenes	Stimulant aromatic, species, dental analgesic
Coriander	Dried fruit	*Coriandrum sativum* (Umbellifeare)	1%–3% volatile oil of which 65%–70% linalol, and pinene	Flavoring agent, carminative
Cumin	Dried fruits	*Cuminum cyminum* (Umbellifeare)	2%–4% volatile oil containing 25%–35% aldehyde, pinene, and α-terpinol	Flavoring agent, carminative
Curry leaf	Fresh leaves	*Murraya koenigii* (Rutaceae)	Volatile oil containing α-phellandre, pinine, terpenol	Stimulant and astringent
Dill	Dried fruit	*Anethum graveolens* (Umbelliferae)	34% volatile oil containing 43%–63% carvone and limonene	Flavoring agent, carminative
Eucalyptus	Fresh leaves	*Eucalyptus globulus* and other species (Myrtaceae)	70%–85% cineol, 5%–15% α-pinene	Antiseptic, stimulating expectorant
Fennel	Dried, ripe fruit	*Foeniculum vulgare* (Apiaceae)	Volatile oil contains 65%–70% anethole, 10%–15% limonene, 6%–7% fenchone	Flavoring agent, carminative
Fenugreek	Seed	*Trigonella foenum* (Leguminoceae)	Sapogenins, mucilage	Cardioactive
Ginger	Dried rhizomes	*Zingiber officinale* (Zingiberceae)	1%–2% volatile oil with mixture of 25 components with monoterpenes, sesquiterpenes	Carminative and stimulant
Large/Black cardamon	Dried fruits	*Amomum subulatum* (Zingiberceae)	3%–7% volatile oil	Flavoring agent, carminative
Lavender	Fresh flowering tops	*Lavandula officinalis* (Lamiaceae)	30%–60% linalyl acetate, linalool, cineole	Perfume
Lemon	Fresh peel of the fruit	*Citrus limon* (Rutaceae)	70%–80% limonene, 8%–10% β-pinene, γ-terpinene, and 2%–4% citral	Flavoring agent carminative
Nutmeg	Dried kernel	*Myristica fragrans* (Myristicaceae)	4%–15% volatile oil containing amyloderxtrin	Condiments, stimulant

Continued

TABLE 7.5 Pharmaceutically Important Volatile Oils and Spices—cont'd

Name	Part of Plant	Biological Source	Active Constituents	Use
Orange oil	Fresh peel of the ripe fruit	*Citrus sinensis* (Rutaceae)	90% limonene, 1%–2% decanal	Flavoring agent
Parsley leaf	Dried or fresh leaves	*Petroselinum crispum* (Apiaceae)	Volatile oil with apiole	Flavoring agent
Pepper	Dried fruits	*Piper nigrum* (Piperaceae)	Oils containing 1-phelladrene, free amino acid like histidine, crystine, etc.	Flavoring agent, bacteriostic
Peppermint	Fresh flowering tops	*Mentha piperita* (Lamiaceae)	45%–60% carvone, limonene	Flavoring agent
Pine oil	Wood	*Pinus palustris* and other species of pinus (Pinaceae)	10% methyl chavicol and related compound and 65% α-terpineol	Disinfectant, deodorant
Rose oil	Fresh flower	*Rosa gallica*, *R. alba*, and other species of Rosa (Rosaceae)	Geraniol, nerol	Perfume
Rosemary	Flowering plant	*Rosmarinus officinalis* (Labiatae)	1%–2% volatile oils with ester and alcohol	Flavoring agent
Spearmint oil	Fresh aerial parts of flowering plants	*Mentha spicata*, *M. cardiaca* (Lamiacaeae)	45%–60% carvone, limonene, cineole	Flavoring agent
Star anise	Dried fruits	*Illicium verum* (Mangnoliaceae)	Volatile oil containing carvone	Carminative, stimulant, diuretic
Tejpat	Dried leaves	*Cinnamomum tamala* (Lauraceae)	Essential oils 0.3%–0.6% comprising linalol, eugenol, etc.	Carminative, antidiarrheal
Thyme	Dried leaves and flowering top	*Thymus vulgaris*, *T. zygis*, and other varieties (Lamiaceae)	Thymol, carvachol, α- and γ-terpinene	Flavoring agent
Turpentine oil	Oleoresin	*Pinus palustris* and other varieties (Pionaceae)	30% β-pinene 65% α-pinene	Counterirritant
Wintergreen oil	Dried plant parts	Leaves of *Gaultheria procumbens*, bark of *Betula lanata* (Betulaceae)	98% methyl salicylate	Flavoring agent, counterirritant, antiinflammatory
Sweet flag or calamus	Rhizomes	*Acorus calamus* (Araceae)	α-Asarone (9.7%), β-asarone (83.2%)	Antidepressant, anxiolytic antibacterial
Ajowan oil	Seeds	*Trachyspermum ammi* (Umbelliferae)	Thymol (63.4%), *p*-cymene (19%), γ-terpinene (16.9%)	Germicide, antispasmodic, and antifungal agent
Bitter orange or marmalade orange	Fruit peel	*Citrus aurantium* (Rutaceae)	DL-limonene (94.81%)	Flavoring agent
Green cardamom	Seed and fruit coat	*Elettaria cardamomum* (Zingiberaceae)	Limonene (4.05% and 3.82%), 1,8-cineole (15.13% and 23.74%), α-terpineol (4.67% and 5.25%), and α-terpinyl acetate (56.87% and 51.25%)	Stimulant, carminative, stomachic, and diuretic
Chinese cinnamon	Stem bark	*Cinnamomum cassia* (Lauraceae)	Cinnamaldehyde (76%), neral (12.82%), 1,8-cineol (11.32%)	Peripheral vasodilatory, antitumor, antifungal
Camphorwood or camphor laurel	Leaves	*Cinnamomum camphora* (Lauraceae)	D-Camphor (40.5%), camphene (1%), 1,8-cineole (11.3%)	Antiinflammatory

oil occurs only in the flower petals and another kind only in the rind. The individual species would be the particular plant organ that contains the volatile oil, such as the bark, bulbs, flowers, fruit, leaves, rhizomes, roots, seed, or in some cases, the entire plant tops.

7.5.1 Chemical Nature of Volatile Oils and Their Analysis

Essential oils are often classified according to the natural orders of the plants in which they occur, but this system has the disadvantage of obscuring the resemblance in composition and properties of various oils. The chemical nature of the pharmaceutically important essential oils of different types is shown in Table 7.6. The important essential oils used in pharmacy are classified according to their main chemical constituents as follows:

(i) Volatile oils containing hydrocarbons.
(ii) Volatile oils containing alcohols.
(iii) Volatile oils containing cineole.
(iv) Volatile oils containing aldehydes.
(v) Volatile oils containing ketones.
(vi) Volatile oils containing phenolic ether Phenols.
(vii) Volatile oils containing esters.

TABLE 7.6 Essential Oils Classified According to Their More Important Constituents

Class	Oil	Botanical Source	Main Constituents
Oils containing hydrocarbons	Turpentine	Species of Pinus	Terpenes (chiefly D- and L-pinene). Resinous oxidation products
	Juniper	*Juniperus communis*	Terpenes (pinene), sesquiterpenes (cadiene), terpene alcohols (juniper camphor)
	Cubebs	*Piper cubeba*	Sesquiterpenes
	Copaiba	Species of *Copaifera*	Sesquiterpenes
	Cade	*Juniperus oxycedrus*	Sesquiterpenes (cadinene, 20%–30%), phenols (5%)
Oils containing alcohols	Rosemary	*Rosmarinus officinalis*	Alcohols (borneol, 10%–16%), esters (bornyl acetate, 2%–5%), cineole, ketones (camphor), terpenes
	Peppermint	*Mentha piperita*	Alcohols, 50%–80% (1-menthol), esters (5%), aldehydes, ketones, terpenes
	Coriander	*Coriandrum sativum*	Alcohols (D-linalol, 45%–70%), terpenes (20%)
	Lavender	*Lavandula vera*	Alcohols (linalol), esters (linalyl acetate), the proportions differing widely in English and French oils. Cineole, 20%–30%
	Rose	*Rosa damascena*	Alcohols, 70%–75% (geraniol, citronellol), solid hydrocarbons
	Nutmeg	*Myristica fragrans*	Alcohols (D-borneol, geraniol, α-terpineol), phenols (eugenol), phenolic ethers, terpenes
	Sandalwood	*Santalum album*	Sesquiterpense alcohols (α- and β-santalos, 92%–98%)
Oils containing cineole	Cajuput	Species of *Melaleuca*	Cineole (45% or more), hydrocarbons, alcohols, aldehydes
	Eucalyptus	Species of *Eucalyptus*	Cineol (55% or more), other constituents varying with the species of eucalyptus
Oils containing aldehydes	Cinnamon	*Cinnamomum zeylanicum*	Aldehydes (cinnamic aldehyde, 55%–70%), phenols (eugenol), hydrocarbons
	Lemongrass	Species of *Cymbopogon*	Aldehydes, 70%–85% (citral), alcohols, esters, terpenes
	Lemon	*Citrus medica* (vr. Limonum)	Aldehydes (citral, 4%–5%; citronellal, esters, hydrocarbons, 90%) (D-liminene)

Continued

TABLE 7.6 Essential Oils Classified According to Their More Important Constituents—cont'd

Class	Oil	Botanical Source	Main Constituents
Oils containing ketones	Dill	*Peucedanum graveolens*	Ketones (D-carvone, 40%–60%), terpenes
	Spearmint	*Mentha spicata* and *M. crispa*	Ketones (91-carvone, 45%–60%), alcohols, esters, terpenes
Oils containing phenols and phenolic ethers	Ajowan	*Carum copticum*	Phenols (themol, 35%–45%; carvacrol), terpenes
	Cloves	*Eugenia caryophyllus*	Phenols, 84%–95% (eugenol), alcohols, ketones, esters, sesquiterpense
	Aniseed	*Pimpinella anisum* and *Illicium verum*	Phenolic ethers (anethole, 80%–90%)
Oils containing esters	Siberian fir	*Abies sibirica*	Esters (bormyl acetate, 30%–40%), terpenes
	Chamomile	*Anthemis nobilis*	Esters (derived from higher aliphatic alcohols and unsaturated acids), hydrocarbons
	Wintergreen	*Gaultheria procumbens* and *Betula lanata*	Esters (methyl salicylate, 99%)
	Mustard	*Brassica sinapoides*	Esters (ally isothiocyante, 92%–99%)

7.5.2 Hydrocarbons Containing Oils

Open-chain hydrocarbons, such as those of the paraffin series, are of rare occurrence in essential oils, but have been detected in a few cases.

It has been observed that terpene hydrocarbons usually occur in most of the volatile oils obtained from natural sources. These are isomeric hydrocarbons of the molecular formula $C_{10}H_{16}$, closely related to which are the more complex sesquiterpenes, $C_{15}H_{24}$ and diterpenes, $C_{20}H_{32}$. Hydrocarbons containing volatile oil may be further classified into *three* classes as follows: (a) unsaturated acyclic hydrocarbons; (b) aromatic hydrocarbons; and (c) alicyclic hydrocarbons.

7.5.2.1 Unsaturated Acyclic Hydrocarbons

Two typical examples of chemical constituents belonging to the category of unsaturated acyclic hydrocarbons are myrcene and ocimene.

(i) β-Myrcene

Chemical structure: It is 7-methyl-3-methylene-1,6-octadiene.

Occurrence: It is found in several essential oils, such as Oil of Bay (or Myrcia oil)—*Myrcia acris* (Fam. *Myricaceae*); Oil of Hops—*Humulus lupulus* Linn. (Fam. *Moraceae*); and Oil of Turpentine—*Pinus longifolia* Roxb. (Fam. *Pinaceae*).
Isolation: Oil of Bay is treated with sodium hydroxide solution and the remaining undissolved portion, which mostly contains myrcene, is repeatedly subjected to fractional distillation under vacuum (it is also obtained by pyrolysis of β-pinene).

Identification:

(a) β-Myrcene, on reduction with sodium and alcohol (absolute), gives rise to dihydromyrcene. ($C_{10}H_{18}$), on subsequent bromination, yields tetrabromo-dihydro myrcene (mp 88°C).

(b) It readily forms addition compounds with α-naphthoquinone (mp 80–81.5°C) and with maleic anhydride (mp 34–35°C).

Use: It is used as an intermediate in the manufacture of perfumery chemicals.

(ii) Ocimene (or *trans*-β-Ocimene)

Chemical structure: It is 2,6-dimethyl-2,5,7-octatriene.
Occurrence: *trans*-β-Ocimene is found in the volatile oil obtained from the leaves of *Ocimum basilium* L. (*Labiatae*); *Boronia dentigeroides* Cheel (*Rutaceae*); *Litsea zeylanica* C & T Nees (*Lauraceae*); and *Homoranthus flavescens* A. Cunn. (*Myrtaceae*).

Isolation: The volatile oil obtained from the fresh leaves of *O. basilium* is treated first with NaOH solution to get rid of the phenolic constituents, namely, eugenol present in the range of 30%–40% of the oil. The undissolved fraction of the oil is taken up in an appropriate solvent, the solvent is removed under vacuum and the resulting volatile oil is subjected to fractionation under vacuum so as to obtain the desired main constituent.

(a) Reduction with sodium and absolute alcohol yields dihydromyrcene, which on bromination yields tetrabromo-dihydromyrcene (mp 88°C).
(b) It yields ocimenol—an alcohol on hydration with sulfuric acid (50%) in glacial acetic acid solution.
(c) Its phenylurethane derivative has mp 72°C.
(d) Ocimene upon oxidation with $KMnO_4$ in alkaline solution affects complete degradation to form acids, the lead salts of which have a rhombic crystalline form, whereas the corresponding lead salts of myrcene, treated in a similar fashion, have a needle form, thereby differentiating between ocimene and myrcene distinctly.

7.5.2.2 Aromatic Hydrocarbons

A typical example of an aromatic hydrocarbon is as follows:

p-Cymene

Chemical structure: It is 1-methyl-4-(1-methylethyl)-benzene.
Occurrence: It occurs in a number of essential oils, such as oils of lemon, nutmeg, coriander, cinnamon, sage, and thyme. *p*-Cymene has been reported in a number of volatile oils, either due to conversion from cyclic terpenes, for example, pinene or terpinene, or from various terpene analogs, for example, citral, carvone, and sabinol.
Isolation: The *p*-cymene fraction obtained by the fractional distillation of volatile oils may be freed from terpenes having identical boiling points by subjecting it to oxidation with a cold $KMnO_4$ solution, whereby the former being resistant to the oxidizing agent is recovered in its pure form. However, pure *p*-cymene may be prepared from thymol.
Identification: *p*-Cymene, on oxidation with hot concentrated potassium permanganate solution, gives rise to *p*-hydroxy-isopropylbenzoic acid having a melting point of 155–156°C.

7.5.2.3 Alicyclic Hydrocarbon

The alicyclic hydrocarbons are also called "monoterpenes" or "true terpenes" and have the empirical formula $C_{10}H_{16}$. Generally, they may be classified into two categories:

A. Monocyclic terpenes

Basically, the cyclic terpenes are the extended structural homologs of cyclohexane usually derived by varying the extent of dehydrogenation. The parent molecule is methyl-isopropyl cyclohexane (or *para*-Menthane). The structure of the monocyclic terpenes is expressed with reference to the saturated parent substance "menthane," that is, hexahydrocymene. Consequently, the three isomeric menthanes, namely, *ortho*, *meta*, and *para*, theoretically yield the monocyclic terpenes, respectively. Examples include limonene and sylvestrene.

B. Bicyclic monoterpenes

The bicyclic monoterpenes, as the name suggests, essentially possess two cyclic rings that are condensed together. This class of compound is relatively more complex in nature in comparison to the monocyclic species. The second ring system usually contains 2, 3, or 4C-atoms in common and the rings may have 3-, 4-, 5-, or 6-membered rings. Examples include sabinene, α-terpenene, camphene, and D-fenchone.

Phenol volatile oil: Two kinds of phenols occur in volatile oils, those that are present naturally and those that are produced as the result of destructive distillation of certain plant products. Eugenol, thymol, and carvacrol are important phenols found in volatile oils. Eugenol occurs in Clove oil, Myrcia oil, and other oils; thymol and carvacrol occur in Thyme oil, Ajowan oil, and creosol; and guaiacol are present in creosote and pine tar. The more important drugs containing phenol volatile oils are Thyme, Clove, Myrcia oil, Creosote, Ajowan, Tulsi, Pine tar, and Juniper tar.

7.5.3 Oils Containing Alcohol

A good number of alcohols occur abundantly in a plethora of volatile oils, which may be judiciously classified into the following categories:

(a) Acyclic (aliphatic) alcohols.
(b) Monocyclic (aromatic) alcohols.
(c) Alicyclic (terpene and sesquiterpene) alcohols.

(d) Acyclic (aliphatic) alcohols: In general, a number of acyclic alcohols, such as methyl, ethyl, isobutyl, isoamyl, hexyl, and other higher alcohols, occur widely in volatile oils, but being water soluble they are usually eliminated during steam distillation.

They may be further subdivided into two important categories, namely:

7.5.3.1 Saturated Aliphatic Alcohols

Saturated aliphatic alcohols normally contain a few saturated monohydroxy alcohols belonging to the paraffin series, most of which are found to be esterified with fatty acids. In the course of steam distillation, these esters undergo hydrolysis to yield the lower members of the saturated aliphatic alcohols together with the lower fatty acids, though rarely. A variety of substances are duly formed on account of the degradation of complex plant constituents, for example, methanol, ethanol (a byproduct of fermentation due to plant starches), furfural, and butanedione (diacetyl), which ultimately are located in the distillation waters of volatile oils.

7.5.3.2 Unsaturated Aliphatic Alcohols

The unsaturated aliphatic alcohols that frequently occur in volatile oils are nothing but terpene derivatives wherein the six-membered carbon ring is found to be broken at one point only; for example, geraniol, nerol, and linalool.

Many of the simple aliphatic and aromatic alcohols occur in essential oils in the form of esters. Most of the important alcohols found in oils in the free state are derivatives of, or closely related to, the terpenes. Phytoconstituents of this class are:

(a) *Borneol*: It is a secondary alcohol closely related to its chloride. It is a crystalline compound, which sublimes readily and melts at 204°C and has an odor resembling that of camphor.
(b) *Menthol*: It is a saturated secondary alcohol derived from hexahydrocymene. It is a colorless, volatile, crystalline compound with a characteristic odor. On oxidation, it yields the corresponding ketone menthenone.
(c) *Terpin*: It is a dihydroxy derivative of hexahydrocymene and is a synthetic product of importance. Its hydrate (terpin hydrate) is a colorless, volatile, crystalline compound with a characteristic odor.

The main distinguishing character of these types of oils is the determination of the alcohol content and, in that way, it is possible to evaluate this group of oils.

Determination of alcohol

The percentage of total alcohol in an essential oil is determined by a process that involves the conversion of the alcohol into acetates, which are subsequently hydrolyzed with standard alcoholic potassium hydroxide. The best form of apparatus for the acetylation process is a flask with a ground-in reflux condenser (an acetylation flask). The total alcohols are calculated together as the equivalent percentage of that alcohol which is present in the greatest proportion. Thus, the alcohols in oil of rosemary are calculated together as borneol.

Heat 10 mL of oil of rosemary with 10 mL of acetic anhydride and 2 g of anhydrous sodium acetate in an acetylation flask for 2 h. Add water, transfer to a separating funnel, and wash the oily layer by shaking well with several successive quantities of water, until the washings are no longer acid. Dry the acetylated oil by adding anhydrous sodium sulfate, shaking well, and allowing it to stand. Filter, and treat a weighed quantity (2.5 g) of the acetylated oil as bornyl acetate, and hence calculate the percentage of borneol in the original oil.

Example: The acetylated oil of rosemary was found to contain 13.6% of esters, calculated as bornyl acetate, $CH_3COOC_{10}H_{17}$.

Now

$$\frac{C_{10}H_{17}OH}{CH_3 \cdot COOC_{10}H_{17}} = \frac{154}{196}$$

Therefore, the percentage of alcohols in the original oil (calculated as borneol)=

$$\frac{13.6 \times 154}{196} = 10.7\%$$

It should be noted that this method gives the total alcohols, free and combined (the results include the borneol present in the original oil and as esters, mainly bornyl acetate). If it is required to determine the free alcohol only, the percentage of esters in the original oil must be estimated as bornyl acetate and this percentage must be subtracted from the 13.6 before multiplying by the factor 154/196.

Different oils of pharmaceutical importance in this class are described below.

(i) Oil of rosemary

It is obtained from the flowering tops of the rosemary (*Rosmarinus officinalis*), which is largely grown in France and Spain. The constituents of oils distilled from rosemary are not completely known, but the more important appear to be borneol (10%–16%), bornyl acetate (2%–5%), camphor (17%), and cineole (about 20%), the remainder consisting principally of terpenes (pinene and camphene).

(ii) Oils of peppermint

They are produced in Europe and America by the distillation of the herb *Mentha piperita* and its varieties. Japanese peppermint oils are obtained from *Mentha arvensis*.

(iii) Oil of coriander

It is obtained by distillation from the ripe fruits of *Coriandrum sativum*, containing oil of 0.2%–1%; the main constituents are coriendrol together with borneol and geraniol.

(iv) Oil of rose

It is obtained from the fresh flower of *Rosa damascena*; it contains mainly geraniol and citronellol.

(v) Oil of lavender

This is obtained from the flowers of *Lavandula vera*. The oils from it mainly consist of 25%–30% of linalol and 20% cineol.

(vi) Oil of nutmeg

It is obtained from the seeds of the Nutmeg tree *Myristica fragrans*. On distillation, it yields 7%–12% of volatile oil.

7.5.4 Oils Containing Cineole

Cineole, or cineol, sometimes known as eucalyptol or cajuputol, has been mentioned previously as a dehydration product of terpin. It is a colorless liquid with a camphor-like odor and behaves chemically like a fairly stable ether.

7.5.4.1 Estimation of Cineole

Cineole combines with acids (phosphoric, arsenic, hydrochloride, etc.) to form loose compounds that are easily decomposed when treated with water. This fact is commonly used in the estimation of cineole in essential oils.

(i) The phosphoric acid process

Cool 10 mL of oil of eucalyptus in a beaker in a freezing mixture, gradually add about 4 or 5 mL of syrupy phosphoric acid (specific gravity 1.75), and mix well. Press the crystalline mass strongly in calico between filter papers, transfer to a small flask with a graduated neck, and add sufficient warm water to bring the liquid near the top of the graduations. Allow to separate and measure the volume of the oily layer (cineole). Cineole has a specific gravity of 0.93, and the percentage by weight in the oil may thus be calculated, although it is more commonly expressed as percentage volume. This process is described in the British Pharmacopeia, which requires oil of eucalyptus to contain not less than 55%, and oil of cajuput not less than 45%, of cineole by volume.

The above method tends to give low results, and the cresineol method is claimed to be more satisfactory. Cocking pointed out that cineole forms a molecular compound with ortho-cresol, which has been termed "cresineol," and he devised a method, based on this fact, for the cryoscopic determination of cineole in oils. Three grams of the previously dried oil are mixed with 1.2 g of ortho-cresol, and the freezing point of the mixture is determined. From a previously constructed graph on which freezing points are plotted against percentages of cineole, the cineole content of the oil may at once be read off. This process is claimed to give a maximum error of only 3%. For details, see Chapter 4.

(ii) Estimation of 1,8-Cineol

As prescribed in European Pharmacopeia, the estimation of 1,8-cineol can be made using the following procedure.

Weigh 3.0 g of the oil, recently dried with anhydrous sodium sulfate R, into a dry test tube and add 2.1 g of melted o-cresol R. Place the tube in the apparatus for the determination of the freezing point and allow to cool, stirring continuously. When crystallization takes place, there is a small rise of temperature. Note the highest temperature reached (t_1). Remelt the mixture on a water bath at a temperature that does not exceed t_1 by more than 5 degree and place the tube in the apparatus, maintained at a temperature 5 degree below t_1. When crystallization takes place, or when the temperature of the mixture has fallen 3 degree below t_1, stir continuously. Note the highest temperature at which the mixture crystallizes (t_2). Repeat the operation until the two highest values are obtained for t_2. If supercooling occurs, induce crystallization by adding a small crystal of the complex, consisting of 3.0 g of cineole R and 2.10 g of melted o-cresol R. If t_2 is below 27.4 degree, repeat the determination after the addition of 5.1 g of the complex. The content of cineole, corresponding to the highest temperature observed (t2) is obtained, where necessary, by interpolation.

7.5.4.2 Oils of Therapeutic Importance

(a) Oil of cajuput

It is obtained by distillation from the leaves of various species of *Melaleuca*, which are principally grown in the East Indies and Northern Australia.

(b) Oil of eucalyptus

It is found principally in Australia and Tasmania. The trees yielding the oils used in medicine are species of *Eucalyptus*; the oils contain a large proportion of cineole, but little or no phellandrene.

7.5.5 Oils Containing Aldehydes

In general, the aldehydes occurring in a large number of volatile oils are mostly either of aliphatic or aromatic nature. The former type (aliphatic aldehyde), with the exception of citral and citronellal, obviously do not exert any significant role in volatile oils. Interestingly, the lower members of this series, such as formaldehyde and acetaldehyde, do occur most frequently in the "distillation water" of volatile oils. Probably the presence of these lower aliphatic aldehydes is due to degradation and decomposition of relatively more complex chemical constituents in plant products. Owing to their solubility in water, these aldehydes invariably get dissolved in the "distillation water" but may generally accumulate in the "oil of cohobation," in case the distillation waters are redistilled (cohobated). On the contrary, the latter type (aromatic aldehyde) plays a vital role in the essential oils; for instance, the volatile oil of bitter almond is almost entirely comprised of benzaldehyde, whereas that of cassia chiefly contains cinnamic aldehyde. In a broader perspective, the "terpene aldehyde" may be classified into four major categories, namely:

(a) Aliphatic terpene aldehydes: e.g., citral, citronellal.
(b) Cyclic terpene aldehydes: e.g., safranal, phellandral, myrtenal.
(c) Aromatic terpene aldehydes: e.g., cumminaldehyde, vanillin.
(d) Heterocyclic terpene aldehydes: e.g., furfural.

Benzaldehyde, C_6H_5CHO, is the product of hydrolysis of the glucosides contained in many plants, and in such cases forms the bulk of the oil obtained by distillation, as in the case of the volatile oil of almonds. These oils have largely been replaced as flavoring agents by synthetic benzaldehyde.

Cinnamic aldehyde, $C_6H_5CH{=}CH{\cdot}CHO$, is the main constituent of oils of cinnamon and cassia. Citral or geranial (present in oils of lemon, lemongrass, etc.) is the aldehyde corresponding to the alcohol geraniol, from which it is produced on oxidation. Further oxidation of citral results in the formation of geranic acid.

Citral is a liquid that boils at 230°C and that has a strong odor of lemon. It is important both on account of its flavoring properties and its use as a starting substance for the preparation of compounds (ionones) widely used in artificial perfumes. Citronellal is the aldehyde corresponding to the alcohol citronellol. The major therapeutically important essential oils of this class are (i) oil of cinnamon and (ii) oil of lemon.

(i) Oil of cinnamon

It is obtained from the bark of *Cinnamomum zeylanicum*, a tree that is principally grown in Ceylon. The yield of oil is about 0.5%–1.0%. The estimation of the oil of cinnamon can be performed by the following procedure, which can be used as an effective tool for the quality control parameters.

Estimation of oil of cinnamon

The aldehyde content of oil of cinnamon may be determined by conversion of the aldehyde into its bisulfite compounds by shaking with sodium bisulfate solution. A more accurate process consists of shaking the oil with a solution of normal sodium sulfite, from which a soluble sulfonate is formed. The sodium sulfite process proceeds as follows:

To 10 mL of oil of cinnamon, contained in a flask with a graduated neck, add 70 mL of a 1-in-5 aqueous solution of sodium sulfite, and sufficient phenolphthalein to give a distinct pink coloration. Heat the mixture on a water bath, shake well, and neutralize with 30% acetic acid. Repeat the heating and neutralization until the pink color fails to return, a process that occupies from 30 to 45 min. After allowing to cool, add sufficient sodium sulfite solution to force the oily layer into the neck of the flask, where its volume may be read. This volume, subtracted from 10, gives the volume of aldehydes present in 10 mL of the oil.

A similar process may be applied to oil of lemongrass. In this case, the formation of the soluble sulfonate derived from citral may be represented by the following equation:

$$C_9H_{15}{\cdot}CHO + 2Na_2SO_3 + 2H_2O = C_9H_{17}\,(SO_2Na)_2{\cdot}CHO + 2NaOH$$

(ii) Oil of lemon

It is obtained from the fresh peel of *Citrus medica* var. *limonum* and grows in the countries bordering the Mediterranean. The oil is extracted from the peel by various mechanical methods. The process of distillation yields inferior oil, owing to partial decomposition. The estimation procedure for the oil of lemon is described below.

Estimation of oil of lemon

Owing to the small percentage of aldehyde in oil of lemon, the sodium sulfite process, as used for oils of cinnamon and lemongrass, cannot be conveniently employed. The following process depends upon the conversion of the aldehydes into their oximes by means of the excess of a standard hydroxylamine solution, the excess being then determined by back titration.

The hydroxylamine process

To 20 g of the oil, add 20 mL of *N*/2 hydroxylamine hydrochloride, 20 mL of alcohol, and 8 mL of *N*/1 alcoholic potassium hydroxide (in order to liberate most of the hydroxylamine from its salt). Boil the mixture under a reflux condenser for 30 min, cool, dilute with about 250 mL of water, add phenolphthalein, and run in *N*/1 alcoholic potassium hydroxide until the liquid just becomes pink. At this point all the excess of hydroxylamine is present in the free state. Add methyl orange and titrate the hydroxylamine with *N*/2 sulfuric acid. Carry out a blank experiment under identical conditions, but omitting the oil of lemon. Calculate the percentage of citral from the difference of the volumes of standard sulfuric acid required in the blank experiment and in the estimation proper.

$$C_9H_{15}\cdot CHO+NH_2\cdot OH=C_9H_{15}\cdot CH{:}N\cdot OH+H_2O$$

$$2NH_2\cdot OH+H_2SO_4=(NH_2\cdot OH)_2\cdot H_2SO_4$$

Hence, 1000 mL of *N*/2 sulfuric acid neutralizes 16.5 g of hydroxylamine, and this is equivalent to 76 g of citral.

7.5.6 Volatile Oils Containing Ketone

Many of the higher aliphatic ketones occur in small quantities in oils, but much more common are ketones derived from the terpenes. Carvone, which occurs in many essential oils, and piperitone, found in some of the eucalyptus oils, are ketones derived from the monocyclic terpenes. Carvone is a simple derivative of limonene. Camphor, an important dicyclic terpene derivative, has been previously mentioned. It is obtained from the wood of the camphor tree (*Cinnamomum camphora*) by steam distillation. From the product, which is a pasty mass, the solid camphor is separated by expression, and is finally purified by sublimation. The residual oil, known as oil of camphor, contains terpenes, phenols, and other compounds, in addition to camphor itself. Generally, the aromatic ketones are classified into two categories, namely:

(i) *Monocyclic terpene ketones*: This class of compounds essentially contains a single cyclic ring structure along with a ketonic function; for example, L-menthone and carvone.

(ii) *Bicyclic terpene ketones*: This class of compounds essentially contains two cyclic ring structures fused with each other along with a ketonic function; for example, camphor and D-fenchone.

The important oils of this class are described in the next section.

(i) Oil of dill

It is distilled from the fruits of *Peucedanum graveolens*, which are principally grown in Central and Southern Europe. European dill fruits yield from 3% to 4% of oil, which contains from 40% to 60% of D-carvone. The rest of the oil consists almost entirely of terpenes (chiefly D-limonene).

(ii) Oil of caraway

It is obtained by distillation from the fruits of *Carum carvi*, a plant that is largely grown in Holland and in Central and Northern Europe. Caraway oil consists almost entirely of D-carvone (45%–60%) and D-limonene, with about 1%–2% of reduction products of carvone (dihydrocarvone, carveol, and dihydrocarveol).

(iii) Oil of spearmint

It is obtained by distillation from the fresh herb *Mentha viridis*, the yield varying from 0.15% to 0.20%.

7.5.7 Oils Containing Phenol

Thymol and carvachol are the most important of the simpler phenols found in essential oils. Eugenol, a phenol found in oil of cloves, is a particularly important compound on account of its use in the manufacture of artificial vanillin, which has replaced the natural substance obtained from *Vanilla* pods to a considerable extent. The estimation of the phenol constituent is a characteristic test for the oils of this class.

Estimation of phenols

The total phenols in such oils as oil of ajowan and oil of cloves are determined by the following simple process:

Heat 10 mL of the oil with 100 mL of a 5% solution of potassium hydroxide in a flask with a graduated neck on a water bath. Shake well until the phenols have passed into solution in the alkali, cool, add water to bring the residual oil into the neck of the flask, allow to stand, and measure the oily layer.

Example: 10 mL of oil of ajowan left 5.8 mL of residual oil. The percentage of phenols by volume was, therefore, $(10-5.8)\times 10$, that is, 42%.

7.5.7.1 Oils of Therapeutic Importance

Some therapeutically important oils in this class are described below.

(i) Oil of ajowan

It is distilled from the fruits of *Carum copticum*, a plant that is principally grown in India. The fruits yield from 2% to 4% of oil, which contains from 35% to 45% of thymol associated with a small amount of carvachol. The nonphenolic portion of the oil is sometimes known as "thymene," and consists almost entirely of hydrocarbons (cymene, dipentene, and terpinene).

(ii) Oil of clove

It is obtained by distillation from the unexpanded flower heads of *Eugenia caryophyllus*. About 90% of the world's supply of this oil is obtained from the Islands of Zanzibar and Pemba, the remainder coming from Sumatra Penang, and the Seychelles.

Cloves yield from 16% to 19% of oil, the phenol content of which varies from 84% to 95%. Those oils with lower phenol content are known in commerce as "opt" and are used in pharmacy, while the "strong" oils may be used for the production of vanillin. In addition to eugenol, the oil contains about 3% of acetyl eugenol, together with sesquiterpenes (α- and β-caryophyllenes), esters (methyl benzoate), ketones (methyl *n*-amyl ketone), and alcohols.

(iii) Oil of aniseed

It is obtained by distillation from the fruits of the anise (*Pimpenella anisum*) and the star anise (*Illicium verum*). The *Pimpinella* oil is mostly obtained from Spain, South Russia, and Bulgaria, the fruits yielding from 1.5% to 3.5%. It contains from 80% to 90% of the phenolic ether anethole, and smaller quantities of methyl chavicole and oxidation products (anisic aldehyde, etc.). The star anise fruits distilled in Southern China yield from 1.7% to 3.5% of oil, of which rather more than 90% is anethole. Methyl chavicole is also present, together with oxidation products (anisi aldehyde and acid) and hydrocarbons (D-pinene and phellandrene).

7.5.8 Oils Containing Esters

The esters of commonest general occurrence in essential oils are the acetates of terpineol, borneol, geraniol, and linalol, all of which are highly odorous substances. Estimation of the ester constituents is the most important parameter for the quality control of the drug.

Estimation of esters

The esters present in essential oils are determined by hydrolysis with standard alcoholic potassium hydroxide and back titration with standard sulfuric acid, a process similar to that employed in the determination of saponification values of fats, fatty oils, and waxes. Most oils contain a number of esters, but they are all calculated together as the equivalent percentage of the ester present in the greatest proportion.

Some pharmaceutically important oils of this class are described below.

(i) Oil of Siberian fir

Also called oil of pine, it is obtained from the leaves *of Abies sibirica* by distillation. It contains from 30% to 40% of esters, methyl bornyl acetate. Terpenes (pinene, camphene, phellandrene, dipentene), sesquiterpenes, and the hydrocarbon santene, C_9H_{14}, have been detected. Santene is a lower homolog of the terpenes and has been found in sandalwood oil.

(ii) Oil of chamomile

It is obtained by distillation from the flowers of *Anthemis nobilis*, cultivated in Europe and to a lesser extent in America. The oil consists mainly of esters of angelic and tiglic acids, two stereoisomeric unsaturated acids of the oleic acid series. The alcohols from which the esters are derived are isobutyl, amyl, and hexyl alcohols and an alcohol, anthemol $C_{10}H_{15}OH$. The hydrocarbon anthemene and azulene are also present. Azulene is a blue compound and the blue color of the freshly distilled oil is attributed to it.

(iii) Oil of wintergreen

The oil of commerce is obtained by distillation from the leaves of *Gaultheria procumbens* or from the bark of *Betula lanata*. These oils are practically identical in composition, both containing about 99% of methyl salicylate. The presence of small quantities of other substances modifies the odor slightly.

(iv) Oil of mustard

It does not preexist in the black mustard seeds of *Brassica nigra*, but it is obtained from the cake remaining after expression of the fixed oil, by macerating with water, and subsequently distilling. The glucoside, sinigrin, undergoes hydrolysis with the formation of allyl isothiocyanate, glucose, and potassium hydrogen sulfate. The oil contains from 92% to 99% of allyl isothiocyanate, together with small quantities of allyl cyanide and carbon disulfide.

7.5.9 Estimation of Oil of Mustard

Oil of mustard is estimated volumetrically by an interesting process that depends upon the fact that allyl isothiocyanate combines with ammonia to form an additive compound, thiosinamine, which reacts with silver nitrate in alcoholic ammoniacal solution to form allyl cyanamide and silver sulfide.

$C_3H_4{\cdot}N{:}C{:}S+NH_3=C_3H_5{\cdot}NH{\cdot}CS{\cdot}NH_2$

Thiosinamine.

$C_3H_5{\cdot}NH{:}CS{\cdot}NH_2+2AgNO_3+2H=C_3H_5{\cdot}NH{\cdot}CN+Ag_2S+2NH_3{\cdot}NO_3$

Allyl cyanamide.

In the actual estimation, an excess of standard silver nitrate is employed, and the excess is determined, after filtering off the silver sulfide, by titration with ammonium thiocyanate.

Weigh 2 g of oil of mustard and dissolve in sufficient alcohol to produce 100 mL. Transfer 5 mL of this solution to a flask, add 30 mL of *N*/10 silver nitrate and 5 mL of dilute ammonia, and heat on a water bath for half an hour with frequent shaking. Cool, dilute to 100 mL, and filter. To 50 mL of the filtrate (representing 0.05 g of the original oil) add excess of nitric acid, and titrate with *N*/10 ammonium thiocyanate, using ferric sulfate as indicator.

From the equations given above, it will be seen that

$2AgNO_3$ is equivalent to $C_2H_5{\cdot}N{:}C{:}S$.

Hence, 1000 mL of *N*/10 $AgNO_3$ is equivalent to 4.95 g of $C_3H_5N{:}C{:}S$.

For volatile oil of mustard, the British and United States Pharmacopeia prescribe limits of 92% and 93% of ally isothiocyanate, respectively, which are determined by the above process.

7.5.10 Physical Characteristics of Volatile Oils

It is a well-known fact that the volatile oils usually differ from each other with regard to their chemical constitutions. However, they invariably possess a number of physical characteristics, which are stated below.

(a) *Odor*: Most volatile oils do possess a very pleasant and characteristic odor, which varies considerably from one specimen to another.

Identification: When a drop of the volatile oil is soaked on a filter paper, an expert may judge its quality and genuineness and may also differentiate the authentic pure sample from an adulterated one by their individual odors.

(b) *Nature*: In general, the volatile oils are mobile liquids at ordinary temperatures.

(c) *Volatility*: The essential oils are mostly completely volatile.

(d) *Color*: Invariably, the color of freshly obtained volatile oils is more or less colorless, but on prolonged storage they usually undergo both oxidation and resinification, thereby rendering them dark in color. A darkened volatile oil can be redistilled to once again obtain a colorless sample.

(e) *Refractive index*: The refractive index of volatile oils varies from 1.42 to 1.61. They are mostly characterized by high refractive indices.

(f) *Optical rotation*: A large number of volatile oils exhibit optical activity by virtue of their chemical composition. It gives some vital information on the source and authenticity of the oil sample, namely:

- **(i)** Both optical rotation and specific rotation offer a fairly dependable and reliable clue to whether the volatile oil is genuine or adulterated.
- **(ii)** It also establishes the source and variety of the volatile oil, for instance: American oil of Turpentine is dextro, whereas the French oil of Turpentine is levo.
- **(iii)** It ascertains whether the chemical constituent is either isolated from the volatile oil or obtained synthetically, for example: Menthol isolated from peppermint oil is exclusively levo rotatory, whereas synthetic menthol could be either racemic or levo. Likewise, the natural camphor is dextro, whereas the synthetic one could be either racemic or levo.

(g) *Specific gravity*: The specific gravity of volatile oils ranges from 0.8 to 1.17.
(h) *Solubility*: The majority of volatile oils are immiscible with water, but are soluble in absolute alcohol and several other organic solvents, for example, ether, chloroform, carbon disulfide, acetone, hexane, and ethyl acetate.

7.5.11 Qualitative and Quantitative Analysis of Volatile Oils

Volatile oils have a strong smell and do not leave a permanent grease spot on paper. Fixed oils do not have a strong odor and leave a permanent translucent grease spot when applied to paper. Following are the most common test procedures for the detection and analysis of volatile oils in general (Kar, 2008).

- Crush a small sample of the crude drug between the thumb and forefinger, and examine for the presence of an odor.
- Extract 7.1 g of the powdered drug by warming with 10 mL petroleum spirit (boiling point range 40–60°C) in a boiling tube heated on a water bath. Do not let the solvent boil dry. Filter the mixture into an evaporating dish and concentrate the filtrate to about 1 mL on a water bath. Using a pipette, apply one drop of the extract to a filter paper. Expose the paper to a current of warm air and note the occurrence of any translucent area. If this is observed, then oils are present.
- Place the paper in an oven at 105°C for 15 min and if the translucent spot can still be observed after that time, then a fixed oil is present.
- The presence of a volatile oil is detected by the disappearance or diminution of the translucent area.
- Drugs containing volatile oils have a strong odor, particularly if they are crushed between the fingers.
- Volatile oils are obtained commercially by steam distillation and not by solvent extraction.

Some distinctive characteristics of a few volatile oils are summarized below:

- With the exception of a few volatile oils, for example, those obtained from the Citrus fruits and those obtained from flower petals (rose), they are obtained by steam distillation from the plant. All the oils are volatile in steam.
- They all have a distinctive odor.
- They are slightly soluble in water, soluble in alcohol (90%), and to varying degrees in alcohol (70%), though the solutions may be slightly opalescent. They are also soluble in the usual organic solvents.
- Because they are volatile, they do not leave a grease spot when a solution of the oil is allowed to evaporate from paper.
- The weights per milliliter at 20°C and the refractive index are some important distinctive criteria for the oils.
- Because many of the constituents of the volatile oils possess asymmetric carbon atoms, the oils themselves rotate the plane of polarized light. Because the proportions of the constituents are not constant in any given oil, the optical rotation varies within certain limits. Chemically, the volatile oils are quite varied, usually being mixtures of compounds that frequently belong to different chemical groups. Most of them, however, contain, either as the most important constituent or a vehicle for other types of compounds, hydrocarbons known as terpenes. Terpenes have the molecular formula $C_{10}H_{16}$, but there are hemiterpenes (C_5H_8), sesquiterpenes ($C_{15}H_{22}$), and even di- and triterpenes. Terpenes may be divided into two main groups—olefinic and cyclic. The cyclic may be further classified into monocycle and bicyclic. In most of the volatile oils, the important constituents are oxygenated derivatives of terpenes, for example, alcohol, aldehydes, and ketones, and in some cases, esters, phenols, ethers, and peroxides (Kar, 2008).

Oil of nutmeg contains a number of alcohols (linalol, terpineol, geraniol, and borneol), phenols (eugenol, isoeugenol, and safrol), the ether myristicin, and about 88% of terpenes, including, D-pinene, dipentene, and camphene. It is possible to make use of the reactions of many of the constituents in qualitative tests for identification purposes or for detecting adulterants as described below:

- Oil of cinnamon contains cinnamic aldehyde and small amounts of eugenol. The latter will give a blue color with ferric chloride, but this color will be modified by the brown color of the oil to give a green coloration.
- Oil of cinnamon leaf, however, contains only eugenol, so that a full blue color will be obtained.
- Oil of cassia contains only cinnamic aldehyde, so that there will be no change in color.
- It is possible in many cases to evaluate the oil by determining the amount of an important constituent or constituents, for example, Oil of lemon should contain not less than 4% (w/w) of aldehydes, chiefly citral.

Aldehydes react with hydroxylamine hydrochloride to form the oxime, releasing an equivalent amount of hydrochloric acid, which can be determined by titration with standard potassium hydroxide. The reaction is not quantitative unless the hydrochloric acid is removed as soon as it is liberated. The determination is carried out in 60% alcohol, in which oil of lemon is insoluble, thus facilitating the separation of the two layers. The solution of hydroxylamine hydrochloride used in

this determination contains some free hydroxylamine base, so that a correction must be made in the calculation of the result to allow for this. The equivalent of citral is multiplied by 1.008.

$R{\cdot}CHO+H_2NOH{\cdot}HCl=R{\cdot}CH{:}NOH+H_2O+HCl$

$R{\cdot}CHO \equiv HCl \equiv KOH$

152.22 g·Citral≡56 g·KOH

(0.07611×1.008) g·Citral≡1 mL·*N*/2 KOH

Oil of clove should contain between 85% and 90% of eugenol. Eugenol reacts with potassium hydroxide to give potassium eugenate, which is soluble in water, thus leaving the unchanged portion of the oil floating on the surface (Shah, 2009).

One of the most important volatile solid substances, which may be obtained from natural sources, is camphor. It is obtained by steam distillation of the trunk and branches of *C. camphora* and fractionation of the product. The camphor obtained from natural sources is dextro-rotatory.

7.6 FIXED OILS, FATS, AND WAXES

They constitute a major class of compounds from plant and animal sources. They were among the earliest organic substances to be investigated and their general chemical nature was elucidated as far back as 1811 by Chevreul. Almost all of them are the main ester derived from trihydric alcohol, glycerin.

Fixed oils and fats (e.g., soybean oil and theobroma oil) consist primarily of triglycerides of fatty acids and a small proportion of sterols. Fixed oils and fats differ solely in their consistency at room temperature (liquid and solid, respectively) which is a function of the degree of unsaturation and chain length of the fatty acids (greater saturation and chain length raise the melting point). Waxes consist of a heterogeneous mixture of fatty acids, long-chain alcohol, both of their esters, and paraffin. Fixed oils, fats, and waxes are all nonpolar in nature and may be extracted expeditiously and somewhat by selection using solvents, such as light petroleum or hexane. Also, they are dissolved in chloroform and alcohol, which are also used to extract out other types of phytochemicals. On an industrial scale, the method of expression instead of solvent extraction may help to obtain these substances. Whenever the presence of fats and oils interferes with the extraction of other constituents, a de-fatting step involving the solvent extraction of fats is helpful (Houghton and Raman, 1998).

7.6.1 Fixed and Volatile Oils

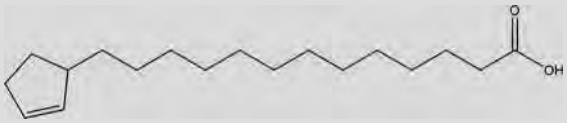
Volatile oils consist mainly of a mixture of small molecules of hydrocarbons containing 10 or 15 carbon atoms. The molecules found in fixed oils, however, are much larger and consist of esters of glycerol and long-chain aliphatic acids. Their solubility properties are similar and thus both types of oil are extracted by nonpolar solvents, such as petroleum ether or dichloromethane. When applied to a piece of filter paper, both oils will leave a translucent grease spot (after evaporation of the extracting solvent). The two types of oil can be distinguished in that volatile oils have a strong smell, whereas fixed oils do not. Additionally, when the filter paper is heated in an oven, a grease spot from a volatile oil will disappear, whereas one from a fixed oil will remain.

7.6.2 Chemical Nature of Fixed Oils, Fats, and Their Analysis

The British Pharmacopeia uses the word "oil" exclusively; oils are usually regarded as being liquids at normal temperatures, whereas fats are solids or semisolids at normal temperatures. All of them exhibit similar chemical and physical properties. Fixed oils and fats are an important group of food reserves, which occur chiefly in seeds, but also to a lesser extent in fruit, leaves, and underground organs. The physical differences between the oils and fats are due in part to the varying proportions of saturated and unsaturated acids in the glycerides. The glycerides of unsaturated acids are usually liquids, for example, olein and linolein, while the glycerides of the saturated acids are usually solids, for example, palmitin and stearin. Many glycerides are common to a large number of oils and fats, for example, olein, palmitin, and searin, while some are confined to specific oils or groups of oils, for example, petroselining occurs only in the oils of the umbelliferous fruits.

Chemically they are mixtures of glycerides—esters of glycerol and various acids. Glycerol, being a trihydric alcohol, can form mono-, di-, and triesters, but in the naturally occurring oils and fats only the triglycerides are found. When the same acid occurs esterified to all three alcoholic groups, the glyceride is said to be a simple glyceride, but when two or three different acids are present, the glyceride is a mixed glyceride. According to Hilditch, oils and fats consist chiefly of mixtures of mixed glycerides and it is only in those oils in which one component acid predominates that there is a relatively high proportion of the simple glyceride (Shah, 2009; Kar, 2008). The fixed oils or fats contain either saturated or unsaturated acids and are usually members of the fatty acid series, as mentioned in Table 7.7.

TABLE 7.7 Series of Fatty Acids With Increasing Carbon Chain Length (Bauer, 1998)

Name	Formula
Saturated monobasic acids	$C_nH_{2n+1}COOH$
Caproic acid	$C_5H_{11}COOH$
Caprylic acid	$C_7H_{15}COOH$
Capric acid	$C_9H_{19}COOH$
Lauric acid	$C_{11}H_{23}COOH$
Myristic acid	$C_{13}H_{27}COOH$
Palmitic acid	$C_{15}H_{31}COOH$
Stearic acid	$C_{17}H_{35}COOH$
Arachidic acid	$C_{19}H_{39}COOH$
Unsaturated monobasic acids	$C_nH_{2n-1}COOH$
Tiglic acid	$C_4H_7COOH\ \Delta^2$
Oleic acid	$C_{17}H_{33}COOH\ \Delta^9$
Petroselinic acid	$C_{17}H_{33}COOH\ \Delta^6$
Erucic acid	$C_{21}H_{41}COOH\ \Delta^{13}$
Unsaturated monobasic acids	$C_nH_{2n-3}COOH$
Linoleic acid	$C_{17}H_{31}COOH\ \Delta^{9,12}$
Hydonocarpic acid	
Chaulmoogric acid	
Unsaturated monobasic acids	$C_nH_{2n-5}\ COOH$
Lenolenic acid	$C_{17}H_{29}COOH\ \Delta^{9,12,15}$
Monobasic hydroxy acids	$C_nH_{2n-2}\ (OH)\ COOH$
Ricinoletic acid	$C_{17}H_{32}\ (OH)\ COOH\ \Delta^9$

Two important aspects of their chemical behavior can be described by the chemical structure of the glycerides:

- In the presence of moisture, hydrolysis is possible, giving free acids. This may be realized by enzyme action or as a result of exposure to air and light.
- Glycerides containing unsaturated acids may become oxidized and then polymerized to form insoluble resin-like substances. The oils may in fact be divided roughly into three groups according to the degree of unsaturation and stability in air. They are the nondrying oils, the partial drying oils, and the drying oils.

A number of oils and fats are used as phytoconstituents and among them the most commonly encountered oils and fats in pharmacy are as follows.

7.6.2.1 Fixed Oils of Therapeutic Importance

- Almond oil, from the kernels of *Prunus amygdalus* var. *amara* or var. *dulcis*.
- Arachis oil, from the seeds of *Arachis hypogaea*.

- Castor oil, from the seeds of *Ricinus communis*.
- Cottonseed oil, from the seeds of *Gossypium* spp.
- Linseed oil, from the seeds of *Linum usitatissimum*.
- Olive oil, from the fruit of *Olea europaea*.
- Palm oil, from the fruit of *Elaeis guineensis*.
- Palm-kernel oil, from the kernels of *E. guineensis*.
- Peach-kernel oil, from the kernels of *Prunus persica*.
- Rape of Colza oil, from the seeds of *Brassica* spp.
- Soybean oil, from the seeds of *Glycine hispida*.
- Sesame oil, from the seeds of *Sesamum indicum*.

7.6.2.2 Fats of Therapeutic Importance

Coconut oil, from the kernels of *Cocos nucifera*.
Hydnocarpus oil, from the seeds of *Hydnocarpus wightiana*.
Japan wax, from the fruits of *Toxicodendron succedaneum*.
Nutmeg butter from the kernels of *M. fragrans*.
Theobroma oil (Cocoa-butter) from the roasted seeds of *T. cacao*.

7.6.3 Main Characteristic Features of Fixed Oils

- Fixed oils are viscous liquids and have a slight odor and bland taste. Their colors vary, for example, olive oil is pale yellow to greenish yellow, linseed oil is yellowish brown.
- They are insoluble in water and alcohol (90%), but soluble in ether, chloroform, petroleum ether, and carbon tetrachloride.
- They leave a grease spot when the solvent is allowed to evaporate from a drop of the solution placed on paper.
- Some degree of the unsaturation present is indicated by the addition of bromine water, the color of the bromine disappearing quite rapidly if there is a high proportion of unsaturated glyceride, as in linseed oil.
- While evaluating the physical properties of the oils, it has been observed that the properties of these oils vary very little. The weight per milliliter and the refractive index are two most important features of oils.
- Theobroma oil is a yellowish-white solid fat with a distinct and characteristic odor. It is somewhat brittle, but when warmed to about 25°C it softens and actually melts between 29 and 34°C.
- Hydrocarpus oil is soft cream-colored fat, which melts between 20 and 25 degrees. Nutmeg also contains solid fat in the endosperm.
- One important feature of a fixed oil or fat is the acid value. This can be defined as the number of milligram of potassium hydroxide required to neutralize the free acid in 1 g of the substance. A high acid value indicates excess acid, which may be present because of faulty preparation or because of the development of rancidity. The acid values of some pharmaceutically important oils are shown in Table 7.8.
- Oils and fats may be hydrolyzed by boiling with excess aqueous alkali. The products are glycerol and the alkali salts of the acids. These are known as soaps and hence the process is called saponification.
- The saponification value of a fixed oil or fat is defined as the number of milligram of potassium hydroxide required to neutralize the fatty acids resulting from the complete hydrolysis of 1 g of the substances. An alcoholic solution of potassium hydroxide is used. The figure includes the amount of free acid that may have been present. It will detect such adulterants as liquid paraffin. Oils and fats also contain a small amount, usually not more than 0.5%, of unsaponifiable matter. This is composed chiefly of steroid material known as phytosterols.
- A very good indication of the degree of unsaturation present in fixed oils or fats may be obtained by ascertaining the amount of bromine or iodine absorbed by the substance. A number of different methods are available, but the one using iodine monochloride is the commonest. It is known as Wij's method. The iodine value of a fixed oil or fat is the weight of iodine absorbed by 100 parts by weight of the substance. The conditions referred to in the details of the method must be followed if constant results are to be obtained. There must be good excess of iodine monochloride present, and if the amount of reagent used up exceeds 50% of that available, then the determination must be repeated using a smaller quantity of the oil.

Chemically, the waxes are esters of the higher fatty acids with monohydric alcohol of high molecular weight. The most common acids and alcohols encountered in the production of waxes are shown in Table 7.9. The waxes may also contain free acids, free alcohol, high-molecular-weight ketones, and high-molecular-weight hydrocarbons.

TABLE 7.8 Acid Value and Saponification Value of Pharmaceutically Important Oils (Pferschy-Wenzig and Bauer, 2015)

Name of Oil	Acid Value	Saponification Value
Almond oil	4.0	187–195
Arachis oil	2.0	188–193
Castor oil	4.0	190–195
Cottonseed oil	0.5	177–187
Hydnocarpus oil	6.0	198–204
Linseed oil	5.0	190–198
Olive oil	2.0	188–195
Sesame oil	2.0	188–196
Theobroma oil	3.0	188–196

TABLE 7.9 The Most Common Acids and Alcohols With Their Molecular Formula Encountered in the Formation of Waxes

Acid	Molecular Formula	Alcohol	Molecular Formula
Palmitic acid	$C_{15}H_{31}COOH$	Cetyl alcohol	$C_{16}H_{33}OH$
Carnaubic	$C_{23}H_{47}COOH$	Carnaubyl	$C_{24}H_{49}OH$
Cerotic acid	$C_{25}H_{51}COOH$	Ceryl alcohol	$C_{26}H_{53}OH$
Melisic acid	$C_{29}H_{59}COOH$	Melissyl alcohol	$C_{30}H_{61}OH$

A common vegetable wax encountered in pharmacy in Carnauba wax, which occurs as a thin coating on both surfaces of the leaves of the palm, *Copernicia cerifera*. It consists chiefly of melissyl cerotate, but contains, in addition, free melissyl and ceryl alcohols and free carnaubic acid. It is soluble in 90% alcohol on warming, as well as being soluble in the usual fat solvents. Waxes differ from fats in as much as they cannot be hydrolyzed with aqueous alkali but only by refluxing with excess alcoholic potassium hydroxide. Beeswax is, of course, the commonest wax encountered in pharmacy, but it is not of plant origin.

7.7 PHENYLPROPANOIDS AND THEIR ANALYSIS

Phenylpropanoids are a diverse group of secondary metabolites, which are different in nature, and they are phenylalanine-derived chemicals that are included in plant phenylpropanoids. Phenylpropanoids assist plants in interacting with the environment. The action of enzymes and enzyme complexes is the major reason for diversity in phenylpolypropanoids; these actions bring about changes in the reactions (Noel et al., 2005). A wide range of natural products are included in phenylpropanoids. These products are derivative of the aromatic amino acids phenylalanine and tyrosine or intermediates of shikimic acid biosynthetic pathways. The structure of phenylpropanoids contains a phenyl ring attached to a three-carbon propane side chain in their compounds and lacks nitrogen (Robbers et al., 1996).

7.7.1 Classification of Phenylpropanoids

The classification of phenylpropanoids is merely based on the nature of the biosynthesis of phenolic compounds, in which cinnamyl alcohols and cinnamic acids play the key role (Kurkin, 2003).

Phenylpropanoids are a large class of natural compounds, which consists the following groups:

I. Simple phenylpropanoids

These are called "simple" because they have low molecular weight and have slight basic modifications form the phenylpropanoid biosynthetic intermediates.

- **(a)** Cinnamyl alcohols and their derivatives (ethers, glycosides)
- **(b)** Cinnamic acids and their derivatives (esters, glycosides, other derivatives)
- **(c)** Cinnamamides
- **(d)** Cinnamaldehydes
- **(e)** Phenylpropanes

II. Complex phenylpropanoids

- **(a)** Phenylpropanoid glycosides based on phenylethanes.
- **(b)** *Lignoids*: Flavolignans; xanthonolignans; coumarinolignans; alkaloido-lignans; neolignans; lignans (dimers and oligomers of phenylpropanoids).

III. Biogenetically related phenylpropanoids

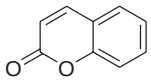
This class includes flavonoids, coumarins (Kurkin, 2003).

7.7.2 Individual Classes of Phenylpropanoids With Examples

Types of phenylpropanoids with example:

(a) Simple phenylpropanoids

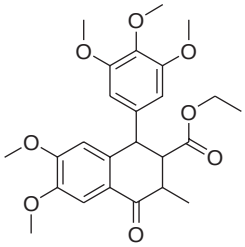
Coumarins



Coumarin

Coumarin is the lactone of o-hydroxyl-cinnamic acid; it is named after a Caribbean tonka tree (coumarou). Coumarins are generally colorless, prismatic crystals with a bitter burning taste and that have a characteristic odor. They are freely soluble in alcohol (Robbers et al., 1996).

(b) Lignans and neolignans



Lignan

Lignins are generally present in woody tissues due to their relation with polymeric lignin present in the plant cells. Lignans are dimericphenylpropanoid derivatives and their formation is manifested by the primary step in their production, which is reduction of ferulic acid to coniferyl alcohol (Robbers et al., 1996). Generally, monolignols are derived from hydroxycinnamic acids (*p*-coumaric, ferulic, andsinapic acids).

Monolignols are either dimerized to lignans in the cell or polymerized into larger lignin structures in the cell wall. Lignans are structurally diverse compounds and are involved in plant defense as antioxidants, biocides, and phytoalexins (Vogt, 2010). They provide protection against diseases and pests, and possibly participate in plant growth control. Generally, lignans are mistaken for lignins and vice versa, but they are very different in nature. Lignans are stereospecific dimers of these cinnamic alcohols (monolignols) bonded at carbon 8 (C8–C8). In the plant, lignans (monolignol dimers) usually occur free or bound to sugars. Diglucosides of pinoresinol, secoisolariciresinol, and syringaresinol are common. Sesaminoltriglucoside and sesaminoldiglucoside occur in sesame seeds. In flax, secoisolariciresinol is present as a diglucoside and is part of an ester-linked complex oroligomer containing 3-hydroxyl-3-methylglutaric acid, a number of cinnamic acidglycosides (usually ferulic or *p*-coumaric acid) and the flavonoid herbacetin. The plant lignans most commonly

distributed in foods are lariciresinol, matairesinol, pinoresinol, and secoisolariciresinol. Several other lignans are present in some foods, including medioresinol (in sesame seeds, rye, and lemons), syringaresinol (in grains), sesamin, and the lignan precursor sesamolin (in sesame seeds). Other lignans found in foods but not often quantified include arctigenin, cyclolariciresinol (isolariciresinol), 7′-hydroxymatairesinol, and 7-hydroxysecoisolariciresinol. (Some cyclolariciresinol occurs naturally and some is formed from lariciresinol during extraction and analysis under acidic conditions.) The nutritional significance of lignans is unknown. Although lignans are not classified as dietary fibers, they share some of the chemical characteristics of lignin, which is an insoluble fiber. Lignins are large plant polymers built from the *p*-coumaryl, coniferyl, and sinapylhydroxycinnamic alcohols. They are racemic (nonstereospecific) polymers, with monolignol units binding at C8 and four other sites (C5–C5, C5–C8, C5–O–C4, C8–O–C4). Lignins are found in vessels and secondary tissues of all higher plants. They are present in a large variety of foods and are particularly abundant in cereal brands. Nutritionally, lignins are considered components of insoluble dietary fiber. Lignins are important in plants because they strengthen the plant cell walls, aid water transport, keep polysaccharides in the plant cell walls from degrading, help plants resist pathogens and other threats, and provide texture in edible plants (Hahlbrock and School, 1989).

7.8 FLAVONOIDS

Flavonoids are naturally occurring phenolic compounds that are structurally derived from flavones, the parent substances occurring in nature as a white mealy farina on *Primula* plants. Flavonoids are mainly water-soluble compounds; they can be extracted with 70% of ethanol remain in the aqueous layer, following partition of this with petroleum ether. Due to changes in the color of flavonoids on treatment with base or ammonia, they are easily detected on chromatograms or in solution. Flavonoids show intense absorption bands in the UV and visible regions of the spectrum as they contain a conjugated aromatic system. Generally, it has been observed that all flavonoids are found bound to sugar in plants, and any one flavonoid aglycone may occur in a plant in several glycosidic combinations. It is always suggested when analyzing flavonoids to examine the aglycones present in the plant extract. There are many classes of flavonoids present in all vascular plants, some more widely distributed than others. Isoflavones and biflavonyls are generally found in few plants (Robbers et al., 1996).

Flavonoids are one of the most widely distributed compounds, with over 2000 different compounds reported. Flavonoids are always present in plants as mixtures and are rarely found as single components. It has also been observed that they are present in mixture form with other flavonoid classes. Classification is primarily based on the study of solubility properties and color reactions. The chemical structures of flavonoids are based upon the C6–C3–C6 carbon skeleton with a chroman ring bearing a second aromatic ring in position 2, 3, or 4. There are five major categories of flavonoids: flavones, flavanones, flavonols, anthocyanidins, and isoflavones. There are some exceptions, such as aurones in which the six-membered heterocyclic ring is replaced by a five-membered ring, and chalcones, which are present in an open-chain isomeric form (Robbers et al., 1996).

7.8.1 Classification

Flavonoids belong to a large group of phenolic plant constituents. They are presented as derivatives of 2-phenyl-benzo-γ-pyrone. In flavonoid molecules, the carbon atoms are assembled in two benzene rings, commonly denoted as A and B, and an oxygen-containing pyrene ring C connects them. A common part in the chemical structure of all flavonoids is one based on the flavan system (C6–C3–C6). Chalcone is formed by condensation of the A and B rings, which undergo cyclization involving isomerase to form flavanone, the initial compound for the synthesis of flavonoids. Due to the structural differences, flavonoids are classified as flavanols, flavanones, flavonols, isoflavones, flavones, and anthocyanins. Other flavonoid compounds include biflavonoids (e.g., ginkgetin), prenylflavonoids, flavonolignans (e.g., silybin), glycosidic ester flavonoids, chalcones, and proanthocyanins (Brodowska, 2017). Individual classes of flavonoids with examples are discussed below.

7.8.1.1 Flavanols

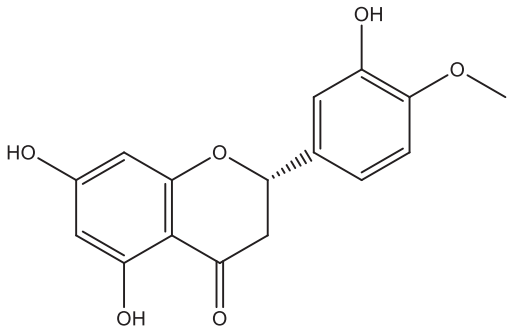
Flavanols constitute a complex group of polyphenols in the range from the monomeric flavan-3-ols (e.g., catechin, epicatechin, gallocatechin) to polymeric procyanidins, known as condensed tannins. Flavanols mainly occur in fruits and derived products, for example, fruit juices or jams. This group also appears in tea, red wine, cocoa, apples, kiwi, and cereals. However, they almost do not exist in vegetables and legumes except for lentils and broad beans. Flavanols can be found in the peels or seeds of fruits and vegetables as well, which are often removed during eating or processing; therefore, their intake is also limited (Brodowska, 2017). This class of polyphenolic phytochemicals occurs in commonly consumed vegetables, fruits, and plant-based beverages. Major sources of these compounds are the parts of grape

berries, apple, tomato, onion, broccoli, and red lettuce. In addition to fruits and vegetables, beverages, such as green tea, black tea, and red wine, also constitute a significant source of flavonols, such as quercetin, kaempferol, or myricetin (Brodowska, 2017).

7.8.1.2 Flavanones

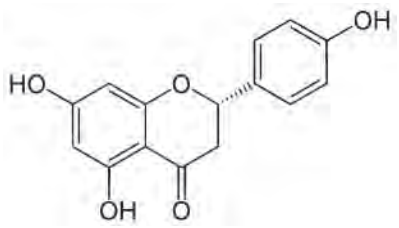
Flavanones are extensively disseminated in around 42 larger plant families, especially in Compositae, Leguminosae, and Rutaceae. Depending on the type of plants, flavanones can be discovered in all of the parts—above and below ground, from vegetative parts to generative organs: branches, bark, stem, leaves, roots, flowers, fruits, seeds, rhizomes, peels, and others. Due to the wide spread of flavanones in foods, naringenin, and hesperetinaglycones seem to be of particular interest.

1. Hesperetin (4′-methoxy-5,7,3′-trihydroxyflavanone) is a distinctive flavanone of lemon, orange, lime, and tangelo. Naringenin (5,7,4′-trihydroxyflavanone) can be found in grapefruit and sour orange. Tomatoes and their products are also rich in this flavonoid.



Hesperetin

2. Naringenin can be described as both an aglycone or a glycoside (Brodowska, 2017).



Naringenin

7.8.1.3 Isoflavones

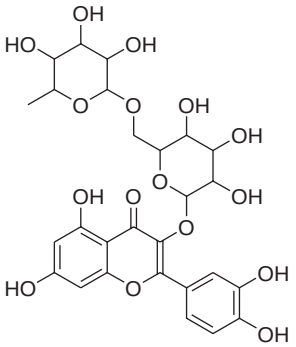
Isoflavones are a distinctive and very important subclass of flavonoid compounds. Their structures constitute the 3-phenylchromen skeletons, which are chemically derived from the 2-phenylchromen skeletons by an aryl-migration mechanism. Isoflavones are mostly found in legumes, especially in soy. However, their presence has also been reported in green split peas, split peas, chickpeas, black beans, lima beans, clover sprouts, and sunflower seeds. Furthermore, these compounds are included in the composition of several foods, vegetarian formulations, and soy products in infant foods, among others. The major isoflavones in the human diet are genistein and daidzein, which exist in four related chemical structures, namely, aglycones, the 7-*O*-glucosides, the 6′-*O*-acetylglucosides, and the 6′-*O*-malonylglucosides (Brodowska, 2017).

7.8.1.4 Flavones

Flavones are very similar structurally to flavonol compounds, having an extra hydroxyl substitution at the carbon 3-position. The major flavones include apigenin and luteolin. Luteolin occurs in vegetables and fruits, such as broccoli, celery, carrots, parsley, onion leaves, cabbages, peppers, chrysanthemum flowers, and apple skins. Apigenin can be found in onions, parsley, wheat sprouts, tea, oranges, chamomile, and in some seasonings (Brodowska, 2017; Salvatore, 2010).

7.8.2 Examples of Classes of Flavonoids

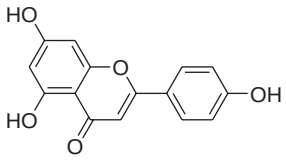
7.8.2.1 Rutin



Rutin

Rutin, the rhamnoglucoside of the flavonoid quercetin, is abundantly found in many plants. Rutin is a citrus flavonoid glycoside found in many plants, such as *Capparis spinosa*, apple, *Lycopersicon esculentum* (Griffith et al., 1959). Tartary buckwheat seeds and common buckwheat seeds contain rutin. Rutin can be found in leaves and flowers of rue, leaves of buckwheat, flowers of pansy, and flowers of rose, as well as the leaves, flower buds, and fruits of *C. spinosa*. Rutin increases intracellular ascorbic acid levels and decreases capillary permeability and fragility, as well as scavenging oxidants and free radicals. It inhibits destruction of bones and lowers the risk of heart disease. It possesses anticancerous and antifungal effects and also antihyperglycemic and antioxidant activity. It is included in some preparations as a dietary supplement.

7.8.2.2 Apigenin

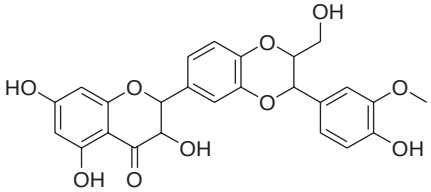


Apigenin

Apigenin is mainly found in herbs, fruits, and vegetables. It is a pigment, yellow in color. Apigenin is found mainly in leaf and flower. Shanghai yellow heart and ventura are two plants indigenous to China that have been observed to have accumulated vast amounts of apigenin (Yan et al., 2014). Apigenin is a yellow-pigmented flavonoid, which is generally found in herbs, fruits, and vegetables. Apigenin is considered a phytoestrogen due to its pharmacological and biological characteristics, which are attributed to the presence of three hydroxyls at 40, 5, and 7 and double bonds at C2 and C3 (Lee et al., 2015). It has antiinflammatory, antioxidant, and anticancer properties.

7.8.2.3 Milk Thistle

The scientific name of milk thistle is *Silybum marianum* (L.) Gaertn. It is an annual or biannual species, which belongs to the Asteraceae family. The species is native to southern Europe, Asia Minor, and northern Africa and it is naturalized in North and South America, Australia, and New Zealand (Martinelli et al., 2017). This herb has been widely used and extensively investigated as a cure or preventive for a wide range of liver problems. A crude mixture of antihepatotoxic principles was first isolated from the plant and designated silymarin (Mukherjee, 2002). Silybin has two diastereoisomers A and B; SILA, SILB; isosilybin (diastereoisomers A and B, ISOA, ISOB); silychristin (SILYC), silydianin (SILYD), silychristin B, isosilychristin (Martinelli et al., 2017).



Silymarin

The plant is native to the Mediterranean and grows throughout Europe and North America. It also grows in India, China, Africa, and Australia (Dixit et al., 2007). Silymarin is a complex mixture of polyphenolic molecules. Seven closely related flavonolignans are included in silymarin, namely, silibin A, silibin B, isosilibin A, isosilibin B, silichristin, isosilichristin, silidianin, and the flavonoid taxifolins (Hellerbrand et al., 2016). Silymarin protects intact liver cells by preventing the entry of toxic substances. Silymarin stimulates protein synthesis, which results in acceleration of the regeneration process, eventually leading to the production of hepatocytes. In chronic inflammatory liver conditions and cirrhosis, it can be used as a supportive treatment (Mukherjee, 2002).

7.8.2.4 Quercetin

Quercetin is found extensively in foods. Plants, starting from the amino acid phenylalanine, synthesize quercetin. Initially, phenylalanine (1) is converted to 4-coumaroyl-CoA (2) in a series of steps known as the general phenylpropanoid pathway using phenyl ammonialyase, cinnamate-4-hydroxylase, and 4-coumaroyl-CoA ligase. Four 4-coumaroyl-CoA and three molecules of malonyl-CoA (3) using 7,2′-dihydroxy,4′-methoxyisoflavanol synthase, form tetrahydroxychalcone (4). Chalcone isomerase catalyzes the conversion of tetrahydroxy chalcone into naringenin (5). Flavonoid 3′ hydroxylase helps in conversion of naringenin to eriodictyol (6) and eriodictyol is then converted to dihydroquercetin (7) with the help of flavanone 3-hydroxylase, which is then converted into quercetin using flavanol synthase (Salvatore, 2010).

7.8.2.5 Hesperetin

Hesperetin was synthesized by the action of sulfuric acid hydrolysis of hesperidin in CH_3OH. In the biosynthetic pathway, acetone and potassium carbonate help in the formation of hesperetin derivatives (Wang et al., 2016).

7.9 RESINS AND RESINOUS PLANT DRUGS AND THEIR ANALYSIS

7.9.1 Resins and Their Importance in Phytotherapy

Lignans and neolignans are typically found as dimeric phenylpropanoid derivatives chemically related to the polymeric lignins of the plant cell and are found in woody tissues. Reduction of ferulic acid to coniferyl alcohol is a primary step in the production of lignans, which are formed via oxidative dimerization of coniferyl alcohol units and linked through the beta carbon atom of the C_3 side chains (Suzuki and Umezawa, 2007). Typically, they are found as single enantiomeric forms, but racemic products are also encountered. They vary substantially in oxidation levels, degree of substitution, and structural complexity. Neolignans are formed by asymmetrical carbon–carbon links in the side chains. Lignans and neolignans play an important role in plant defense as antimicrobial, antifungal, and antiviral agents. Lignans are of considerable pharmacological interest. Some of the important resins in phytotherapy, along with their constituents, are shown in Table 7.10.

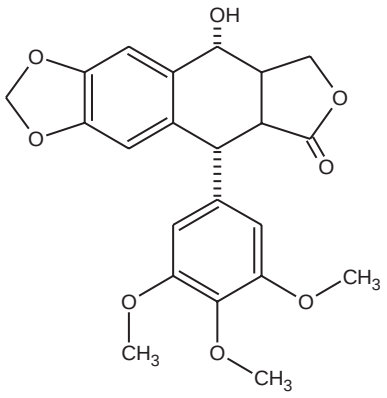
Resins form an ill-defined group of plant products, which are produced normally during growth or secreted as a result of an injury to the plant. As a class, resins are related to one another more by their physical properties and appearance than by any similarity in chemical composition. They are usually brittle amorphous solids, which fuse readily on heating after passing through a preliminary stage of softening. They are insoluble in water, but dissolve in alcohol or other organic solvents, forming solutions, which, on evaporation, deposit the resin as a varnish-like film. Resins burn with a characteristic smoky flame (Mukherjee, 2002).

7.9.1.1 Podophyllum

Podophyllum consists of the dried rhizome and roots of *Podophyllum peltatum* (Fam. Berberidaceae). It is also known as mayapple or American Mandrake. *Podophyllum* contains 3.5%–6% of a resin whose active principles are lignans. These include podophyllotoxin (20%), alpha-peltatin (10%), and beta-peltatin (5%). A number of lignan glycosides are in the plant, but because of their water solubility, they are lost during the normal preparation of the resin. The antimiotic and purgative properties of these compounds depend on a lactone ring in the *trans* configuration. Treatment with mild alkali produces epimerization with formation of the stable *cis* isomers, which are physiologically inactive. Picropodophyllin is an inactive *cis* isomer produced in this way from the active *trans* podophyllotoxin. *Podophyllum* yields not less than 5% of *Podophyllum* resin. *Podophyllum* possesses drastic purgative properties. Its resin is also employed as an antimitotic and caustic. Indian *Podophyllum*, the rhizome of podophyllin, is the powdered mixture of resins removed from *Podophyllum* by percolation with alcohol and by subsequent precipitation from the concentrated percolate when added to acidified water. The precipitated resin is washed twice with water and is dried and powdered. It is an amorphous powder that varies in color from light brown to greenish yellow and that turns darker when subjected to temperatures exceeding 25°C or when exposed to light. It has a slight, peculiar, bitter taste and is highly irritating to the eye and to mucous membranes in general.

TABLE 7.10 Resins of Therapeutic Importance and Their Constituents

Resin	Botanical or Zoological Source	Constituents		
		Resin Alcohols	Resin Acids	Resenes
Burgundy pitch	*Picea excelsa*	—	α- and β-picea-pimarolic acids, picea-pimarinic acids, picea-pimaric acid	Juroresene
Colophony	Species of *Pinus*	—	82%–90% chiefly abietic acid $C_{20}H_{30}O_2$	5%
Dragon's blood	Species of *Daemonorops*	Dracoresinotannol as its benzoic and benzoyl acetic esters (57%)	—	Dracoresene (14%)
Guaiacum	*Guaiacum officinale* and *G. sanctum*	—	α- and β-guaiaconic acids (70%), guaiaretic acid (11%), guaiacic acid	—
Mastich	*Pistacia lentiscus*	—	α- and β-masticonic acids (38%), α- and β-masticic acids (4%), masticolic acid (0.5%)	α- and β-masticoresenes (50%)
Sandarac	*Callitris quadrivalvis*	—	Sandaracopimari acid (85%), sandaracinic acid, sandaracinolic acid	Sandaracoresene
Shellac	*Coccus lacca*	—	Aleuritic acid, $C_{15}H_{28}(OH)_3$. COOH, and shelloic acid, probably combined together	—

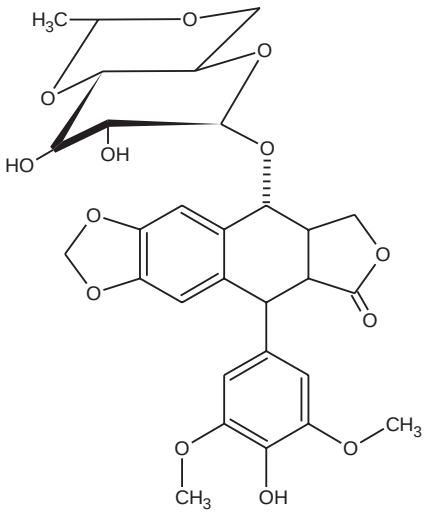


Podophyllotoxin

Podophyllum resin is a caustic for certain papillomas. It is applied topically as a 25% dispersion in compound bezoin tincture or in a 70%–96% solution of alcohol. *Podophyllum* resin has also been used as a drastic purgative and as a hydragogue cathartic. A number of lignans with lactone rings in the *trans* configuration are tumor-inhibiting constituents of *Podophyllum* resin. Such compounds include podophyllotoxin, several podophyllotoxin derivatives, and alpha- and beta-peltatin. The peltatins are responsible for most of the purgative effects of the drug.

7.9.1.2 Etoposide

Etoposide is a semisynthetic podophyllotoxin derivative used as an antineoplastic agent. It differs structurally from podophyllotoxin by having an ethylidene glucoside attached at the C-1 position; it has an epimeric configuration at the C-4 position of ring C, and there is a hydroxyl group at the C-4′ position rather than a methoxy group. This hydroxyl group is associated with etoposide's ability to induce single-stranded DNA breaks, and the ethylidene glucoside moiety is associated with the drug's inability to inhibit microtubule assembly, a property which may decrease the toxic effects associated with podophyllotoxin. Etoposide is used in combination with other chemotherapeutic agents for refractory testicular tumors and as a first-line treatment in small-cell lung cancer.



Etoposide

It has also been used in the treatment of acute nonlymphocytic leukemias, Hodgkin's disease, non-Hodgkin's lymphomas, Kaposi's sarcoma, and neuroblastoma.

7.9.1.3 Teniposide

It is a semisynthetic derivative of podophyllotoxin, which is used as a component of multidrug antineoplastic regimens for induction therapy in childhood acute lymphoblastic leukemia that is refractory to induction with other therapy. Teniposide differs structurally from etoposide by the addition of a thenylidene ring on the glucopyranoside ring. Its mechanism of action is postulated to be similar to that of etoposide.

7.9.2 Chemical Classes of Resins and Their Analysis

Many resins are believed to be oxidation products of the terpenes. They are usually more-or-less complex mixtures, and their main constituents may be conveniently classified as follows:

Resin alcohols

Complex alcohols of high molecular weight are distinguished as "resinotannols" or "resinols," according to whether they do or do not give a tannin reaction with iron salts. Resin alcohols are present in resins both in the free state and as "resin esters" in combination with simple aromatic acids (such as benzoic, salicylic, cinnamic, and umbellic).

Resin acid

Substances of exceedingly complex composition, which usually combine the properties of carboxylic acids and phenols, are called resin acids. They occur in resins both in the free state and as esters. The free acids are soluble in aqueous alkali, usually forming soap-like solutions or colloidal suspensions. The metallic salts of resin acids are known as resinates. Some of them find extensive applications in the manufacture of cheap soaps and varnishes.

Resenes

Complex neutral substances, practically devoid of characteristic chemical properties, are called resenes. They do not form salts or esters, and are insoluble in, and resist hydrolysis by, alkalis.

Test for colophony

(i) Dissolve in petroleum spirit and shake with a 0.1% solution of cupric acetate. A slowly developing emerald-green color should be noted.

(ii) Pour about 20 drops of a 1% solution of colophony in acetic anhydride into a porcelain dish. Add 2–3 drops of concentrated sulfuric acid. A wine-red color, slowly changing to brown, should be noted.

Gum-resins and oleo-resins

Gum-resins, such as gamboge, are simple mixtures consisting chiefly of resin and gum. The gum is usually a glucosidal substance similar in composition to gum acacia.

Oleoresins are more-or-less homogeneous mixtures of resins and volatile oils. The term "oleo-gum-resin" is sometimes applied to substances, such as myrrh, which consist of mixtures of resins, gums, and volatile oils.

There is no sharp line of demarcation between these various types of resinous substances, and classification is sometimes difficult; thus, guaiacum resin, which is not usually regarded as a gum-resin, may contain as much as 10% of gum, while small proportions of volatile oil are present in many resins, such as mastich and sandarac. Some important gum and oleo-resins of therapeutic importance are shown in Table 7.11. Oleo-resins are sometimes spoken of as balsams (e.g., Canada balsam), but this term is more properly restricted to resinous substances that contain large proportions of free or combined benzoic or cinnamic acids.

Gluco-Resins

Substances known as gluco-resins occur in such drugs as jalap, scammony, and turpeth roots, from which they may be obtained by extracting with alcohol and precipitating the resin by pouring the alcoholic solution into water. As thus obtained, the gluco-resins are complex mixtures. On hydrolysis, they yield sugars and complex resin acids, which appear to be combined in the drug in the form of glucosides. *Orizaba jalap* root (*Ipomoea orizabensis*) yields 12%–18% of resin, about 65% of which is ether soluble. The resin is a complex mixture and from the products of hydrolysis of the ether-soluble portion have been isolated jalapinolic acid and its methyl ester and sugars (glucose and a methyl p*ent*ose). The resin thus appears to consist largely of the glucosides and methyl pentosides of jalapinolic acid and methyl jalapinolate.

Seammony root (*Convolvulus scammonia*) yields 3.13% of resin, which is almost entirely soluble in ether. In chemical composition and therapeutic action, it is very similar to the ether-soluble portion of the resin of *O. jalap* root, and for this reason, the British Pharmacopeia permits the use of the resin from either *O. jalap* or scammony under the name of scammony resin, provided that not less than 75% of it is soluble in ether.

The products of hydrolysis show that scammony resin consists mainly of the glucosides and methyl pentosides of jalapinolic acid and its methyl ester. The methyl pentose has been identified as rhamnose and appears to be different from the methyl pentose isolated from the products of hydrolysis of *O. jalap* resin. Jalap root (*Ipomoea purga*) yields 2.22% of resin, only 10%–15% of which is ether soluble. The ether-soluble portion closely resembles the ether-soluble portions of scammony and *O. jalap* resins, and is therefore sometimes called "scammonin." The portion insoluble in ether is termed "jalapin" and is a complex mixture, which appears to consist partly of the glucosides of convolulinolic and ipurolic acids.

Kaladana resin (*Ipomoea hederacea*) was formerly believed to be identical with the ester-insoluble resin of jalap, but it has been shown to consist of a purgative nonglucosidal resin mixed with large proportions of glucosidal matter.

TABLE 7.11 Gum-Resins and Oleo-Resins of Pharmaceutical Importance

Drug	Botanical Source	Resin	Gum	Volatile Oil	Constituents of the Resin
Scammony	*Convolvulus scammonia*	70%–90%	10%–15%	—	Resin identical with that obtained from the dry root (see Gluco-resins)
Gambose	*Garcinia hanburyi*	70%–80%	15%–20%	—	"Garcinolic acids," resin esters and a resene
Myrrh	*Commiphora myrrha*	25%–40%	50%–60%	2.5%–8.0%	α-, β-, and γ-commiphoric acids, other resin acids, resenes, complex phenolic compounds
Olibanum	*Boswellia carterii* and other species	60%–70%	20%–30%	5%–10%	Boswellic acid (free and combined), olibanoresene
Ammoniacum	*Dorema ammoniacum* and other species	65%–70%	20%	0.5%	Ammonresinotannol combined with salicylic acid, free salicyle acid (traces) and a resene
Galbanum	*Ferula galbaniflua* and other species	60%–65%	20%	5%–15%	Galbaresinotannol combined with umbelliferone, free umbelliferone (traces)
Asafetida	*Ferula foetida*, *F. rubricaulis*, and other species	40%–65%	25%	4%–20%	Asaresinotannol combined with ferulic acid. On boiling with acids, yields free umbelliferone
Canada turpentine	*Abies balsamea* and *A. canadensis*	70%–80%	—	18.24%	α- and β-andinolic acids (48%–60%), canadinic acid (13%–20%), canadolic acid (0.5–3.0), canadoresene (1%–20%)
Capaiba (maracaibo)	Species of *Copaifera*	40%–60%	—	40%–50%	β-Meta-copaivic acid, $C_{10}H_{16}O_2$, illuric acid, $C_{20}H_{28}O_3$, copaivic acid, copaiboresenes

Turpeth root (*Ipomoea turpethum*) yields 5%–10% of resin, which is partly soluble in ether. The resin has not been thoroughly investigated, but the ether-soluble portion seems to consist of a rhamnoside (α-turpethein) and a glucoside (β-turpethein). The portion insoluble in ether is known as "turpethin."

7.9.3 Quantitative Chemical Analysis of Some Resinous Compounds

Test for myrrh

Shake a little myrrh with ether, decant the liquid on a watch-glass, and allow to evaporate. To the residual film, add a few drops of 50% nitric acid. A violet color is produced. A similar coloration is formed by exposure of the film to a vapor of concentrated nitric acid or of bromine.

Distinction between ammoniacum, galbanum, and asafoetida:

(i) Test for salicylic acid

Add a drop of ferric chloride solution to a clear ethereal extract of the drug. A faint violet color is produced with ammoniacum, but not with galbanum or asafoetida.

(ii) Test for free umbelliferone

Boil a small quantity of the drug with 90% alcohol, cool, and decant the solution through a filter into alcohol containing ammonia. A blue fluorescence is given with galbanum, but not with ammoniacum or asafoetida.

(iii) Test for combined umbelliferone

Boil a small quantity of the drug with concentrated hydrochloric acid for a few minutes, dilute the liquid with an equal volume of water, and filter as in the previous test into alcoholic ammonia. A blue fluorescence is given by asafoetida and galbanum, but not by Persian ammoniacum.

(iv) Distinction between Persian and African ammoniacums

Grind up with water, and add sodium hypochlorite solution. Persian ammoniacum gives an orange-red color, but African does not. Also, note that African ammoniacum gives a positive result in the test for combined umbelliferone. African ammonicum is not used medicinally and is rarely met with in commerce.

(v) *Test for sumatra benzoin*

Warm gently with potassium permanganate solution. An odor of benzaldehyde is evolved, owing to oxidation of the cinnamic acid.

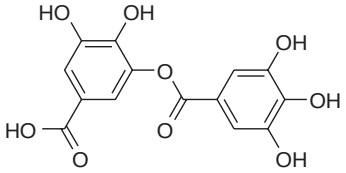
7.10 TANNINS OF THERAPEUTIC IMPORTANCE AND THEIR ANALYSIS

The tannins are amorphous, rarely crystalline substances, soluble in water and alcohol, and having an astringent and bitter taste. Chemically, they are complex phenolic substances and may be classified according to the nature of the products of hydrolysis and some of their chemical reactions. The true tannins will convert animal hides into leather (Baker and Ollis, 1961).

7.10.1 Chemical Classification of Tannins and Their Quantitative Analysis

(i) Depside or pyrogallol tannins

Based upon pyrogallol, they give gallic acid as the final product of hydrolysis and consist of a glucose molecule in which all the hydroxyl groups are combined with digallic acid residues.



Digallic acid

They occur in rhubarb, cloves, logwood, bearberry leaves, red rose petals, and witch-hazel leaves. They can be extracted easily with 45% alcohol, which does not extract chlorophyll, and are also soluble in water. They give the following color reactions:

- blue colors or precipitates with ferric chloride;
- precipitated by heavy metals;
- precipitated by gelatin;

- precipitated by alkaloids quite slowly;
- yield bulky precipitates with phenazone, especially in the presence of sodium acid phosphate;
- with iron and ammonium citrate or iron and sodium tartarate, they give a water-soluble iron–tannin complex that is insoluble in a solution of ammonium acetate. This is known as the Metchell's test for tannins; and
- do not produce any precipitate with bromine water.

(ii) Ellagic acid tannins

These are very similar to the depside tannins but give ellagic acid on hydrolysis. They occur in oak bark, pomegranate rind, pomegranate root bark, and eucalyptus leaves. They give the same types of color reactions after hydrolysis as the ellagic acid tannins described before.

(iii) Catechol or phlobatannins

These tannins give catechol or pyrocatechuic acid on alkaline hydrolysis, but with acids or by boiling in air they are oxidized to red water and alcohol-insoluble substances known as phlobaphenes. They occur in cinnamon, wild cherry bark, cinchona bark, oak bark, acacia bark, witch-hazel bark, krameria root, male fern rhizome, as well as in association with depside tannins in witch-hazel leaf and tea. These tannins are also present in catechu.

The tannins from krameria root can quite easily be extracted with 45% alcohol. Some may be converted to phlobaphenes during the process, in which case the extractive will give a turbid solution when passed into water, though it should remain clear when poured into dilute glycerin. Krameria tannic acid can quite easily be converted to krameria red by boiling with acid. The phlobaphene is insoluble in water but soluble in glycerin. Cinchotannic acid extracted with water will respond chemically as a typical phlobatannin and give the following special color reactions:

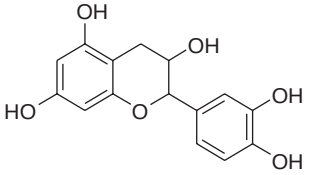
- Green color with ferric chloride.
- Precipitated by heavy metals.
- Precipitated by gelatin.
- Precipitated by alkaloids quite slowly.
- Yield bulky precipitates with phenazone, especially in the presence of sodium acid phosphate.
- With iron and ammonium citrate or iron and sodium tartarate, they give a water-soluble iron–tannin complex, which however, is insoluble in solution of ammonium acetate. This is known as the Metchell's test for tannins.
- Do not produce any precipitate with bromine water.
- The phlobatannins may also be distinguished from the depside tannins by the fate that the phlobatannins produce a leather without a bloom, whereas the leather produced by the depside tannins has a distinct bloom.

(iv) Pseudotannins

These are tannin-like substances that respond to most of the chemical tests of the true tannins but which will not produce leather. Neither is it possible to hydrolyze them, and they are often referred to as condensed tannins. There are four groups:

- Gallic acid itself, found in rhubarb.
- The crystallizable catechins found in catechu, cutch, and guarana.
- Caffeotannins found in coffee, cinchona bark, and nux vomica seeds.
- Ipecacuanha tannins based on ipecacuanhic acid and found in ipecacuanha root.

The principal tannin in catechu is the condensed tannin acacatechin, though some phlobatannin also occurs.



Acacatechin

The characteristic reactions of this class of tannins are similar to those for phlobatannins, except the following reaction.

The presence of phloroglucinol nuclei in the molecule will cause a matchstick to stain red when dipped in a catechin solution followed by concentrated hydrochloric acid and then warmed.

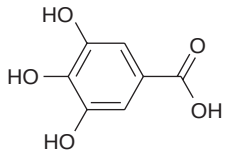
(v) Tannic acid

Tannic acid, gallotannic acid, or tannin is usually obtained from nutgall. The powdered galls are extracted with a mixture of ether, alcohol, and water, and the liquid separates into two layers. The aqueous layer contains gallotannin, and the

ethereal layer contains the free gallic acid present in the gall. After separation, the solution of gallotannin is evaporated, and the tannin is purified in various ways.

Tannic acid occurs as an amorphous powder, glistening scales, or spongy masses that are light brown to yellowish white. The odor is faint and the taste is strongly astringent. Tannic acid is soluble in water, alcohol, and acetone and insoluble in ether, chloroform, and benzene.

Tannic acid is an astringent. It was formerly used in the treatment of burns, but this application has been discontinued. Its topical use is now restricted to the treatment of bed sores, minor ulceration, and the like. As an alkaloidal precipitant, it has been employed in cases of alkaloidal poisoning.



Gallic acid

Gallic acid is 3,4,5-trihydroxybenzoic acid that crystallizes with one molecule of water. It occurs in nutgall and can be prepared from tannic acid by hydrolysis with dilute acids. Bismuth subgallate is used by ostomates to help control odors.

(vi) Hamamelis leaf

Hamamelis leaf, or witch-hazel leaf, is the dried leaf of *Hamamelis virginiana* Linn. (Fam. Hamamelidaceae). Hamamelis leaf contains hamamelitannin and tannin that appears to be derived from gallic acid, as well as a hexose sugar, a volatile oil, a bitter principal, gallic acid, and calcium oxalate. Hamamelis leaf possesses astringent and hemostatic properties. Hamamelis water or distilled witch-hazel extract is prepared by steam distillation of the recently cut and partially dried dormant twigs of *H. virginiana*. Essentially, it is a hydroalcoholic solution of the volatile oil of the plant.

Analysis of the volatile oil reveals that it contains 2-hexen-1-al (9.7%), acetaldehyde (3.2%), alfa-ionone (3.5%), beta-ionone (1.0%), and safrole (0.2%). Although tannins are not carried over in the distillation process, hamamelis water is nevertheless widely utilized for its astringent properties resulting from the 14% to 15% alcohol to which the final product is adjusted. It is incorporated in hemorrhoidal products, preparations for treating insect bites and stings, and even teething preparations. The contained volatile oil does confer a pleasant fragrance to such products.

(vii) Nutgall

Nutgall is the excrescence obtained from the young twigs of *Quercus infectoria* and allied species of Quercus (Fam. Fagaceae). The oak galls are obtained principally from Aleppo in Asiatic Turkey.

The excrescence (gall) is caused by the puncture of a hymenopterous insect, *Cynips tinctoria* and the presence of the deposited ovum. Several stages in the development of the gall correspond to the development of the insect:

(i) When the larva begins to develop and the gall begins to enlarge, the cells of the outer and central zones contain numerous small starch grains.
(ii) When the chrysalis stage is reached, the starch near the middle of the gall is replaced in part by gallic acid, but the peripheral and central cells contain masses of tannic acid.
(iii) As the winged insect develops, nearly all of the cells contain masses of tannic acid with a slight amount of adhering gallic acid.
(iv) When the insect emerges from the gall, a hole to the central cavity is formed. Thus, the tannic acid, owing to the presence of moisture and air, may be oxidized in part into an insoluble product, and the gall becomes more porous, thereby constituting the so-called white gall of commerce.

The principal constituent is tannic acid, which is found to the extent of 50%–70%; the drug also contains gallic acid (2%–4%), ellagic acid, starch, and resin. Nutgall, the chief source of tannic acid, is used in the tanning and dyeing industry, and formerly, in the manufacture of ink. Medicinally, it has astringent properties.

7.11 TERPENOIDS AND THEIR ANALYSIS

Terpenoids are widely distributed in nature and are found in abundance in higher plants. In addition, fungi produce a range of interesting terpenoids. Marine organisms are a prolific source of unusual terpenoids, and terpenoids are found as insect pheromones and in insect defense secretions. Terpenoids are defined as natural products whose structures may be divided into isoprene units; hence, these compounds are also called isoprenoids. In addition, this class of compounds is

also collectively called the terpenes; however, the -old suffix is more logical, as used for alkaloids, flavonoids, and others. The -ene suffix should be restricted to the unsaturated hydrocarbons of the class. The isoprene units arise biogenetically from acetate via mevalonic acid and are branched-chain, five-carbon units containing two unsaturated bonds (Glasby, 1982).

During the formation of terpenoids, the isoprene units are usually linked in a head-to-tail manner, and the number of units incorporated into a particular unsaturated hydrocarbon terpenoid serves as basis for the classification of these compounds. Monoterpenoids are composed of two units and have the molecular formula $C_{10}H_{16}$. Sesquiterpenoids, $C_{15}H_{24}$, contain three isoprene units. Diterpenoids, $C_{20}H_{32}$, have four isoprene units. Triterpenoids, $C_{30}H_{48}$, are composed of six isoprene units, and tetraterpenoids or carotenoids, $C_{48}H_{60}$, have eight units (Harborne and Tomas-Barberan, 1991). The concept that terpenoids are built from isoprene units is known as the "biogenetic isoprene rule."

7.11.1 Monoterpenoids

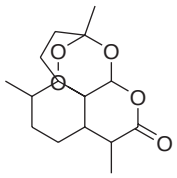
More than 1000 naturally occurring monoterpenoids are presently known, most of which have been isolated from higher plants. More recently, however, a number of halogenated monoterpenoids have been isolated from marine organisms, and monoterpenoids are occasionally found in defense and pheromonal secretions of insects. Characteristic features of monoterpenoids are their volatility and intensely pungent odors, and they are the most common components in plants responsible for fragrance and flavor. Consequently, there is a great commercial interest in these compounds for the production of volatile oils in the perfume and fragrance industry and in the production of spices and culinary herbs for the food and seasoning industry (Guenther, 1948).

7.11.2 Sesquiterpenoids

The sesquiterpenoids have a wide distribution in nature and form the largest class of terpenoids. Among the first sesquiterpenoids isolated from nature were β-cardinene from oil of cade (juniper tar) and β-caryophyllene from clove oil. Abscisic acid is a sesquiterpenoid that is an essential plant hormone controlling growth and development.

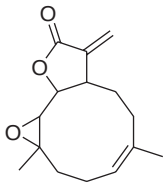
Sesquiterpenoid lactones are chemically distinct from other members of the sesquiterpenoid group through the presence of an α-methylene-γ-lactone system. Many of them also contain α,β-unsaturated carbonyls as well as epoxides. These functional groups represent reactive receptor sites for biological nucleophiles, such as the thiol and amino groups of enzymes. Consequently, a wide spectrum of biological activities has been reported for these compounds, for example, antimicrobial and antitumor activity, and some are highly toxic to mammals.

- Artemisinin, or quinhaosu, is a sesquiterpenoid lactone obtained from the leaves and flowering tops of *Artemisia annua* Linn. (Fam. Asteraceae), an herb that has traditionally been used in China for the treatment of malaria for over a 1000 years. Artemisinin and its derivatives are potent and rapidly acting blood schizontocides in the treatment of malaria. They are active against *Plasmodium vivax* and against both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. They produce a more rapid clearance of parasites than quinine or chloroquine, which has been encouraging in the treatment of cerebral malaria.



Artemisinin

- The dried flower heads of *Matricaria chamomilla* (Fam. Asteraceae) constitute the drug known as German chamomile. A related plant, *A. nobilis*, known as Roman chamomile, contains similar constituents and is similarly employed. The main constituent of chamomile is α-bisabol, α-bisaboloxide A and B. It is extensively cultivated in Europe, where it is widely utilized in folk medicine for its carminative, spasmolytic, and antiinflammatory effects.
- The leaves of *Tanacetum parthenium* (Fam. Asteraceae), known as feverfew, have been used for centuries as an antipyretic or febrifuge. Recently, however, double-blind placebo-controlled clinical trials have shown that feverfew is effective in the prophylaxis of migraine by substantially reducing the frequency and severity of the headache. The principal primary responsible is parthenolide, a sesquiterpenoid lactone of the germacranolide type, with the chemical name 4α,5β-epoxygermacra-1(10),11(13)-dien-12,6α-olide. Parthenolide acts as a serotonin antagonist, resulting in an inhibition in the release of serotonin from the blood platelets.



Parthenolide

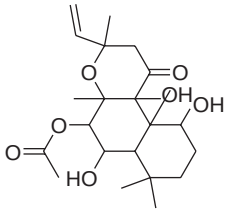
- Valerian consists of the dried rhizome and roots of *Valeriana officinalis* Linn. (Fam. Valerianaceae). It has been employed as an antianxiety agent and sleep aid for more than a thousand years. The drug contains from 0.3% to 0.7% of an unpleasant-smelling volatile oil containing bornyl acetate and the sesquiterpenoids, valerenic acid, valerenolic acid, and acetoxyvalerenolic acid. Also present is a mixture of lipophilic iridoid principals known as valeportriates. These bicyclic monoterpenoids are quite unstable and occur only in the fresh plant or in material dried at temperatures under 40°C. Although the specific active principal(s) of valerian have not been determined, it is possible that a combination of the sesquiterpenoids and the valepotriates may be involved.

7.11.3 Diterpenoids

The diterpenoids comprise a large group of nonvolatile C_{20} compounds derived from geranyl pyrophosphate. Although mainly of plant or fungal origin, they are also formed by some marine organisms and insects. Some acyclic diterpenoids are known, but the vast majority are carbocyclic compounds containing as many as five rings. Diterpenoids at many different states of oxidation are known, ranging from hydrocarbons to highly oxygenated compounds, and are usually isolated as optically active solids that can exist in both the normal and the antipodal stereochemical configurations. The normal series has an A/B ring fusion, stereochemically related to that of the steroids, and those in which the ring fusion is antipodal are known as the ent series; example of each series may occur in the same plant species.

The diterpenoids have a wide range of different biological activities. The resin acids, such as abietic acid, are exuded from the wood of trees, especially conifers, and protect the tree from infection and insect attack. The gibberellins are plant hormones that stimulate plant growth, whereas prodolactones are plant growth inhibitors. Diesters of phorbal have been extensively used in pharmacological investigations on carcinogenesis because of their ability to promote tumor formation. In contrast to the triterpenoids, the diterpenoids are rarely combined with sugars to form glycosides; however, an exception is stevioside, which is a glycoside about 300 times sweeter than sucrose and used as a sweetening agent in Japan. Many therapeutically potent diterpenoids are available for treating deadly ailments, among which a few are described below.

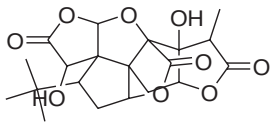
- Forskolin or colforsin is a labdane diterpenoid isolated from the roots of the Indian herb *Coleus forskohlii* (Fam. Lamiaceae). *Coleus* is from the Greek coleos meaning sheath, referring to the fused filaments of the flower that form a staminal sheath around the style. Forskolin is 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydoxylabh-14-en-11-one and was discovered via a screening program in India of plants used in Hindu and Ayurvedic traditional medicine. It has been shown to be a prototype for a novel class of drugs that activates cyclic AMP-generating systems through an ability to stimulate adenylate cyclase in a receptor-independent manner. As a consequence, it has become an important research tool in cyclic AMP-related studies. In addition, forskolin has a high therapeutic potential in diseases such as congestive cardiomyopathy and bronchial asthma, in which the repeated use of β-adrenergic agonists leads to desensitization of the receptors and a loss of drug efficacy. It also has potential for use in glaucoma and hypertension.



Forskolin

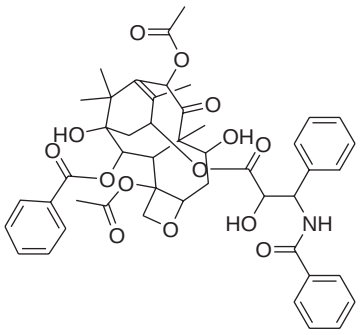
Ginkgo is a potent source of therapeutically important diterpenoid. A concentrated acetone–water extract of the dried leaves of *Ginkgo biloba* (Fam. Ginkgoaceae) is currently a popular drug in Europe for the treatment of peripheral vascular disease, particularly cerebral circulatory disturbances and other peripheral arterial circulatory disorders. The extract is

a complex mixture of constituents, and more than one agent may be responsible for the therapeutic effect. The flavone glycosides of the rutin type reduce capillary fragility and reduce blood loss from the capillary vessels, which may prevent ischemic brain damage. Probably more important is the presence of diterpenoid lactones, known as ginkgolides. Ginkgolides A, B, C, and M have been shown to inhibit platelet-activating factor (PAF). PAF produced by a variety of body tissues not only induces aggregation of the blood platelets, but also causes broncho-constriction, cutaneous vasodilation, chemotaxis, hypotension, and the release of inflammatory compounds. All of these are blocked by the ginkgolides, resulting in an increase in blood fluidity and circulation.



Ginkgolide A

- Taxol or paclitaxel is a diterpenoid obtained from the bark of *Taxus brevifolia* Nutt. (Fam. Taxaceae), also known as the pacific yew. The plant is a small, slow-growing evergreen tree native to the northwestern United States. Taxol is one of the most important antineoplastic drugs to emerge from the antitumor screening of natural products in recent years. The mode of action is unique in that it enhances the polymerization of tubulin, the protein subunit of the spindle microtubulin and induces the formation of stable, nonfunctional microtubules. As a consequence, taxol disrupts the dynamic equilibrium within the microtubule system and blocks cells in the late G2 phase and M phase of the cell cycle, inhibiting cell replication. Taxol is a complex diterpenoid with a taxane ring system and a four-membered oxetane ring. An ester side chain at position 13 of the taxane ring is essential for the cytotoxic activity of the drug. In addition, the presence of an accessible hydroxyl group at position 2 of this ester side chain enhances the activity. Taxol is hydrophobic; therefore, the injectable concentrate preparation for intravenous infusion is solubilized in polyoxyethylated castor oil. Before injection, it must be diluted in sodium chloride or dextrose solution or combinations thereof. Taxol is used in the treatment of metastatic carcinoma of the ovary after a failure of first-line or subsequent chemotherapy and in the treatment of breast cancer after a failure of combination chemotherapy for metastatic disease or relapse within 6 months of adjuvant chemotherapy.



Taxol

7.11.4 Triterpenoids

The triterpenoids are formed biosynthetically from six isoprene units and share in common the C_{30} acyclic precursor squalene. Different types of ring closure in squalene can give rise to many different skeletal types of triterpenoids. In fact, over 4000 natural triterpenoids have been isolated, and more than 40 skeletal types have been identified. The triterpenoids can be divided into two main classes: the tetracyclic compounds and the pentacyclic compounds. Also, in the later stages of biosynthesis, small carbon fragments may be removed to produce molecules with less than 30 carbon atoms, for example, the C_{27} steroids. Most triterpenoids are alcohol and can combine with sugars to form glycosides, which is the case with saponins. Free triterpenoids are often components of resins, latex, or the cuticle of plants. Triterpenoids that are ecologically significant include the cucurbitacins and quassinoids, which have a bitter taste that may be a defense against herbivory, and the saponins, which are toxic to cold-blooded animals, such as mollusks. The limonoids, a group of tetranortriterpenoids, have insect antifeedant properties. For example, the neem tree *Azadirachta indica* produces the limonoid azadirachtin, which is one of the most potent insect antifeedant compounds known. Another group of triterpenoids is the phytoecdysones

that disrupt metamorphosis in insects that are feeding on plants. Besides these, there are triterpenoids that are used as drugs, which have been discussed in the glycosides section.

7.11.5 Tetraterpenoids and Carotenoids

The carotenoids comprise an important and ubiquitous class of C_{40} tetraterpenoids. Many of the yellow, orange, red, and purple colors seen in living organisms are due to the presence of these compounds. Their biosynthesis takes place in the chloroplasts of the plants or chromatophores of bacteria and fungi. The precursor, geranyl pyrophosphate, undergoes a head-to-head dimerization to give prephtoene pyrophosphate and then phytoene, the central intermediate in the formation of C_{40} carotenoids. In some microbes, additional isopentenyl groups may be added into the tetraterpenoid backbone to produce C_{45} and C_{50} carotenoids, the homocarotenoids. The C_{40} carotenoids may also be oxidized to carotenoids containing less than 40 carbons, the apo-carotenoids. Approximately 600 naturally occurring carotenoids have been described. The largest structural variety is found in those isolated from marine organisms, including some with acetylenic groups. Well-known carotenoids are either simple unsaturated hydrocarbons based on lycopene or their oxygenated derivatives, known as xanthophylls. The chemical structure of lycopene consists of a long chain of eight isoprene units joined head to tail, giving a conjugated system of alternate double bonds, which is the chromophore responsible for imparting color to the molecule. Cyclization of lycopene at both ends of the molecule produces the bicyclic hydrocarbon β-carotene, the most common carotenoid in higher plants.

In plants and microorganisms, carotenoids function as photoprotective agents, as phytosynthetic accessory pigments, and as membrane stabilization components. In animals, carotenoids serve as a source of vitamin A and other retinoids and as photoprotective and cancer prevention agents. These protective functions of carotenoids appear to be due to their ability to quench free radicals and singlet oxygen, acting as antioxidants and preventing oxidation damage to cells. There is a large commercial market for carotenoids as colorants for foods, animal feeds, pharmaceuticals, and cosmetics. Carotenoids for this purpose are obtained by chemical synthesis, extraction from natural products, such as carrots, and fermentation. Biotechnology is now playing a major role in improving carotenoid production with the use of directed mutagenesis, carotenogenic enzymes, immobilized enzyme systems, and bioreactors (Britton, 1983).

Beta-carotene, in addition to being a provitamin A substance, is effective in reducing photosensitivity in individuals with erythropoietic protoporphyria. It does not act as a sunscreen in normal individuals and should not be used for that purpose. However, β-carotene does provide a novel and safe approach to the treatment of a specific type of photosensitivity.

7.12 GLYCOSIDAL COMPONENTS AND THEIR ANALYSIS

"Glycoside" is a general term covering a large variety of drugs whose common feature is that they contains a minimum of one sugar molecule that is linked via its anomeric carbon to a different moiety. In common usage, the term organic compound refers to heterosides, and the nonsugar moiety is noted as aglycone or genin. Different classes of glycosides—anthracene derivative, flavonoid, cardenolide, cyanogenic, and others—are decided by the aglycone structure. Due to the presence of one or more sugars in the molecule, glycosides are relatively polar. The absolute polarity is determined by the number and types of sugar (e.g., acetylated or not) and also the structure of the aglycone. Most glycosides are extracted with polar solvents, such as acetone, ethanol, methanol, water, or mixtures of these. However, cardiac glycosides, with bulky steroidal aglycones, have considerable solubility in chloroform. While extracting with water, it is sometimes possible to get enzyme breakdown of the glycoside to the aglycone due to the action of glycosidases coextracted from the plant material. This will not occur if boiling water is used or if a considerable amount of alcohol or ammonium sulfate is added to the extract. In some cases, it could be the aglycone instead of the glycoside that is to be extracted, and this needs hydrolytic separation of the aglycone and sugar before or after extraction. Glycosides, in which the aglycone-sugar link is formed via an oxygen, nitrogen, or sulfur atom (*O*-, *N*-, or *S*-glycosides) are often hydrolyzed by boiling with dilute acid (e.g., 10% sulfuric acid). The glycoside is resistant to acid hydrolysis, wherever there is a C–C link between the aglycone and sugar, but is often attenuated by the action of iron (III) chloride and acid reflux. However, this is a strong oxidizing agent and hence can produce other effects on the aglycone structure, for example, oxidization of monoanthrones to anthraquinones. Aglycones of glycosides may also be obtained by hydrolyzing extracted glycosides in aqueous media, as mentioned earlier, followed by extraction of the aglycone with a less polar solvent, for example, diethylether or dichloromethane. If the aglycone is appreciably soluble in hot water, then the initial extraction itself can utilize warm aqueous acid (which hydrolyzes glycosides and extracts aglycones). On cooling the extract, the aglycones can be partitioned as they become less water soluble (Houghton and Raman, 1998). Different glycosides of therapeutic importance and their hydrolysis products are shown in Table 7.12.

TABLE 7.12 Some Pharmaceutically Important Glycosides and Their Hydrolysis Products

Drug	Botanical Source	Glucosides and Their Hydrolysis Products	Use
Alderbuckthorn bark	*Rhamnus frangula*	Frangulin → frangula emodin + rhamnose	Cathertic
Bearberry leaves	*Arctostaphylos uva-ursi*	Arbutin → quinol + glucose	Flavoring agent
Bitter almonds	*Prunus amygdalus* var. *amara*	Amygdalin → benzadehyde + hydrocyanic acid + glucose (2 moles)	Cardiac stimulant
Black haw bark	*Viburnum prunifolium*	Viburnin	Flavoring agent
Black hellebore rhizome	*Helleborus niger*	Helleborein (a saponin) → acid and neutral helleboretins + acetic acid + glucose. Hellebroin	Cardiac stimulant
Black mustard seeds	*Brassica nigra*	Singrin helleborein (a saponin) → acid and neutral helleboretins + acetic acid + *gluco*se. Helleborin → allyl isothiocyanate + potassium hydrogen sulfate + glucose	Condiment
Buchu leaves	*Barosma betulina*	Hesperdin → hesperetin + rhamnose + glucose	Flavoring agent
Cherry-laurel leaves	*Prunus laurocerasus*	Prulaurasin helleborein (a saponin) → acid and neutral helleboretins + acetic acid + glucose. Helleborin → benzaldehyde + hydrocyanic acid + glucose	Flavoring agent
Digitalis leaves	*Digitalis purpurea*	Digitoxin helleborein (a saponin) → acid and neutral helleboreteins + acetic acid + glucose. Helleborin. Digitoxigenin + digitoxose + another sugar. Gitalin { Bigitalin / Digitonin }	Cardiac tonic
Gentian root	*Gentiana lutea*	Gentiopicrin helleborein (a saponin) → acid and neutral helleboretins + acetic acid + glucose. Helleborin. Gentiogenin + glucose (in the fresh root). Gentin helleborein (a saponin) → acid and neutral helleboretins + acetic acid + glucose. Helleborin. Gentienin + glucose + xylose. Gentiamarin	Bitter tonic
Horseradish root	*Cochlearia armoracia*	Sinigrin → allyl isothiocyanate + potassium hydrogen sulfate + glucose	Condiment
Lily of the valley flowers	*Convallaria majalis*	Convallamarins → convallamaretin + glucose. Convallarins (acid and neutral saponins)	Cardiotonic
Linseed	*Linum usitatissimum*	Linamarin (a cyanogenetic glucoside)	Condiment
Nux vomica seeds, picrorhiza rhizome, quillaja bark	*Strychnos nux-vomica*, *Picrorhiza kurroa*, and *Quillaja saponaria*	Loganin Picrorhizin Picrorhizin Quillajic acid → quillajic sapogenin + glucose. Quillajic sapotoxin → quillaja sapotoxin sapogenin + glucose	CNS stimulant
Red poppy petals	*Papaver rhoeas*	Macocyanin (isomeric with cyanin)	Coloring agent
Red rose petals	*Rosa gallica*	Cyanin → cyanidin + glucose	Perfume
Saffron	*Crocus sativus*	Crocin → crotein + a sugar. Picrocrocin	Coloring agent
Sarsaparialla (Jamaica)	*Smilax ornata*	Sarasasaponin → sarasasapogenin + glucose	Rheumatism and skin diseases
Senega root	*Polygala senega*	Polygalic acid Senegin → senegenin + a sugar	Stimulant expectorant
Squill	*Urginea scilla*	Scillaren	Cardiotonic
Strophanthus seeds	*Strophanthus kombe*	k-Strophanthin → strophanthidin + a sugar	Cardiotonic

TABLE 7.12 Some Pharmaceutically Important Glycosides and Their Hydrolysis Products—cont'd

Drug	Botanical Source	Glucosides and Their Hydrolysis Products	Use
Urginea	*Urginea indica*	Probably similar to squill	Expectorant, cardiac stimulant
White mustard seeds	*Brassica alba*	Sinalbin → acrinyl isothiocyanate + sinapin hydrogen sulfate + glucose	Condiment
Wild cherry bark	*Prunus serotina*	Prunasin → benzaldehyde acid + glucose	Flavoring agent
Willow bark	*Salix alba*	Salicin → saligenin + glucose	Antipyretic
Wintergreen leaves	*Gaultheria procumbens*	Gautherin → methyl salicylate + glucose	Antiinflammatory

Glycosides also belong to a wide range of chemical subgroups. The majority of these share common properties, such as solubility in water or aqueous alcohol and susceptibility to acid hydrolysis to yield the aglycone. The general test for the presence of glycosides involves parallel testing of a water extract and an acid extract of the organic material. Water extracts intact glycosides and any free reducing sugars already present in the material. Treatment with acid results in the hydrolysis of *O*-, *N*-, and *S*-glycosides to one or more reducing sugars and the free aglycone. The newly released sugar, along with any free sugars preexisting in the plant material, will be extracted into the acid. Both extracts are tested with Fehling's reagent, which contains copper ions. In the presence of reducing sugars, Cu^{+2} ions are reduced to Cu^{+} and a red precipitate of copper oxide (Cu_2O) is produced. A heavier precipitate will be produced with the acid extract if glycosides are present in the sample material. However, this method does not apply to *C*-glycosides.

7.12.1 Chemical Nature of Glycosides and Their Analysis

Chemically, the glycosides are usually mixed acetals in which the hydroxyl group on the anomeric carbon atom is replaced by a moiety possessing a nucleophilic atom. In the case of *O*-glycosides, the nucleophilic atom is most commonly oxygen present in an alcohol, a phenol, or a carboxyl. The sugar hemiacetal may react with a hydroxyl group of another sugar to form a disaccharide, and the reaction can be repeated to yield polysaccharides. When the nucleophilic atom is sulfur, such as in a thiol group, *S*-glycosides are obtained. *N*-glycosides, such as nucleosides, are formed when the nucleophilic atom is carbon in the form of a carbanion, giving rise to *C*-glycosides. The nonsugar component of a glycoside is known as the aglycone; the sugar component is called the glycone. Both α- and β-glycosides are possible, depending on the stereo configuration at the anomeric carbon atom. However, primarily β forms occur in plants. Emulsion and most other natural enzymes hydrolyze only the β varieties.

In the nomenclature of glycosides, the trivial names have an "in" ending, and the names indicate the source of the glycoside, for example, digitoxin from *Digitalis*, salicin from *Salix*, and prunasin from *Prunus*. The systematic names are usually formed by replacing the "ose" suffix of the parent sugar with "oside." The anomeric prefix (α or β) and the configurational prefix (D or L) immediately precede the sugar stem name, and the chemical name of the aglycone precedes the name of the sugar. For example, the systematic name of salicin is *O*-hydroxymethylphenyl β-D-glycopyranoside. From the biologic viewpoint, glycosides play an important role in the life of the plant and are involved in its regulatory, protective, and sanitary functions. Among such a variety of compounds, one finds many therapeutically active agents. Some of our most valuable cardiac glycosides are from *Digitalis*. Laxative drugs, such as senna, aloe, rhubarb, *Cascara sagrada*, and frangula, contain anthraquinone glycosides, and sinigrin, a glycoside from black mustard, yields allyl iosothiocyanate, a powerful local irritant.

A number of semispecific tests have been devised, which rely mostly on the special properties conferred by the aglycone moiety. Anthracene derivative glycosides (e.g., frangulin A) may be detected by the pink color produced by dihydroxyanthraquinone aglycones in alkaline solution. Acidic ferric chloride may be used to oxidize reduced anthracene forms to the anthraquinone structure. This reagent also hydrolyzes *C*-glycosides, which are common among this group and are resistant to hydrolysis by acid alone. Cardenolides are a group of cardioactive glycosides in which the aglycone consists of a steroid linked to an α,β-unsaturated lactone and the sugar chain generally includes deoxy-sugars (e.g., digoxin). These two structural features give color reactions in the Kedde and Keller–Kiliani tests, respectively. Alcohol is often used to extract

cardenolide glycosides (which also display solubility in chloroform). This may result in the coextraction of chlorophyll and phenolic pigments, which can be removed by lead subacetate precipitation before the chemical tests are carried out.

Saponin glycosides are of two types: those based on a triterpenoid aglycone and those with a steroidal aglycone (e.g., glycyrrhizin and dioscin, respectively). The combination of a bulky, lipophilic aglycone with hydrophilic sugar moieties confers surface-active properties on the molecules (saponin = soap like) and, consequently, their presence in aqueous extracts can be inferred by the formation of stable froths on shaking. A related property is their ability to hemolyze red blood corpuscles (resulting from disruption of the cell membrane). This can also be used as a basis for a test, although it works better with steroidal saponins than triterpenoid derivatives that have a slower hemolyzing action. The presence of cyanogenic glycosides (e.g., prunasin) can be investigated by a method based on their decomposition to yield hydrogen cyanide. Plant materials containing these glycosides commonly contain glycosidase enzymes, which are capable of cleaving sugars from the aglycone. Applying a small quantity of water to the plant material can activate such enzymes, which then break the glycosides to release hydrogen cyanide. This reacts with sodium picrate to form the reddish-purple compound sodium isopurpurate.

The classification of glycosides is a difficult matter. If the classification is based on the sugar group, a number of rare sugars are involved; if the aglycone group is used as a basis of classification, one encounters groups from probably all classes of plant constituents. A therapeutic classification, although excellent from a pharmaceutical viewpoint, omits many glycosides of pharmacognostic interest. Some glycosides contain more than one saccharide group, possibly as di- or trisaccharides. Under proper conditions of hydrolysis, one or more of the saccharide groups can be removed from such compounds, resulting in glycosides of simpler structure. All natural glycosides are hydrolyzed into a sugar and another organic compound by boiling with mineral acids; however, they vary in the ease with which this hydrolysis is performed. In most cases, the glycoside is hydrolyzed easily by an enzyme that occurs in the same plant tissue, but in different cells from those that contain the glycoside. Injury to the tissues, the germination process, and perhaps other physiologic activities of the cells bring the enzymes in contact with the glycoside, and the hydrolysis of the latter takes place. A large number of enzymes have been found in plants. Many of these enzymes hydrolyze only a single glycoside; however, two enzymes, namely emulsion of almond kernels and myrosinase of black mustard seeds, both hydrolyze a considerable number of glycosides. Glycosides that are derivatives of rhamnose require a special enzyme known as rhamnase for their hydrolysis.

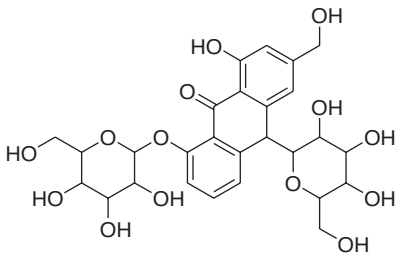
7.12.2 Anthraquinone Glycosides

A number of glycosides with aglycones related to anthracene are present in such drugs as *C. sagrada*, frangula, aloe, rhubarb, and senna. These drugs are used as cathartics. The glycosides, upon hydrolysis, yield aglycones that are di-, tri-, or tetrahydroxy anthraquinones or modifications of these compounds. A typical example is frangulin A, which hydrolyzes to form emodin (1,6,8-trihydoxy-3-methylanthraquinone) and rhamnose. Glycosides of anthranols, dianthrones, and oxanthrones (reduced derivatives of anthraquinones) also occur in plant materials, and they make significant contributions to the therapeutic action of these natural products. The anthraquinone and related glycosides are stimulant cathartics and exert their action by increasing the tone of the smooth muscle in the wall of the colon and stimulate the secretion of water and electrolytes into the large intestine.

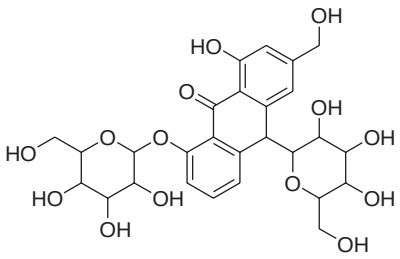
After oral administration, the anthraquinone glycosides are hydrolyzed in the colon by the enzymes of the microflora to the pharmacologically active free aglycones, which usually produce their action in 8–12 h after administration. These agents are indicated for constipation in patients who do not respond to mild drugs and for bowel evacuation before investigational procedures or surgery. Stimulant laxatives are habit forming, and long-term use may result in laxative dependence and loss of normal bowel function. Glycosides of anthranols and anthrones elicit a more drastic action than do the corresponding anthraquinone glycosides, and a preponderance of the former constituents in the glycosidic mixture can cause discomforting griping action. The drugs of choice are *C. sagrada*, frangula, casanthranol, and senna. Aloe and rhubarb are not recommended because they are more irritating, which increases the chance for the griping action. Different therapeutically potent anthraquinones are as follows.

7.12.2.1 C. sagrada *or* Rhamnus purshiana

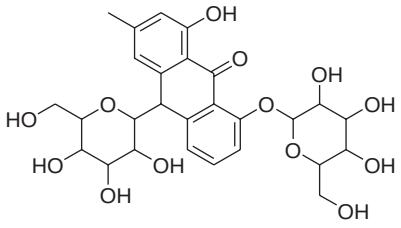
It is the dried bark of *R. purshiana* (Fam. Rhamnaceae). It should be aged for at least 1 year before use for medicinal preparation. Reduced forms of emodin-type glycosides are present in the fresh bark; during the minimum 1 year storage period, these glycosides are converted to monomeric oxidized glycosides, which exhibit milder cathartic activity. Two types of anthracene compounds have been reported. Normal *O*-glycoside (based on emodine), about 10%–20% and aloin-like *C*-glycosides, representing about 80%–90% of the total.



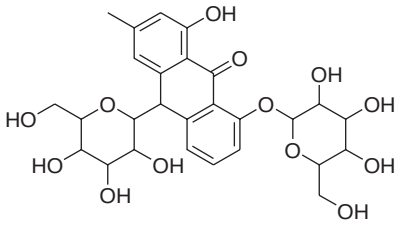
Cascaroside A



Cascaroside B



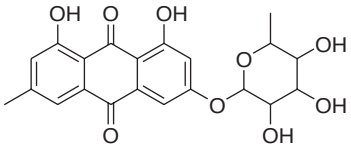
Cascaroside D



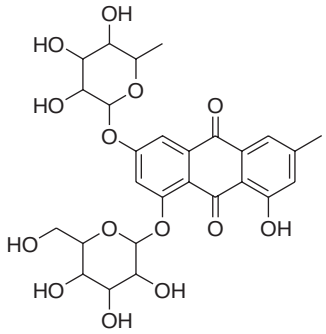
Cascaroside C

7.12.2.2 Frangula or Buckthorn Bark

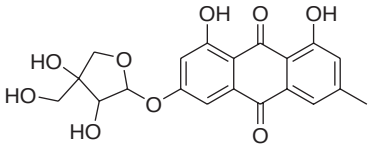
Frangula or Buckthorn bark is the dried bark of *R. frangula* Linn. Its laxative activity is due to the presence of anthraquinone glycosides—fragulin A and B and related gluco frangulins A and B. Just like *Cascara*, the bark should be aged 1 year before use to allow the reduced form of the glycoside with harsh action to be oxidized to the milder form.



Frangulin



Glucofrangulin

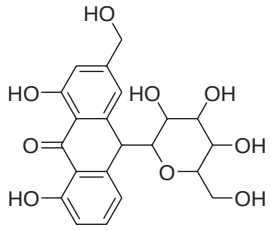


Frangulin B

7.12.2.3 Aloe

Aloe are the dried latex (juice) of the leaves of *Aloe barbadensis* known as Curacao aloe or different other varieties of aloe, of the family Liliaceae. It yields not less than 50% of water-soluble extractives. Over 300 species of Aloe are known, most of which are indigenous of Africa. Aloe is a typical xerophytic plant having fleshy leaves. Aloe occurs in the market as

opaque masses that range from reddish black to brownish black to dark brown in color. The taste of each variety of aloe is nauseating and bitter and has a disagreeable odor. The *Aloe vera* gel is used as a wound-healing agent and against burns, abrasions, and other skin irritations. It contains anthraquinone glycosides aloine A and B (barbaloin and isobarbaloin). The active content of aloe varies qualitatively and quantitatively according to the species from which the drug is obtained. Besides the physiologically active compounds of 10%–30%, other inactive compounds present are 16%–63%, comprising a large amount of resinous material and a volatile oil. Aloe is a cathartic. It elicits a drastic cathartic action.



Aloin A = (10R)

Aloin B = (10S)

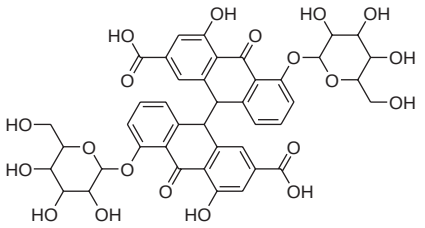
Aloins

7.12.2.4 Rhubarb, Rheum, or Chinese Rhubarb

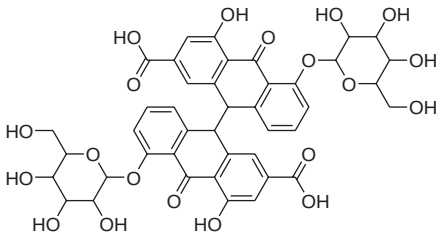
Rhubarb, rheum, or Chinese rhubarb consists of dried rhizomes and root that are deprived of periderm tissues of *Rheum officinale* or *Rheum palmatum* or other species (family Polygonacecea). Indian rhubarb or Himalayan rhubarb is the dried rhizomes or root of *Rheum emodie* or *Rheum webbianum* or of some related rheum species that are native to India, Pakistan, or Nepal. The principal constituent is the rhein anthron. Rhubarb is used in cathartic preparations and the action is drastic.

7.12.2.5 Senna Leaf

Senna leaf consists of dried leaflets of *Cassisa angustifolia*, known as Tinnevelly senna, and *Cassia acutifolia*, known as Alexandrian senna of the family Fabaceae. The principal active constituents of senna are dimeric glycosides whose aglycones are composed of aloe emodine and rhein. Those present in the highest concentration are sennoside A and B, a pair of stereo isomers, whose aglycones are rhein dianthrone (sennidin A and B). Senna pods also contain useful active glycosides. A concentrate of the active constituents and the isolated mixture of sennosides, which may be prepared from either the leaves or the pods, are used in various products, some of which contain a senna component combined with a hydrocolloid or a surfactant.



Sennoside A



Sennoside B

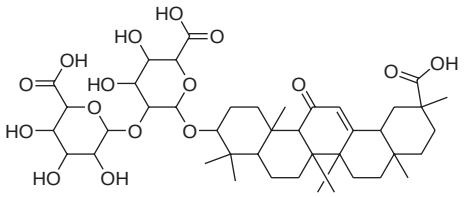
7.12.3 Saponin Glycosides

This group of glycosides is widely distributed in the higher plants. Saponins form colloidal solutions in water that foam upon shaking; they have a bitter, acrid taste, and drugs containing them are usually sternutatory and otherwise irritating to the mucous membrane. They destroy red blood corpuscles by hemolysis and are toxic, especially to cold-blooded animals. Many saponins are used as fish poisons. Upon hydrolysis, they yield an aglycone known as a "sapogenin." The sapogenin portion can be one of two possible chemical classes, either steroid or triterpenoid. Triterpenoid saponins are the most widely distributed in nature with more than 360 different sapogenins representing 750 different glycosides. Because these aglycones have a large number of carbon atoms (C_{27}–C_{30}) making them lipophilic, the saponin molecule due to the water-soluble sugars has a hydrophilic/hydrophobic asymmetry that results in a lowering of the surface tension in aqueous

solution, hence, the foaming upon shaking. The sapogenins form readily crystallizable compounds upon acetylization. This process can be used to purify sapogenins. Much of the research conducted on the saponin-containing plants was motivated by the attempt to discover precursors for the partial synthesis of steroid hormones. Because animal sources for the steroid hormones are in limited supply, academic, industrial, and governmental research agencies have examined many species of plants, particularly those containing steroidal sapogenins.

7.12.3.1 Dioscorea

After many years of investigation, the most important plant steroids for the partial synthesis of steroid hormones are diosgenin and botogenin from the genus *Dioscorea*: hecogenin, manogenin, and gitogenin from the species of Agave; sarsasapogenin and smilagenin from the genus Smilax; and sitosterol from crude vegetable oils. The more poisonous saponins are often called "sapotoxins." Many are toxic to insects and mollusks, and some have been used for controlling schistosomiasis snails. From the medicinal viewpoint, the most widely used saponin-containing drugs are glycyrrhiza and ginseng.



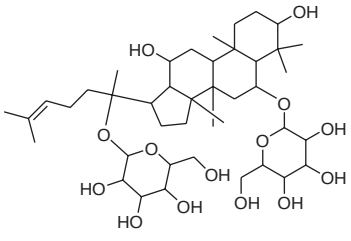
Glycyrrhizin

7.12.3.2 Licorice

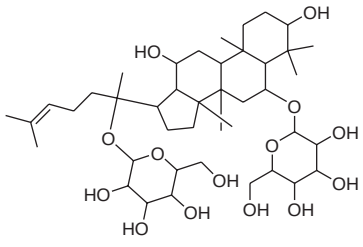
Glycyrrhiza is the dried roots and rhizomes of *Glycyrrhiza glabra* Linn., known as licorice of the family Fabaceae. Glycyrrhiza possesses demulcent and expectorant properties. It contains a saponin-like glycoside, Glycyrrhizin (glycyrrhizic acid), which is 50 times as sweet as sugar. Upon hydrolysis, the glycoside losses its sweet taste and is converted to the aglycone glycyrrhetic acid with two molecules of glucuronic acid. It is used as a flavoring agent and to mask the taste of some drugs, such as aloes, ammonium chlorides, quinine, and others. The surfactant property of the saponin also facilitates the absorption of poorly absorbed drugs, such as anthraquinone glycosides. Commercially, licorice is added to chewing gum, chocolate, candy, cigarettes, smoking mixtures, chewing tobacco, snuffs, and others to impart good flavor. It is even added to beer as it increases foaming. Glycyrrhetic acid is used in dermatological practice for its dermatological properties and licorice root extract is employed in the treatment of peptic ulcer and Addison's diseases (chronic adrenocortical insufficiency). Glycyrrhizin increases fluid and sodium retention and promotes potassium depletion.

7.12.3.3 Ginseng

It is the root of a perennial herb known as *Panax quinquefolius* Linn. and *Panax ginseng* of the family Araliaceae. Ginseng contains a complex mixture of triterpenoid saponins, which can be either steroidal triterpenes or pentacyclics related to oleanolic acid. Ginseng is a favorite remedy in Chinese medicine and is considered to have considerable tonic, stimulant, diuretic, and carminative properties. It reportedly has adaptogenic (antistress) action and also acts favorably on the metabolism, the central nervous system, and on endocrine secretion. It is employed in the treatment of anemia, diabetes, insomnia, neurasthenia, gastritis, and especially for sexual impotence (Shibata et al., 1985). Because of the high cost of Ginseng products and almost total lack of quality control in the health food industry, there is a great variation in the ginsenoside content of various preparations.



Ginsenoside RG 3



Ginsenoside RG 1

7.12.4 Cyanogenic Glycosides

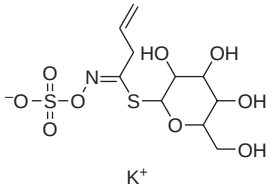
Several glycosides yielding hydrocyanic acid (HCN) as one of the products of hydrolysis are commonly found in plants. They are designated as cyanogenic glycosides and are accompanied in the plant by enzymes (β-glucosidases) that catalyze the hydrolysis. Some common cyanogenic glycosides are derivatives of mandelonitrile (bencaldehyde-cya-nohydrin). The group is represented by amygdalin, which is found in large quantities in bitter almonds, in kernels of apricots, cherries, peaches, plums, and in many other seeds of the Rosaceae, and also by prunasin, which occurs in *Prunus serotina*. Both amygdalin and prunasin yield mandelonitrile as the aglycone. Sambunigrin from *Sambucus nigra* liberates (*S*)-mandelonitrile as its aglycone. The enzymatic hydrolysis of amygdalin takes place in three steps. The β-glucosidase emulsion has been separated into three different enzyme activities: amygdalin hydrolase that catalyzes the breakdown of amygdalin to glucose, prunasin and prunasin hydrolase that hydrolyzes prunasin to glucose and mandelonitrile, and mandelonitrile lyase that catalyzes the dissociation of mandelonitrile to HCN and benzaldehyde. Cyaogenesis has been demonstrated in over 3000 plant species belonging to 110 plant families. In these plants, β-glucosidases and the cyanogenic glycosides are present in different cellular compartments. The compartmentalization has to be disrupted for the enzyme and the substrate to come into contact and lead to cyanogenesis. This suggests that cyanogenesis is a chemical defense response to organisms damaging the plant tissue when feeding on intact plant parts or attacking the plant through a site of injury. Preparations from plant materials containing cyanogenic glycosides are widely employed as flavoring agents. Anticancer claims have also been made for an amygdalin-containing preparation known as laetrile and the possibility for control of sickle cell anemia with cyanogenic glycosides has been noted. Some of the important types of glycoside are described in what follows.

Wild cherry is the dried stem bark of *P. serotina* (Fam. Rosaceae). It contains the cyanogenic glycoside prunasin (R-mandelonitrile glycoside). This is formed by the partial hydrolysis of amygdalin. The yield of hydrocyanic acid in wild cherry bark varies from 0.23% to 0.32% in inner bark and 0.03% in trunk bark and varies even in bark of the same thickness from the same tree. In syrup form, it is used as a flavored vehicle in cough remedies in which it is considered a sedative expectorant. Apricot pits are the kernels of varieties of *Prunus armeniaca* (Fam. Rosaceae). They are used as a source of laetrile or amygdalin that exists to the extent of about 3% (Poulton, 1983).

7.12.5 Isothiocyanate Glycosides

The seeds of several mustard family plants contain glycosides, the aglycons of which are isothiocyanate. These glycosides are also termed as glucosinolates and represent a group of bound toxins, such as the cyanogenic glycoside. Upon hydrolysis by the enzyme myrosinase, glucosinolate yields D-glucose and a labile aglycone that spontaneously rearranges with the loss of sulfate to give an isothiocyanate as the major product. The glucosinolates have a restricted distribution to a few plant families and are characteristic constituents of the mustard family (Brassicaceae), which includes oil seeds (rape seed), condiments (mustard and horse radish), and vegetable (broccoli, cabbage, and turnips). In plant species, they act as a feeding deterrent against both insects and mammals. The plant families have been found to have anticarcinogenic properties. Indole 3-carbinol derived from indolyl methyl glucosinolate isolated from mustard family vegetables and 4-methyl sulfenyl isothiocyanate isolated from broccoli have been shown to possess anticarcinogenic potential in the sense that they reduce the risk of estradiol-linked mammary cancer and induce anticarcinogenic enzymes, respectively. The principal isothiocyanate glycosides of pharmaceutical import are described below.

- Black mustard or brown mustard is the dried ripe seed of varieties of *B. nigra* or *Brassica juncea* (Fam. Brassicaceae). It contains 30%–35% of fixed oils. The principal constituent is sinigrin, which is present by the enzyme myrosinase. It is a local irritant and emetic and commercially used as a condiment.



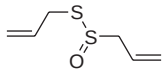
Sinigrin

White mustard is the dried seeds of *Brassica alba*. It contains the enzyme myrosinase and a glycoside sinalbin, which upon hydrolysis yields *para*-hydroxybenzyl isothiocyanate, a pungent testing oil component along with 20%–25% of fixed oil.

7.12.5.1 Other Organosulfur Drugs

7.12.5.1.1 Garlic

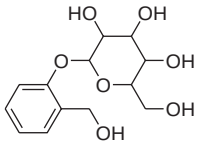
Garlic consists of the bulb of *Allium sativum* (Fam. Liliaceae) and has been consumed both as a food and as a medicine since the time of the pharaohs. The intact cells of garlic contain the odorless, sulfur-containing amino acid derivatives (+)-*S*-allyl-L-cysteine sulfoxide. This compound is commonly known as alliin and occurs in garlic in a concentration of up to 1.2% of fresh weight. When the cells are crushed, it comes into contact with the enzyme alliinase, which is stored in vacuoles within the cell, and it gets converted to allicin (diallyl thiosulfinate). Allicin has potent antibacterial activity. Unfortunately, it is also the compound responsible for the characteristic odor and flavor of garlic, decomposing readily in the presence of air and water to yield diallyl disulfide, diallyl trisulfide, and the corresponding polysulfides, all of which are strong-smelling compounds. In addition to antibacterial activity, garlic possesses antihyperlipidemic activity, enhances blood fibrinolytic activity, and inhibits platelet aggregation. (E) Ajoene and (Z) ajoene are formed from allicin and have antithrombotic properties, with the Z isomer being more bioactive. Studies indicate the mode of action involves inhibition of fibrinogen receptors on blood platelets.



Allicin

7.12.6 Alcohol Glycosides

Salicin is a glycoside obtained from several species of *Salix* and *Populus*. Most willow and populas barks yield salicin, but the principal sources are *Salix purpurea* and *S. fragilis*. The glycoside populin (benzoylsalicin) is also associated with salicin in the barks of the Salicaceae. Salicin is hydrolyzed into D-glucose and saligenin (salicyl alcohol) by emulsion. Salicin has antirheumatic properties. Its action closely resembles that of salicylic acid, and it is probably oxidized to salicylic acid in the human system. Recognition of the properties of salicin clarifies many folklore uses of populas and willow barks.



Salicin

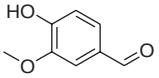
7.12.7 Aldehyde Glycosides

Vanilla is a drug that has an aldehyde aglycone as its chief constituent. Vanillin is the aglycone developed during the curing of vanilla beans. Vanillin is methylprotocatechuic aldehyde. Vanilla bean is the cured, full-grown, unripe fruit of *Vanilla planifolia* Andrews, often known in commerce as Mexican or Bourbon vanilla, or of *V. tahitensis* J.W. Moore, known in commerce as Tahiti vanilla (Fam. Orchidaceae).

Natural vanillin is a complex flavor containing aromatic aldehyde obtained from crude Vanilla capsules. Vanilla is a genus of orchids that grow in tropical climates, which extract flavor obtained from the fruit pods or beans of several orchids of this species. Out of these, the best and most important commercial extract of vanillin is obtained from *Vanilla fragrans* also known as *V. planifolia*. Out of the 110 vanilla species described, *V. planifolia* Andr. is the normally cultivated species for commercial vanilla flavor production. Relating to biosynthesis, it has been suggested that ferulic acid, a central intermediate of the general phenylpropanoid pathway, is converted to vanillin by a reaction analogous to that of the β-oxidation of fatty acids.

V. planifolia is an herbaceous, perennial orchid that grows mainly in wet tropical lowland forests. In fact, the green vanilla pods are essentially flavorless due to the glycoside form of the major aroma components. For commercial purpose, the fruits are harvested in their green premature state, about 6–9 months after flowering and subjected to a curing process in which the final flavored components are formed by a combination of hydrolytic enzymes. This liberates the different aglycons, as well as secondary enzymatic and nonenzymatic modifications, resulting in a complex mixture of products. For the rich overall vanilla flavor, volatile constituents of the cured beans are most important. More than 160 compounds have

been identified in the beans of *V. planifolia* and many of them contribute to the overall organoleptic property. Out of these, only 26 compounds have been found in a concentration higher than 1 ppm. The most important constituents present are 4-hydroxy-3-methyoxy benzaldehyde (vanillin), 4-hydroxy-3-methoxy benzoic acid, *p*-hydroxyl benzoic acid, and *p*-hydroxy benzaldehyde. These compounds occur in the green bean and are broken down during the process of curing the beans. Beside the whole cured vanilla beans, there are several processed vanilla products in the market, including vanilla extract, vanilla oleoresins (a conc. solvent extract), and vanilla powder. All of these products have potential demand in the world market and the production of vanillin by tissue culture can never be overruled by a synthetic one. Vanillin is one of the most high-valued phytoproducts for its universally appreciated aroma chemical. The flavor and odor of vanilla developed during the curing process and the vanilla beans, extracts, and tinctures are universally accepted food flavors. Cured vanilla beans are produced at the level of more than 2000–2400 metric tons (Mts) per year. Vanilla is the second most expensive species after saffron, with a global demand for 2000 Mts per year. Based on climatic conditions and variation in the natural conditions, the production of vanilla varies in different countries. To cope with the high demand, biotechnological approaches for the production of vanilla through the use of different tissue culture techniques have been developed. Several workers have reported vanilla formation in plant cell culture in which the productivity was enhanced by selecting phytohormones and elicitors, and by immobilization and adsorptive vanilla recovery. Vanillin precursors, such as ferulic acid, phenyl alanine, dehydroshikimic acid, and vanillyl alcohol, proved to be suitable for the same.



Vanillin

Artificial vanillin is produced from eugenol (derived from clove oil) or acid hydrolysis of lignin (wood) and represents the sensory backbone of vanilla. It constitutes more than 90% of the U.S. vanilla flavoring market and about 50% of the French market. One ounce of artificial vanilla has the same flavoring power as a gallon of natural extract. Moreover, synthetic vanilla costs 1/100th the price of the natural product. In this way, it acts not only as a substitute for vanilla but also as a supplement to the adulterated vanilla extracts. Despite the strong competition from synthetic vanilla, the factors responsible for strengthening the production of natural vanilla are:

- Increased health awareness and performance for natural products among the people.
- Escalating consumer demand for processed foods that exclusively use pure natural flavor and spices.
- An explosion in the popularity of gourmet ice creams, which tend to use pure natural products exclusively.

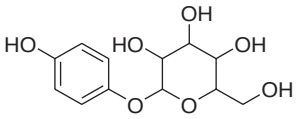
There are several grading standards for the export quality of vanilla, which differ among producing countries and are relatively complex. Classification is based on the bean length, aroma, color, moisture content, consistency and freedom from blemishes, insect infestation, and mildew. Vanilla produced from *V. planifolia* has been classified into five different grades, each preferred by different countries where the beans are exported. In the United States, a minimum of 2% of vanilla is preferred. In Europe, French imports are required to have minimum vanillin content of 2% and a moisture content between 15% and 17%.

Based on these concepts, the market demand for natural vanillin, particularly for the developed countries, is rising. From 1991 to 1993, 80% of the world imports of vanilla beans occurred in the United States, France, and Germany. Thus, based on the world market, vanillin has a great market potential that can never be supplemented by artificial vanillin, but only by natural vanilla produced from plant tissue culture technology through the use of various biotechnological approaches.

7.12.8 Phenol Glycosides

The aglycone groups of many of the naturally occurring glycosides are phenolic in character. Arbutin, found in Uva Ursi, chimphila, and other ericaceous drugs, yields hydroquinone and glucose upon hydrolysis. Hesperidin, which occurs in various citrus fruits, may be classified as a phenol glycoside. Phloridzin, found in the root bark of rosaceous plants, baptisin from Baptisia, and iridin from Iris species are additional examples of phenol glycosides.

Uva ursi or bearberry is the dried leaf of *A. uva-ursi* or its varieties *coatylis* and *adenotricha* (Fam. Ericaceae). The plant is a procumbent evergreen shrub indigenous to Europe, Asia, and the northern United States and Canada. In addition to the glycoside arbutin, the leaves contain corilagin, pyroside, several esters of arbutin, quercitin, gallic acid, elagic acid, and ursolic acid.

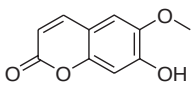


Arbutin

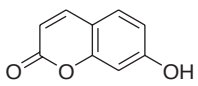
7.13 FLUORESCENT SUBSTANCES AND THEIR ANALYTICAL PARAMETERS

In the near-ultraviolet region of the spectrum (300–400 nm), some of the phytoconstituents show more or less brilliant coloration when exposed to radiation. This phenomenon of emitting visible wavelengths as a result of being excited by radiation of a different wavelength is known as fluorescence. Sometimes the amount of ultraviolet light normally present with visible light is sufficient to produce the fluorescence, but more often a more powerful source of ultraviolet light is necessary, for example, a mercury vapor lamp. It is often possible to make use of this phenomenon for the qualitative examination of herbal drugs. The following examples elaborate on this.

- Quinine, in oxyacid solution, exhibits a bright purple fluorescence when exposed to strong ultraviolet light and a very pale-blue fluorescence may be observed in ordinary light.
- β-Methyl aeseculetin or scopoletin is a common phytoconstituent present in herbal drugs, for example, wild cherry bark. This gives a violet fluorescence when an alkaline solution is exposed to ultraviolet light. Scopoletin is a derivative of coumarin. Belladonna herb also contains β-methylaesculetin, but this is masked by the red fluorescence of the chlorophyll.



Aesculetin-6-methyl ether (scopoletin)



Umbelliferone

- Umbelliferone is a derivative of coumarin, which exhibits a blue fluorescence in ammoniacal solution when radiated by very weak ultraviolet light, so that it is readily visible when examined by reflected daylight. It occurs in free condition in galbanum.
- Quassia wood contains a substance that gives a bright whitish-blue fluorescence in acid solution when subjected to strong ultraviolet radiation.
- The presence of constituents with these properties often gives the drug itself quite a characteristic appearance when it is examined in ultraviolet light, which can be explained by the examples given in Table 7.13.
- These fluorescence techniques can be useful for the detection of the adulteration in crude drugs. For example, it is often possible to detect adulteration of olive oil by examination under ultraviolet light.

"Virgin" olive oil gives a deep, golden-yellow fluorescence, but refined olive oil usually gives a pale-blue fluorescence. Arachis oil, sesame oil, and tea-seed oil also give blue fluorescence. By treating the oil with decolorizing charcoal, the blue fluorescence of the refined olive oil is removed, so that, if none of the other oils are present as adulterants, the fluorescence should be a golden yellow. A faint dull-blue fluorescence may remain, but it is nothing like the deep blue fluorescence shown by the other oils mentioned.

7.14 COLORING MATTERS AND THEIR ANALYSIS

The colored organic compounds are widely distributed in nature. Leaves of most plants contain green chlorophyll, which has an important catalytic effect on the process of plant metabolism. Besides this, other natural coloring matters are of varied chemical nature. The color of many flowers and fruits are due to flavones. Anthocyans, which produce the violet blue

TABLE 7.13 Fluorescence Characteristics of Some Herbal Drugs

Plant Drug Name	Fluorescence Nature
Shensi rhubars	Velvety-brown
Rhapontic rhubarb	Violet
Cinchona bark	Purplish-blue with yellow patches
Wild cherry bark	Mauve
Quassia wood	Whitish-blue points
Derris root (*D. elliptica*) (*D. mallacensis*)	Gray Orange-yellow
Gentian root	Whitish-blue
Vera cruz jalap	Pale pinkish-violet
Ipomoea	Deep purplish-violet

or red colors of many flowers, are glucosides derived from anthocyanidines and various sugars. The anthocyanidines are very closely related to flavones. Many plant products containing coloring matters have been used in the past in dyeing and in the manufacture of pigments. Among these are indigo, madder, log wood, fustic, weld, gamboge, cutch, and gambier; some coloring matters, such as litmus, turmeric, and cochineal, are used as indicators. Some coloring matters of pharmaceutical importance are red rose petals, red poppy petals, log wood, sappan wood, red sanders wood, alkanet root, saffron, and cochineal.

The coloring matters in plants occur in two forms:

(i) Those present as pigments in cell bodies, for example, plastids.
(ii) Those present in the solution of the cell sap.

7.14.1 Plastid Pigments in Plants and Their Analysis

Chlorophyll is the main pigment and almost all plant leaves contain this pigment. It has specific characters, which can be considered for its extraction and characterization as follows:

- It can be extracted from leaves when treated with strong alcohol but will not be extracted if the leaves are treated with weak alcohol, for example, with 45% alcohol the tannins can be extracted without hindrance to the chlorophyll.
- They are insoluble in water but soluble in organic solvents, though a trace of water is necessary in order to extract chlorophyll from dried plant material. The color of an acetone solution of chlorophyll is deep blue-green with a red fluorescence, and, when diluted with a large volume of water, the fluorescence disappears.

Chlorophyll, as extracted from leaves, consists of at least four pigments: chlorophyll A, chlorophyll B, carotene, and xanthophyll. It has been observed that by transferring the pigments to petroleum ether, it is possible to affect a partial separation of them by taking advantage of their solubility in methyl alcohol and petroleum ether. Chlorophyll A remains in a solution of petroleum ether, while chlorophyll B goes into solution in methyl alcohol. Chlorophyll A imparts a greenish-blue color to the organic solvents and chlorophyll B gives a bright green color, but the colors are somewhat masked by the carotene in petroleum ether and xanthophyll in the methyl alcohol.

If chlorophyll B is transferred to an ethereal solution, then both chlorophyll A and B can have the phytol part of their molecules removed by treating the dilution with a methanolic solution of potassium hydroxide. Potassium salts of chlorophyll A and B are formed. At the beginning, the color of the interface becomes yellow (in case of chlorophyll A) or brownish red (in case of chlorophyll B), but after about 10 min it changes back to green, without the fluorescence present with the chlorophyll itself.

- The potassium salts of the acid chlorophyllins are insoluble in ether but soluble in water so that by shaking the ethereal solution with water after treatment with alkali, the saponified chlorophylls are separated from the carotene, and xanthophyll remains in solution in the ether. The ethereal solution will be colored yellowish orange and the aqueous solution will be dull green.

- Magnesium in the molecule can easily be replaced by copper or zinc to give the most stable compound, which has an intense green color. These are the water-soluble chlorophylline of commerce, and under certain conditions, they act as catalysts in the oxidation of some sulfur-containing compounds.
- Carotene and xanthophyll differ in their solubility in methyl alcohol and petroleum ether. Carotene is insoluble in methyl alcohol and soluble in petroleum ether, while xanthophyll is soluble in methyl alcohol and insoluble petroleum ether. Carotene imparts an orange-red color to the solution and xanthophyll imparts a yellow color.
- When chlorophyll A or B in ethereal solution is treated with acid, it loses its magnesium and is converted into pheophytin, which is olive green in color. By heating this with a trace of copper or zinc acetate, a stable metallic pheophytin is formed, which is brilliant green in color but which lacks the fluorescence of the original magnesium chlorophyll.
- When an acetone solution of chlorophyll is subjected to column chromatography separation with adsorbents, separation of the four pigments takes place and they manifest themselves as colored bands on the adsorbents.

Other colored pigments associated with plastids are the carotenoid red or yellow substances, for example, capsanthins from capsicum. Other pigments are also present in capsicum and can be separated by using different adsorbents in the column. For the separation of the capsanthins by column chromatography, the usual adsorbent can be calcium carbonate, which should contain one-half of the column and the other half by calcium hydroxide, which will be on the top of the column. Thus, by means of adsorption chromatography, they can be separated.

7.14.2 Pigments of the Cell Saps in Plants

The cell sap soluble pigments are predominant in plant drugs. They are of two groups, which can be further classified into different subgroups based on their chemical nature. They are described further in what follows.

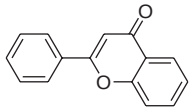
7.14.2.1 Anthoxanthins

The anthoxanthins are based upon the benzopyran nucleus and occur either in the free condition or as glycosides. The free anthoxanthins are pale yellow and their glycosides are colorless. The main characteristic feature is that both of them give an intense yellow color when made alkaline. The subgroups of this class of pigments and their distinctive tests are described below.

(i) Flavones

They occur in different plant drugs in different structures. For example, 5,7,3′,4′-tetrahydroxy flavone occurs as luteolin in *Digitalis purpurea* leaves and as diosmin in buchu (*Barosma betulina* or round buchu, *Barosma crenulata* or oval buchu leaves, and *Barosma serratifolia* or long buchu of the family Rutaceae). Similarly, 5,7,4′-trihydoxy flavone occurs as apicin in parsley flowers (*Petroselinum sativum*) and as apigenin in chamomile flower (*A. nobilis* of the family compositae). The tests for them are:

- Apigenin, when treated with ammonia solution, develops an intense yellow color.
- These flavones are soluble in weak alcohol and have the following specific test:
  - When treated with alkali, an intense yellow color is produced, which disappears when the anthoxanthins are made acidic again by the treatment of mild acid.
  - They give yellowish-red precipitate with lead acetate.
  - When treated with ferric chloride, a reddish-brown precipitate results.



Flavone

(ii) Flavanols

They are also abundantly present in plant drugs in different forms and can be characterized by different reactions. Among this class of pigments, quercetin, rhamnetin, isorhamnetin, and rutin occur in oak, cascara (*R. purshiana* of family Rhamnacecae), senna (*C. angustifolia*), and rue, respectively.

- Quercetin is 5,7,3,4-tetrahyxdroxy flavanol. Rhamnetin and isorhamnetin are the 7- and 3-monomethyl ethers, respectively. Rutin is quercetin with two sugars, one of which is rutinose, attached to form a diglycoside. It is obtained commercially from buckwheat.
- Kaempferol is 5,7,4′-trihydroflavanol and occurs as the glycoside kaempferin in senna leaves.

(iii) Flavanones

The main constituent of the flavanones is hesperidin, which is chemically the 4-monomethylether of 5,7,3′,4′-tetrahydroxyflavanone and occurs in lemon and orange peel.

(iv) Isoflavanones and xanthones

Isoflavanones and xanthones are also the bases of anthoxanthins, for example, gentisin, the 9-methyl ether of the 4,7,9-trihydroxy xanthone occurs in gentian root.

7.14.2.2 Anthocyanins

The anthocyanins are red, blue, or violet pigments, all of which are glycosidic in nature. They form stable oxonium salts that are amphoteric and give different colors at different pH values. There are three subgroups in this class:

- Pelargonidins
- Delphinidins
- Cyanidins

Of these classes, the main anthocyanins that occur in pharmacy belong to cyanidins. Mecocyanin, a genetobiocyde, occurs in red poppy petals and cyanin, a 3,5-diglycoside, occurs in red rose petals. There are a few distinctive tests for cyanin, which is soluble in dilute alcohol, but the color may disappear, which can be explained by the following tests:

(i) When treated with acids, the red color is intensified.
(ii) When treated with alkali, a green color is produced and, on acidification again, the original red color returns.
(iii) When treated with lead acetate, insoluble, colored lead salts are produced.
(iv) When mixed with amyl alcohol, no color is seen in the amyl alcohol layer, thus indicating the glycosidal nature of the coloring matter.
(v) When hydrolyzed, the colored free anthocyanidin is soluble in amyl alcohol.

7.15 PROTEINOUS COMPONENTS AND THEIR ANALYSIS

Proteins are complex nitrogenous organic substances of natural origin that have a high molecular weight. They are levo rotatory and, in addition to nitrogen, contain carbon, hydrogen, and oxygen and, in some cases, they also contain sulfur or phosphorous. They may occur in colloidal solution in cell sap or in solid form, for example, aleurone grains in seed; more complex protein, in combination with lecithin and nucleic acid, is the basis of protoplasm itself. Plant proteins are classified as follows:

(i) Simple proteins

These are a single entity and can be further grouped into:

- Albumin
- Globulin
- Prolamines
- Glutelins
- Histones
- Protamins

(ii) Conjugated proteins

Here, the proteins are in combination with other groups or metal. They often constitute a part, or the whole, of enzymes. The typical conjugated proteins are:

- Metallo protein
- Lipo proteins
- Hemo protein
- Necleo protein

(iii) Derived proteins

These are not true proteins, but products of like complexity derived from proteins, for example, peptides and peptones. Chemically, proteins are built up from a number of amino acids and characterized by:

- Polypeptide (–CONH–) linkages.
- At least one free NH_2 group and one free COOH group is present in protein structure.

Thus, they are amphoteric substances. The main amino acids occurring in plant proteins are of different types, which are shown in Table 7.14.

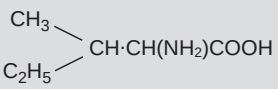
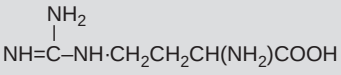
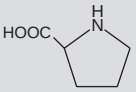
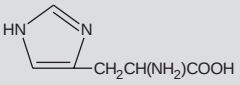
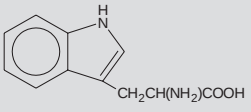
7.15.1 Therapeutically Important Plant Proteins and Their Analysis

(i) Albumin

Albumin proteins can be obtained in large amounts from fennel fruits after extracting with water, in which they are quite soluble. The main distinguishing characters of these proteins are:

- They are coagulated by heat.
- They are precipitated from aqueous colloidal solution by adding absolute alcohol.
- On treatment with concentrated nitric acid followed by excess ammonia, an orange color is produced, which can be a distinguishing character for the proteins and is known as the xanthoproteic reaction.
- They are precipitated by heavy metals, such as mercury and lead, and with Millon's reagent, a white precipitate, which, if formed turns brick red on warming.
- Substances that have two –CONH– groups joined together and attached to the same nitrogen atom give a delicate violet or pink color if, to a few milliliter of aqueous solution, 1 mL of 10% sodium hydroxide solution is added, followed by one drop of dilute copper sulfate solution. This is known as the biuret reaction.

TABLE 7.14 Amino Acids Occurring in Plant Proteins

Amino Acid	Chemical Structure
Glycine	CH_2NH_2COOH
Valine	$CH_3CH(NH_2)COOH$
Leucine	$(CH_3)_2CH{\cdot}CH(NH_2)COOH$
Isoleucine	$(CH_3)2CH{\cdot}CH_2{\cdot}CH(NH_2)COOH$
Serine	
Aspartic acid	$CH_2OH{\cdot}CH\ (NH_2)COOH$
Glutamic acid	$COOHCH_2CH_2CH(NH_2)COOH$
Arginine	
Lysine	$CH_2(NH_2)CH_2CH_2CH_2CH(NH_2)COOH$
Cystine	$COOHCH(NH_2)CH_2S{\cdot}SCH_2CH(NH_2)COOH$
Phenylalanine	$C_6H_5CH_2CH(NH_2)COOH$
Tyrosine	$(p)\ OHC_6H_4CH_2CH(NH_2)COOH$
Proline	
Histidine	
Tryptophane	

- Proteins are precipitated by picric acid and tannic acid.
- Plant albumins are precipitated from aqueous solution by saturating the solution with an electrolyte, for example, ammonium sulfate, magnesium sulfate, or sodium chloride.
- Proteins are precipitated by sulfosalicylic acid and phosphotungstic acid.

(ii) Globulin

Globulins are insoluble in water but soluble in salt solution. A good source is the protein-amandine from sweet or bitter almonds. Their main distinguishing characters are:

- All plant globulins are not coagulated by heat.
- They are precipitated from aqueous colloidal solution by the addition of absolute alcohol.
- On treatment with concentrated nitric acid followed by excess ammonia, an orange color is produced, which can be a distinguishing character for the proteins and is known as the xenthoprotic reaction.
- They are precipitated by heavy metals, such as mercury and lead, and with Millon's reagent, with which a white precipitate is formed that turns brick red on warming.
- Substances having two –CONH– groups joined together and attached to the same nitrogen atom, give a delicate violet or pink color if, to a few milliliter of aqueous solution 1 mL of 10% sodium hydroxide solution is added, followed by one drop of dilute copper sulfate solution. This is known as the biuret reaction.
- Proteins are precipitated by picric acid and tannic acid.
- Globulins are precipitated from aqueous solution by half saturating with ammonium sulfate.
- Proteins are precipitated by sulfosalicylic acid and phosphotungstic acid.

(iii) Prolamines

Prolamines and glutelins occur chiefly in graminaceous seeds and can be obtained from flour dough, after washing away all the starch with water, in which they are quite insoluble. The residue known as gluten is brown and extremely sticky when moistened. The main distinguishing characters of this protein are:

- The prolamines are soluble in 70%–90% alcohols, but the glutelins are insoluble.
- They are also insoluble in salt solution and in dilute alkalis.
- On treatment with concentrated nitric acid followed by excess ammonia, an orange color is produced, which can be a distinguishing character for the proteins. This is known as the xenthoproteic reaction.
- They are precipitated by heavy metals, such as mercury and lead, and with Millon's reagent a white precipitate is formed, which turns brick red on warming
- Substances that have two –CONH– group joined together and attached to the same nitrogen atom give a delicate violet or pink color if, to a few milliliter of aqueous solution 1 mL of 10% sodium hydroxide solution is added followed by one drop of dilute copper sulfate solution. This is known as the biuret reaction.

Nucleoproteins are quite an important group of conjugated proteins because they constitute a large proportion of the protoplasm of cells. On hydrolysis with enzymes or acid, they split up into various proteins and nucleic acids. The nucleic acid of yeast can be easily prepared. When tested for phosphate, it is found to give a yellow-colored precipitate of condensation of four nucleotides, each of which consists of phosphoric acid, D-ribose, and a purine. The four purines differ in each nucleotide: they are adenine, cytosine, guanine, and uracil.

7.16 STEROIDS

Steroids constitute a class of compounds that is widely distributed throughout nature. The diversity of biologic activities of steroids includes the development and control of the reproductive tract in humans (estradiol, progesterone, testosterone), the molting of insects (ecdysone), and the induction of sexual reproduction in aquatic fungi (antheridiol). In addition, steroids contribute to a wide range of therapeutic applications, such as cardiotonics (digitoxin), vitamin D precursors (ergosterol), oral contraceptive agents (semisynthetic estrogens and progestins), antiinflammatory agents (corticosteroids), and anabolic agents (androgens).

7.16.1 Sterols

The first steroids isolated from nature were a series of C_{27}–C_{29} alcohols that are found in the lipid fractions of many tissues. These compounds were solids and, therefore, named sterols from the Greek stereos, meaning solid. The most widely occurring sterol is cholesterol. It was first isolated from human gallstones and because it is a constituent of animal cell

membranes, it has been found in all animal tissue. It is one of the chief constituents of lanolin and, therefore, is found in many drug products. Much has been written about cholesterol and human health. Cholesterol is present in atherosclerotic plaques, and feeding of cholesterol to susceptible animals has induced atherosclerosis. In humans, atherosclerosis and coronary heart diseases are frequently associated with conditions in which the blood cholesterol is elevated. Recently, the results of numerous clinical studies have indicated that the lowering of serum cholesterol levels lowers the risk of coronary heart disease and may produce a regression of atherosclerotic lesions.

The principal sterol in fungi is ergosterol. Thus, C_{28} sterol arises biosynthetically through a transmethylation reaction of the cholestance side chain involving *S*-adenosyl methionine. Ergosterol is also known as provitamin D_2 because, upon ultraviolet irradiation, a series of isomerizations and the subsequent opening of ring B results in the formation of vitamin D_2. Vitamin D_2 is formed in the same manner from 7-dehydrocholesterol. This compound occurs in small quantities with cholesterol in animal tissue, including human skin, in which irradiation from the sun catalyzes the formation of vitamin D.

The most common sterol in plants is β-sitosterol (stigmast-5-en-3-β-ol), a C_{29} compound. It has been shown that a second transmethylation from methionine accounts for the C_{29} atom. In general, sitosterols are widely distributed throughout the plant kingdom, and may be obtained from wheat germ oil, rye germ oil, corn oil, cottonseed oil, and other seed oils. Closely related to β-sitosterol is the sterol, stigmasterol, which was first isolated from calabar beans, but is also found in soybean oil. The double bond at position 22 of stigmasterol allows it to be more readily converted into the pregane-type steroid hormones than is β-sitosterol; consequently, the extraction of stigmasterol from soybean oil is an important commercial process.

7.16.2 Steroidal Compounds of Therapeutic Importance and Their Analysis

7.16.2.1 Steroidal Saponins and Cardiac Glycosides

These saponins are less widely distributed in nature than the pentacyclic triterpenoid type. Phytochemical surveys have shown their presence in many monocotyledonous families, particularly the Dioscoreaceae (e.g., *Dioscorea* spp.), Amaryllidaceae (e.g., *Agave* spp.), and Liliaceae (e.g., *Yucca* and *Trillium* spp.). In the dicotyledons, the occurrence of diosgenin in fenugreek (Leguminosae) and of steroidal alkaloids in *Solanum* (Solanaceae) is of potential importance. Some species of *Strophanthus* and *Digitalis* contain both steroidal saponins and cardiac glycosides.

Steroidal saponins are of great importance and interest, owing to their relationship with such compounds as the sex hormones, cortisone, diuretic steroids, vitamin D, and the cardiac glycosides. Some are used as starting materials for the synthesis of these compounds. Diosgenin is the principal sapogenin used by industry, but most yams, from which it is isolated, contain a mixture of sapogenins in the glycosidic form.

Although total synthesis of some medicinal steroids is employed commercially, there is also a great demand for natural products, which serve as starting materials for their partial synthesis. The demand for steroidal compounds continually increases. In 1978, 600–700 t of diosgenin were used annually, with the worldwide sale of hormones estimated at $1500 million per annum. Efforts are constantly being made to discover new high yielding strains of plant and to assure a regular supply of raw material by the cultivation of good-quality plants. Some steroids present in nature are characterized by the highly specific and powerful action that they exert on the cardiac muscle. These steroids occur as glycosides with sugars attached at the 3-position of the steroid nucleus. Because of their action on the heart muscle, they are named cardiac glycosides. The steroid aglycones or genins are of two types, namely, cardenolide and bufadienolide.

The more prevalent in nature are the cardenolides, which are C_{23} steroids that have a 17-β side chain and an α,β-unsaturated five-membered lactone ring. The bufadienolides are C_{24} homologs of the cardenolides and carry a doubly unsaturated six-membered lactone ring at the 17-position. The bufadienolides derive their name from the generic name for the toad, bufo (the prototype compound bufalin was isolated from the skin of toads). An unusual aspect of the chemistry of both cardenolides and bufadienolides is that the C/D ring junction has the *cis*-configuration. To obtain optimum cardiac activity, the aglycone should possess an α,β-unsaturated lactone ring that is attached at the 17-position of the steroid nucleus and the A/B and C/D ring junctions should have the *cis*-configuration. Metabolic reduction of the double bond in the lactone ring of digoxin to form dihydro digoxin may explain why certain individuals are refractory to digoxin therapy. If the glycoside is cleaved, the glycone retains cardiac activity; however, the sugar portion of the glycoside confers on the molecule solubility properties that are important in its absorption and distribution in the body, and the stereochemistry and confirmation of the sugar moiety influence in binding affinity to a sugar-binding site on the receptor protein. Oxygen substitution on the steroid nucleus also influences the distribution and metabolism of glycosides. In general, the more hydroxy groups on the molecule, the more rapid the onset of action and the subsequent dissipation from the body. The use of the cardiac glycosides in therapeutics stems from the ability of these compounds to increase the force of systolic contraction (positive inotropic action). An increase in contractility in the failing heart results in a more complete emptying of the ventricle and shortening in the length of the systole.

(i) Digitalis

Digitalis or foxglove is the dried leaf of *D. purpurea* (Fam. Scrophulariaceae). The leaves of the other Digitalis species, *D. dubia*, *D. ferruginea*, *D. grandiflora*, *D. lanata*, *D. lutea*, *D. mertonensis*, *D. nervosa*, *D. subalpina*, and *D. thapsi*, also show the presence of cardiac glycosides. The drug contains a large number of glycosides, of which the most important from a medicinal viewpoint are digitoxin, gitoxin, and gitaloxin. More of their chemistry and analysis has been explained under the glycoside section. The total concentration of these three glycosides varies appreciably with the plant source and the conditions of growth. Also, because all are secondary glycosides derived by hydrolysis of some of the sugars from the primary or parent glycosides occurring in the leaf, their concentration depends on the manner of treatment of the plant material following harvesting (Wright, 1960).

The pharmacological effectiveness of the cardioactive glycosides is dependent on both the aglycones and the sugar attachments; the inherent activity resides in the aglycones, but the sugars render the compounds more soluble and increase the power of fixation of the glycosides to the heart muscle. It appears that the key grouping for the attachment of the molecule through a hydrogen bond to the phosphorylated receptor enzyme is the Δα,β-carbonyl function of the lactone. All of the active aglycones feature hydroxyl at C-3 and C-14 and the presence of a third hydroxyl group, at C-12 or C-16, will modify the activity and toxicity of the compound. The stereochemical requirements for activity include the *cis*-configuration between rings C and D, the β-orientation of the unsaturated lactone ring at C-17, and the three β-orientation of the glycosidic linkage.

The overall action of the *Digitalis* glycosides is complicated by the number of different effects produced. *Digitalis* probably acts in competition with K ions for specific receptor enzyme (ATPase) sites in the cell membranes of cardiac muscle and is particularly successful during the depolarization phase of the muscle when there is an influx of Na ions. The clinical effect in case of congestive heart failure is to increase the force of myocardial contraction (the positive inotropic effect). Arising from their vagus effects, the digitalis glycosides are also used to control supraventricular (atrial) cardiac arrhythmias. The diuretic action of digitalis, important in the treatment of dropsy, arises from the improved circulatory effect.

Digitoxin

Digitoxin is a cardiotonic glycoside obtained from *D. purpurea*, *D. lanata*, and other suitable species of Digitalis. On hydrolysis, digitoxin yields one molecule of digitoxigenin and three of digitoxose. It is a highly potent drug and should be handled with exceptional care. Digitoxin occurs as a white or pale buff, odorless, microcrystalline powder. It is a bitter substance that is practically insoluble in water and slightly soluble in alcohol. It is the most lipid-soluble of the cardiac glycosides used in therapeutics.

(ii) *Digitalis lanata*

D. lanata or Grecian foxglove is the dried leaves of *D. lanata*, a plant indigenous to southern and central Europe. It is the source of digoxin; however, nearly 70 different glycosides have been detected in the leaves of *D. lanata*. All are derivatives of five different aglycones, three of which (digitoxigenin, gitoxigenin, and gitaloxigenin) also occur in *D. purpurea*. The other two types of glycosides derived from digoxigenin occur in *D. lanata* but not in *D. purpurea*.

Digoxin

Digoxin (Lanoxin) is the most widely used of the cardiotonic glycosides, and it is obtained from the leaves of *D. lanata*. On hydrolysis, digoxin yields one molecule of digoxigenin and three of digitoxose. It is a highly potent drug and should be handled with exceptional care. Digoxin occurs as a white, crystalline powder.

7.16.2.2 Other Cardioactive Drugs

A number of plants contain cardioactive glycosides, and some of them have been employed for many years as cardiac stimulants and diuretics. Several are more potent than digitalis, but they are less reliable because their dosage cannot be controlled properly. Although most of these drugs were recognized officially for years and were considered efficacious, they have been superseded by the digitalis glycosides.

(i) Convallaria or lily-of-the-valley root

It is the dried rhizome and roots of *Convallaria majalis* Linn. (Fam. Liliaceae). More than 40 cardioactive glycosides have been isolated from the drug. Principal among these is convallatoxin, a monoglycoside composed of the genin of K-strophanthin (strophanthidin) and the sugar of G-strophanthin (rhamnose). Other minor glycosides include convallatoxol and convalloside.

(ii) Apocynum, black Indian hemp, dog bane, or Canadian hemp

This consists of the dried rhizome and roots of *Apocynum cannabinum* Linn. (Fam. Apocyanaceae). The chief constituent is cymarin, although apocannoside and cyanocannoside have also been isolated from *A. cannabinum*.

(iii) Adonis or pheasant's eyes

This is the dried over ground portion of *Adonis vernalis* Linn. (Fam. Ranunculaceae). Cardioactive glycosides identified in the drug include adonitoxin, cymarin, and K-strophanthin.

(iv) Black hellebore or Christmas rose

This is the dried rhizome and roots of *Helleborus niger* Linn. (Fam. Ranunculaceae). The chief constituent is hellebrin. Black hellebore possesses cardiac stimulant properties in contrast to green hellebore (*V. viride*), which is a cardiac depressant.

(v) Oleander

It is another plant that contains cardiac glycosides. The leaves of *Nerium oleander* (Fam. Apocynaceae) have been used to treat cardiac insufficiency. The chief constituent is oleandrin, a 3-glycoside-16-acetyl derivative of gitoxigenin.

(vi) Strophanthus

This is the dried, ripe seed of *Strophanthus kombe*, or of *S. hispidus* (Fam. Apocyanaceae). Strophanthus seeds have long been used by native Africans in the preparation of arrow poisons. K-strophantoside, also known as stroposide, is the principal glycoside in both *S. kombe* and *S. hispidus*. It is composed of the genin, strophanthidin, coupled to a trisaccharide consisting of cymarose, β-glucose, and α-glucose. Alpha-glucosidase removes the terminal α-glucose to yield K-strophantin-β, and the enzyme, strophantobiase, contained in the seed converts this to cymarin plus glucose. A mixture of these glycosides, existing in the seed in concentrations of up to 5%, was formerly designated strophanthin or K-strophanthin.

Ouabain is a glycoside of ouabagenin and rhamnose. It may be obtained from the seeds of *Strophanthus gratus* or from the wood of *Acokanthera ouabai* (Fam. Apocyanaceae). It is extremely poisonous. Ouabain is also known as G-strophanthin.

(vii) Squill or squill bulb

This consists of the cut and dried, fleshy, inner scales of the bulb of the white variety of *Urginea maritima* Linn., known in commerce as white or Mediterranean squill; or of *U. indica* known in commerce as Indian squill (Fam. Liliaceae). The central portion of the bulb is excluded during processing. Squill contains about a dozen cardioactive glycosides. The principal one, scillaren A, comprises about two-thirds of the total glycoside fraction. On hydrolysis, it yields the aglycone scillarenin, a bufadienolide, plus rhamnose and glucose. Other minor glycosides include glucoscillaren A (scillarenin + rhamnose + glucose) and proscillaridin A (scillarenin + rhamnose).

Squill is an expectorant, but it also possesses emetic, cardiotonic, and diuretic properties. Red squill consists of the bulb or bulb scales of the red variety of *U. maritima*, which is imported for use as a rat poison. It should not be present in the medicinal squill and may be detected by the presence of red, pink, or purple epidermal or paranchymal tissues. Most of the squill imported into the United States is of the red variety. Mostly it is used as a rodenticide. Rodents lack the vomiting reflex, which makes red squill particularly lethal to these animals. The inadvertent ingestion by humans of plant materials that contain cardiac glycosides induces the vomiting reflex and reduces the life-threatening aspects of the toxic manifestations.

(viii) *Dioscorea* species

Tubers of many of the dioscoreas (yams) have long been used for food, as they are rich in starch. In addition to starch, some species contain steroidal saponins and alkaloids. The sapogenins are isolated by acid hydrolysis of the saponin. Previous fermentation of the material often gives a better yield. The water-insoluble sapogenin is then extracted with a suitable organic solvent. Both wild and cultivated plants are used. Cultivation requires attention to correct soil and drainage, support for the vines and freedom from weeds, virus, fungus, and insect attack. According to the species, the tubers reach maturity in 3–5 years. On average, yams yield 1%–8% of total sapogenin according to species.

(ix) Sisal

Hecogenin is obtained commercially as the acetate in about 0.01% yield from sisal leaves (*Agave sisalana*). The sludge produced contains about 80% of the hecogenin originally present in the leaves; steam at 200 lb/in^2 pressure is employed to complete the hydrolysis of the original glycoside. By filtration and drying, a concentrate containing about 12% hecogenin and varying amounts of other sapogenins is produced.

A. sisalana also contains the dihydroxy steroid rockogenin, sometimes in appreciable quantity; this compound appears to be an artifact formed during processing and should be avoided. Agave hybrids with a high hecogenin content and relatively free of tigogenin, with which it is usually associated, have been developed.

(x) Fenugreek seeds

A spice cultivated in many parts of the world and derived from *Trigonella foenum-graecum* (Fam. Leguminosae). They are of pharmaceutical interest as containing a number of steroidal sapogenins—particularly diosgenin, which is contained in the oily embryo. Although the diosgenin yield is lower than that of the dioscoreas, fenugreek is an annual plant, which also gives fixed oil, mucilage, flavoring extracts and high protein fodders as side products.

(xi) *Solanum* species

This large genus (over 1000 spp.) is characterized by the production of C_{27} steroidal alkaloids in many species. Some of these alkaloids are the nitrogen analogs of the C_{27} sapogenins. Another series of C_{27} compounds contain a tertiary nitrogen in a condensed ring system, for example, solanidine. These compounds can also be employed in the partial synthesis of steroidal drugs. Species so exploited are *Solanum laciniatum*.

7.16.3 Quantitative Test Procedure and Assay Methods for Cardioactive Drugs

The tests and assays available for cardioactive medicinals depend on biological activity, reactions of the sugar side chain of the glycosides and properties of the butenolide side chain. Traditionally, the British Pharmacopeia has employed a biological assay for Digitalis (and formerly Strophanthus) on the basis that, compared with chemical and physical assays, it offers the best indication of the combined activity of the complex mixture of glycosides present. It is currently official for the assay of prepared Digitalis BP, a preparation of powdered digitalis leaves adjusted to strength of 10 units per gram. In the assay, the volumes of extracts of the digitalis under test required to stop the hearts of guinea pigs are compared with the volumes needed of similar extracts of a standard preparation of prepared digitalis. Pigeons may also be used for the assay. The results are evaluated statistically. Frogs and cats have also featured as test animals.

For digitalis leaves, the European Pharmacopeia and the BP utilize the red-violet color at a λ_{max} of 540 nm produced by the interaction of cardenolides and 3,5-dinitrobenzoic acid. Other color reactions based on the butenolide moiety are the red-orange with λ_{max} of 495 nm given with an alkaline sodium picrate reagent (EP assay for digitoxin and digoxin), the red color with xanthydrol reagent (EP test for digitalis leaf), and the red color (λ_{max} about 470 nm) produced with sodium dinitroprusside. In the ultraviolet region, the butenolide side chain exhibits a λ_{max} of 217 nm and, with a purified substance—for example, the eluted glycoside zones produced by TLC, which contain little extraneous material—this can be used for rapid evaluation. These spectroscopic tests do not, in themselves, distinguish between glycosides and their corresponding aglycones.

A color test specific for the digitoxose moiety is the Keller–Kiliani test, which has been described under steroids. The test is employed by the EP for the identification of digitoxin and digoxin and by the BP as an assay for digoxin injection and tablets with a λ_{max} of 590 nm.

7.17 CARBOHYDRATES AND THEIR ANALYSIS

Carbohydrates are the group of naturally occurring compounds, which are either sugars or compounds, that produce sugar on hydrolysis. The difference between sugars and nonsugars is chiefly a physical one. The sugars are crystalline, water soluble, and sweet, whereas the nonsugars are amorphous, water insoluble, and tasteless substances. Those containing 3–8 carbon atoms are known as monosaccharides and consist of a single unit of which the pentose and hexoses are of importance. Out of these, the carbohydrates having an aldehyde group are known as aldoses, while others having ketone groups are known as ketoses (Bugg, 1999).

Carbohydrates, as the name suggests, were defined as a group of compounds composed of carbon, hydrogen, and oxygen in which the latter two elements are in the same proportion as in water and are expressed by a formula $(CH_2O)_n$, that is, hydrates of carbon. It should be kept in mind that all organic compounds containing hydrogen and oxygen in the proportion found in water are not carbohydrates. For example, formaldehyde HCHO, for the present purpose written as $C(H_2O)$, acetic acid CH_3COOH written as $C_3(H_2O)_2$, and lactic acid $CH_3CHOHCOOH$ written as $C_3(H_2O)_3$, are not carbohydrates. Also, a large number of carbohydrates, such as rhamnose ($C_6H_{12}O_5$), cymarose ($C_7H_{14}O_4$), and digitoxose ($C_6H_{12}O_4$), which do not contain the usual proportions of hydrogen to oxygen, are known. Finally, certain carbohydrates are also known that contain nitrogen or sulfur in addition to carbon, hydrogen, and oxygen. From the above discussion, it can be concluded that the definitions described above are not correct; however, carbohydrates are now defined chemically as polyhydroxy aldehyde or polyhydroxy ketones or compounds that, on hydrolyses, produce either of the above. Carbohydrates are among the first products to arise as a result of photosynthesis. They constitute a large proportion of the plant biomass and are responsible, as cellulose, for the rigid cellular framework and, as starch, for providing an important food reserve. Of special pharmacognostical importance is the fact that sugars unite with a wide variety of other compounds to form glycosides and secondary metabolites. Mucilage, as found in marshmallow root and psyllium seeds, acts as a water-retaining vehicle, whereas gums and mucilage, which are similar in composition and properties, are formed in the plant by injury or stress and usually appear as solidified exudates; both are typically composed of uronic acid and sugar units. The cell walls of the brown seaweeds and the middle lamellae of higher plant tissues contain polysaccharides consisting almost entirely of uronic acid components. Low-molecular-weight carbohydrates are crystalline, soluble in water and sweet in taste, for example, glucose, fructose, and sucrose. The high-molecular-weight carbohydrates (polymers) are amorphous, tasteless, and relatively less soluble in water, for example, starch, cellulose, and inulin (Pigman et al., 1984).

7.17.1 Classification

In a broader sense, the polysaccharides or glycan may be classified into two major groups, namely, homoglycans and heteroglycans.

(i) *Homoglycan*: A polysaccharide is called a homoglycan when it contains only one type of monosaccharide unit, for example, cellulose. A large number of plant products belong to this particular category, namely, honey, starch, hetastarch, inulin, lichenin, dextran, cyclodextrins, cellulose, cotton, and dextrin.

(ii) *Heteroglycan*: A polysaccharide is known as a heteroglycan when it involves more than one kind of monosaccharide unit, for example, D-gluco-D-mannose is made up of D-glucose and D-mannose. In general, gums represent a heterogenous group of acidic substances, which essentially possess in common the characteristic property of swelling in water to form either gels or viscous, sticky, solutions. It has also been advocated that gums are the resulting products obtained from normal plant metabolism (Franz, 1989).

7.17.2 Inulin

It is a carbohydrate made up of 25–30 D-fructofuranose units. It is insoluble in cold water but soluble in hot water, giving a colloidal solution that does not deposit inulin unless cooled to a low temperature.

- Iodine produces no color with inulin.
- As inulin contains few free carbonyl groups, it will reduce Fehling's solution very slightly.
- Upon hydrolysis it will give fructose, which can reduce Fehling's solution. It can also be identified by Pinoff's and Seliwanoff's tests.

7.17.3 Cellulose

Cellulose is the principal constituent of the cell walls of higher plants, though in many secondary tissues it is impregnated with other substances, for example, lignin and suberin. It occurs in an almost pure form in the trichomes of cotton seed (*Gossypium* species). It is a straight chain polysaccharide of glucopyranose units linked through the 1–4 linkages. The molecules are joined by the beta glycosidic linkage so that the disaccharide residues are cellobiose. They have the following distinguishing features:

- Cellulose is insoluble in cold and boiling water.
- It does not reduce Fehling's solution.
- Cellulose is not colored with iodine. The further addition of strong sulfuric acid colors cellulose blue or bluish green.
- Cellulose is insoluble in dilute caustic alkali; stronger alkalis cause mercerization.
- Cellulose is slowly soluble in a freshly prepared ammoniacal solution of cupric oxide (Cuoxam or Schweitzer's reagent). At first the cellulose threads absorb the cuoxam and swell up, gradually losing their shape and finally dissolving. By adding strong acid, the cellulose is reprecipitated, which, after thorough washing, can be tested colorimertically.
- Cellulose is soluble in 80% (v/v) sulfuric acid. It is insoluble in 66% (v/v) sulfuric acid in the cold, but is soluble while warming. It is insoluble in dilute sulfuric acid.
- Cellulose can be hydrolyzed with an acid to the constituent sugars, which being glucose, can reduce Fehling's solution and form osazones.

7.17.4 Agar

It is a dried gelatinous substance obtained by evaporating to dryness the aqueous extractive of some bleached sea weeds, for example, *Gelidium amansii*, *Gelidium cartilaginium*, *Gelidium pristoides*, *Gracilaria confervoides*, *Pterocladia lucida*, *P. capillacea*, and other members of Rodophyceace. Chemically, agar consists essentially of the calcium salt of the sulfuric ester of a polysaccharide, the basic sugar being galactose. It has the following features:

- Agar is insoluble in cold water, but swells up in hot water to form a colloidal solution, which on cooling sets to a gel.
- The polysaccharides are precipitated with alcohol.
- When treated with iodine solution, no color is produced; however, if 1 mL of 0.02 N iodine solution is added to 0.1 g of powdered agar, a crimson color is obtained.
- Agar does not reduce Fehling's solution.
- Agar can be hydrolyzed with acids to galactose, which reduces Fehling's solution and forms an osazone. If hydrochloric acid is used to bring about the hydrolysis, the presence of sulfuric acid may be demonstrated by adding barium chloride, from which a slight precipitate of barium sulfate is obtained.

7.17.5 Irish Moss and Iceland Moss

Irish moss is a red-colored algae, *Chondrus crispus*, which contains a polysaccharide, and which is very similar to agar except that all the galactose units are joined through the 1–3 linkages and the sulfuric ester through the carbon atom.

Iceland moss is the lichen *Cetraria islandica*, which contains two complex polysaccharides, lichenin and iso lichenin, both as part of the cell wall and distributed as cell content. Lichenin consist of a D-glucose unit, of which about 80%–90% are joined through the 1–4 linkages and the reminder through the 1–3 linkages. It is considered intermediate between starch and cellulose in its properties. Isolicenine consists of glucose, galactose, and mannose units. They have the following distinguishing characteristics.

- Both of these substances swell up with cold water and finally dissolve to form a viscous colloidal solution.
- When treated with iodine solution, a blue color is produced because of the isolichenin, which is colored blue. Lichenin is not so colored.
- None of the above reduces Fehling's solution, but the sugars formed by hydrolysis will reduce Fehling's solution.

7.17.6 Pentosans

The most widely distributed pentosans are those made up of xylose and arabinose units. They occur chiefly in the secondary cell wall, particularly the hardwoods, coniferous woods and grass straw. The xylans consist of a xylopyranose chain joined through the 1–4 linkages, while arabinans are considered to be arabopuranose units, joined through the 1–5 and 3–1 linkages.

When bran and saw dust is washed free from soluble sugar, it can then be tested for pentoses by hydrolyzing the pentosans with strong hydrochloric acid, which converts the pentoses to furfural, giving a yellow color with aniline and a red color with phloroglucinol (Phillips et al., 1992).

7.17.7 Polyuronides

They are polysaccharide substances containing uronic acid in addition to condensed sugar residue. They occur both as cell content and cell wall material. They consist of a wide variety of substances, which can be described as follows:

- Hemicelluloses are complex polyuronides consisting of four or more polysaccharides associated with uronic acid. They are soluble in 4% sodium hydroxide solution.
- The thickened cell wall of the endosperm of the *Nux vomica* seeds consists chiefly of hemicelluloses, the concerned sugars being galactose, mannose, arabinose, and xylose, which can be tested by the same procedure described above.

7.17.8 Pectic Substances

They occur chiefly in the cell wall but are also found in a solution of the cell sap of a number of plants. They occur in three forms. Pectic acid and pectin are soluble in water and can be extracted with water from suitable plant materials, for example, black currants and apples. The other component is protopectin, which is insoluble in water and is found only in the cell wall (Walter, 1991).

Pectin is the methyl ester of pectic acid. Protopectin differs from pectin in its insolubility in water and it is thought that this is due to the chains of the polyuronides being much longer. Commercial pectin is chiefly obtained from fruits and consists of mostly pectin with traces of pectic acid. The pectic substances can be characterized by the following properties:

- Pectin dissolves in water to give a colloidal solution, which, when dehydrated, forms a solid gel. Dehydration may be brought about by 65%–70% sucrose; the pH range should be 3–3.5.
- Pectic acid has no gelling properties but calcium pectate has the property of gelling in certain conditions.
- When treated with hydrochloric acid, pectin is not precipitated from aqueous solution.
- Excess calcium chloride solution does not precipitate pectin.
- When an aqueous solution of pectin is made alkaline with sodium hydroxide and allowed to stand for 15 min, the pectin is demethylated to pectic acid.
- Excess calcium chloride precipitates calcium pectate.
- When treated with lime water, a gelatinous precipitate will separate out after some time.
- The tests performed for pentoses are also characteristic of pectin.

7.17.9 Gums

They are the polyuronides formed in plants as abnormal products from normal cell wall constituents. They are formed as the result of unfavorable conditions of growth or injury. Chemically, they are the calcium, magnesium, or potassium salt of the uronic acid part of the polyuronides.

- Most of the gums are soluble in water and form a sticky colloidal solution.
- They are all insoluble in alcohol.
- In the solid state, they are amorphous and translucent (Davidson, 1980; Dea, 1989).

7.17.10 Acacia

Acacia is the gummy exudation obtained from the stems of the *Acacia senegal* or other species of *Acacia*. The pharmaceutical quality has been bleached, with the result that it is opaque due to small cracks and fissures on its surface. It consists of the calcium, potassium, and magnesium salts of arabic acid. Hydrolysis of the arabic acid shows that it consists of L-rhamnose, D-galactose, and L-arabinose combined with a uronic acid. The characteristic features of acacia can be studied through use of the following points:

- It dissolves in water to form a viscous colloidal solution.
- The colloidal solution gets precipitated when treated with alcohol.
- When treated with iodine, no color is produced.
- It does not reduce Fehling's solution.
- Hydrolysis is not complete even after prolonged heating with acid; uronic acid is still joined with some of the sugars.
- As one of the sugars is arabinose, the tests for the pentoses as described earlier can be taken into consideration.
- A mixture of osazones is produced when the hydrolyzed products are treated accordingly.

7.17.11 Tragacanth

It is the gummy exudation obtained from the stems of *Astragalus gummifer*. The best quality gum (Persian) consists of translucent flakes, but other qualities, for example, *Smyrnan*, contain starch from the plants that is quite opaque. Chemically, tragacanth consists of two fractions:

(i) Water soluble, known as tragacanthin, which dissolves in water to give a slightly viscous colloidal solution. It is known as polyuarabinan tetragalactan geddic acid.
(ii) Water insoluble, known as bassorin, which swells up in water to form a colloidal gel.

It is a methylated tragacanthan xylan bassoric acid. The gelling power depends upon the number of hydroxy groups that are methylated. Good-quality tragacanth should contain not less than 60% bassorin. Mucilage of tragacanth consists of a suspension of the colloidal gel of bassorin dispersed throughout the colloidal solution of tragacanthin. The suspending power of "mucilage" depends upon the bassorin content and the methoxy value of the bassorin.

- Both bassorin and tragacanthin are precipitated by alcohol.
- No color is given with iodine (*Note*: Poor samples of the drug may contain starch, which will stain blue, and which are modified to a green color by iodine).
- Tragacanth does not reduce Fehling's solution.
- Hydrolysis of tragacanth may be brought about, at least partially, by heating with dilute acids. The hydrolyzed gum will:
  - Reduce Fehling's solution.
  - Contain arabinose and xylose, and will, therefore, respond to the tests for pentoses.
  - Yield a mixture of osazones.

7.17.12 Mucilages

Mucilages are normal constituents of many plants, usually occurring in special cells. They rapidly absorb water and swell into viscous colloidal solutions. Chemically, they resemble the gums. They are of different varieties, which can be explained further based on their characteristics as follows:

(i) The mucilage of linseed can quite easily be obtained in colloidal solution by allowing linseed to stand in water overnight.
  - It is precipitated by alcohol.
  - No color is given with iodine.

- It will not reduce Fehling's solution.

(ii) When hydrolyzed with dilute acids, it will give its constituent sugars, which are glucose, galactose, arabinose, rhamnose, and xylose, and the sugar acids.

- These sugars will reduce Fehling's solution.
- There will be a positive result to the test for pentoses.

There are of course a number of examples in which the therapeutic effects of the herbal drug differ (to some extent) from that of the concentration of its active constituents. This may be due to a synergistic effect of several constituents. Alternatively, it may be due to a modifying effect by some constituents upon the physical properties of the others. In many cases, it is economically unsound to synthesize or to isolate the active constituents because of their uncertain therapeutic value. Thus, from the pharmaceutical point of view, it is necessary to have a knowledge not only of the pharmaceutically active substances of an herbal drug but also of their analytical profiles. Such knowledge would enable maximum isolation and evaluation of suitable methods for purification of the constituents.

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Chapter 8

Thin-Layer Chromatography for Evaluation of Herbal Drugs

Chapter Outline

8.1 PRINCIPLES OF TLC

Thin-layer chromatography (TLC) was accepted as a separation method within the analytical laboratory after suitable sorbents became available for self-preparation of thin-layer plates. This development took place at the beginning of the 1960s. The Russian botanist Michail S. Tswett first reported the separation of plant dyes at a conference held in 1903. In the

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conference, he demonstrated the separation of leaf dyes by using calcium carbonate. Because of the nonhomogeneity in the packing structure, density, and surface structure of thin-layer plates coated by hand, quantitative analysis was not possible because of the inherently poor reproducibility and inadequate accuracy. The desire to obtain high-quality thin-layer plates combined with improved reproducibility of the layer properties led to the development of machine-made precoated layers for TLC.

TLC is widely used in the quality control of compounds by individually separating each component. Both qualitative and quantitative evaluation can be performed with this technique for the presence or absence of any particular secondary metabolite. A well-known example in support of this is the diterpene taxol from the endophytic fungus *Taxomyces andreanae*, which showed identical R_f values to taxol isolated from the Pacific Yew (*Taxus brevifolia*) when compared in four different solvent systems. This comparative study among different solvent systems helped to validate the finding. TLC analysis is also used for the classification of organisms on the basis of chemicals present in them, especially the filamentous bacteria of the genus Actinomycetes. Another important genus, *Streptomyces*, consists of an L-stereo isomer of the metabolite diaminopimelic acid and its meso form in its rarer genera.

The invention of efficient instruments for sample application, development, and evaluation as one prerequisite for quantitative thin-layer chromatography (QTLC) led to an increase in the demands made on the quality of the precoated layers available. After these increased quality requirements had been met as a second prerequisite for QTLC, in particular by the development of precoated layers for high-performance thin-layer chromatography (HPTLC), TLC could be employed as a quantitative analytical method comparable with other such techniques. TLC can be compared well with other chromatographic methods. The following parameters are characteristic of the TLC technique:

- To see the entire chromatographic separation at a glance, with an opportunity to judge the quality of the separation and to estimate the rough proportion of substances present and to recognize them properly.
- To avoid contamination with previous samples by only using the separation system once.
- To choose from a maximum possible number of solvents for the separation of desired substances, because the solvent evaporates completely before the actual separation.
- To choose from a wide range of commercially available stationary phases, which can be easily modified and which will not cause any "packing problems."
- To get just the desired components to "run" while others that are unimportant stay behind at the start.
- To optimize the separations quickly and at low cost by a straightforward change of the mobile and stationary phases.
- To subject a chromatogram to another chromatographic at right angles to the first direction, and thus improve the separation, as in two-dimensional TLC (2D-TLC).
- To develop a screening method and thereby to develop a test procedure for preselection of suitable separation systems, which can be transferred to a thicker bed or column for preparative applications.
- To separate as many samples as possible simultaneously and thus save time and money and also to cochromatograph standard substances under identical conditions.
- To have a choice of a maximum possible number of detection systems, possibly in series.
- To use a simple detection method valid for numerous substances (fluorescence quenching).

8.2 SEPARATION OF PHYTOCONSTITUENTS IN TLC

TLC separation is affected by the utilization of the blend or concentrate as a spot or thin line onto a sorbent supported on a backing plate. Among the TLC plates available (thickness 0.1–0.2 mm), the most common silica gel plate has dimensions of $20 \times 20\,cm^2$, supported by plastic or aluminum, and having 0.2 mm thickness of silica sorbent (Gibbons and Gray, 1998). The plate is then put into a tank with adequate solvent to simply wet the lower edge of the plate just below the space where the spot was applied. The mobile phase then travels up the plate by capillary action, known as development. The information provided by a finished chromatogram includes the "migrating behavior" of the separated substances. It is given in the form of the R_f value (relative to front):

$$R_f = \frac{\text{Distance}\left(\text{starting zone-substance zone}\right)}{\text{Distance}\left(\text{starting zone-solvent front}\right)}$$

The site of greatest substance density is considered to be the location of a zone. Owing to the numerous effects influencing the migration of a substance, which are very difficult to control at the same time, the R_f value should be regarded as an

approximate value. A substance that has migrated under identical conditions on the same plate as the spot of interest can serve as a standard in order to obtain a relative R value, known as the R_{st} value.

$$R_{st} = \frac{\text{Distance}\left(\text{starting zone-substance zone}\right)}{\text{Distance}\left(\text{starting zone-zone of reference substance}\right)}$$

$$R_f = \frac{\text{Compound distance from origin}\left(\text{midpoint}\right)}{\text{Solvent front distance from origin}}$$

In contrast to the R_f value, which is always <1, the R_{st} value can be >1.

8.2.1 Efficiency of TLC System

The separating efficiency of a TLC system can be seen from the zone spreading experienced by the applied substance spot as it migrates along the separation distance. It is described by the number of theoretical plates, n, or the plate height, h

$$N = 16\left(X / W\right)^2 \; H = W^2 / 16X$$

Here, X indicates the distance of a substance spot from the origin and the diameter of the spot over a distance of 10 cm. The height of a theoretical plate for a TLC plate is about 12 µm. The smaller the height of a theoretical plate, the more separation processes takes place over a given separation distance, and the higher is the separation efficiency.

8.2.2 Resolution of TLC System

Of decisive importance for practical applications, the resolution is the extent to which two different substances are separated by a given TLC system (solvent system + stationary phase). This depends both on the separating efficiency and on the selectivity of the separation system. The relevant value is the resolution R_S.

$$R_S = \frac{d}{1/2W_1 + 1/2W_2}$$

The values W indicate the spot diameters of two substances 1 and 2, while d is the distance between the centers of the spots. In other words, the greater the distance and the smaller the average spot diameters of the substances, the better the resolution. If $1/2W_1 + 1/2\,W_2 = D$, then $R_S = 1$ and the substances are just baseline separated, that is, the chromatographic signal just reaches the baseline between the spots.

8.2.3 Separation Number of TLC System

The separation number (SN) indicates the maximum number of substances that can be separated over a given separation distance by a TLC system in the ideal case.

The experimental determination of the SN can be described according to the following steps:

- A mixture of substances having different R_f values spread over the entire range of a chromatogram is subjected to chromatography.
- With the aid of a densitometer a densitogram is recorded and the width of the spots as measured at half peak height is plotted on an expanded scale against the distance of migration.
- The points obtained are joined by a straight line and extrapolated to spot width b_0 at $Z_f = 0$ and b_1 at $Z_f = 1$.
- SN (dimensionless number) is calculated from the simplified expression

$$\text{SN} = Z_f / b_0 + b_1 - 1$$

where Z_f is the migration distance to the front.

An important factor in quantifying the migration of a compound on a particular sorbent and solvent system is the R_f value. The R_f value is in the form of a ratio that is always <1 and changes according to the variation of the stationary phase and the mobile phase. This is sometimes known as hR_f, that is, relative to solvent front = 100, $hR_f = R_f \times 100$. In such a case,

when the stationary phase is silica (normal phase—NP), polar compounds are strongly attracted by the stationary phase, thus getting attached to the plate and traveling slowly through the plate. Therefore, these compounds have low values for R_f (retardation factor) (Gibbons and Gray, 1998).

Nonpolar compounds are less attached to the sorbent and will thus travel more quickly in the plate. This leads to higher R_f values. Because of this, a mixture of compounds will be retained at different places in the sorbent according to their respective polarities. Polarity is characteristic of the type and quantity of the functional groups present, which are capable of forming hydrogen bonds with the sorbent molecules (Gibbons and Gray, 1998).

When a substance is applied in dissolved form to the thin-layer plate during the separation process, it is transported along by the mobile phase, resides for a certain time on the stationary phase, and is then carried along again. In this way, the substance is slowed down relative to the velocity of the mobile phase; the more it preferentially resides on the stationary phase, the greater the slowing effect will be. Thus, substances are also separated that resemble each other in their affinity for the two phases: even small differences lead to differences in the chromatographic run if only they can act frequently enough. It depends on two basic underlying principles of the differences in affinity: adsorption and partition equilibria. The events can be described physicochemically in the following terms. During adsorption, substances dissolved in the mobile phase are adsorbed on the surface of a sorbent, such as alumina. Partitioning results from differences in solubility in two immiscible phases. In partition chromatography, substances dissolved in the mobile phase are partitioned between this phase and a second liquid phase attached to a solid substrate (such as reverse-phase (RP)-silica gel or cellulose). At low concentrations of substance A (samples of the order of a few micrograms), the simplified relation describing both equilibria is $C_s/C_m = K$, where C_s is the equilibrium concentration of substance A in the stationary phase, and C_m the equilibrium concentration of substance A in the mobile phase. At high concentrations of the substance in the mobile phase (close to saturation), this simple relation no longer applies, and the curve, called an isotherm, shows a discontinuity. The larger the value of K, the higher the affinity of the substance for the stationary phase and the more slowly it will travel. In general, adsorptive processes have proved suitable for the separation of compounds in herbal drugs differing in polarity (esters, alcohols, acids) and of structural isomers exhibiting different energies of interaction with an adsorbent. Partitioning processes support the separation of substances differing in their solubility, for example, in a homologous series. The processes named so far are based on adsorption and partitioning. Four main mechanisms are involved in the separation of a mixture of compounds and more than one mechanism may be involved at a time.

8.2.4 Adsorption Chromatography

Silica and alumina are two of the most widely used stationary phases in this form of chromatography. As the compounds travel along, their relative rates of movement are influenced by their individual affinities for the sorbent (Gibbons and Gray, 1998). Partition happens when one compound is more firmly adsorbed by the sorbent than the other. If the sorbent is silica or alumina, polar compounds move slowly compared with nonpolar common items. Adsorption happens as a result of the attraction between the compound and the compound associated with the stationary phase. On account of silica, which has silanol groups, interaction happens between the compound and the free hydroxyls on the sorbent. In this specific case, adsorption includes hydrogen bonding between compound molecules and adsorbent surface hydroxyl groups.

8.2.5 Partition Chromatography

This system deals with the solubility of the compound between the stationary phase and the mobile phase. Compounds that are more soluble in the mobile phase will travel more quickly through the plate than other components that are more soluble in the stationary phase. RP-TLC consists of stationary phases that distribute the compound between a hydrophobic stationary phase and an aqueous mobile phase. The most widely utilized RP (stationary phase) is silica, after it has been treated with a straight-chain 18 carbon alkyl unit to form an octadecasilyl (ODS) moiety. Nonpolar compounds, for example, the sesquiterpene artemisinin, are readily soluble in stationary phases, for example, OD; during solvent migration a partition is made between the two stages. Separation occurs due to the difference in partitioning capacity of compounds between the mobile and stationary phases.

8.2.6 Size-Inclusion/Exclusion Chromatography

Compounds may be isolated by their relative sizes and by their incorporation (or avoidance) into the sorbent. The most normally utilized size-inclusion sorbents are the dextran gels, especially the lipophilic forms, such as Sephadex LH-20, which are mostly used for the partition of small hydrophobic compounds from their "contaminants," generally chlorophylls, etc.

In natural solvents, for example, chloroform and methanol, these gels swell to shape a matrix. As compounds travel with the solvent through the gel, small particles are incorporated into the gel matrix, while bigger particles are not retained and they travel at a much faster rate. It should be kept in mind that partitions on gels, for example, Sephadex LH-20, may include the mechanism of adsorption, partition, and ion exchange. Once in a while the pattern of bigger particles eluting first and smaller particles eluting last might be reversed. This type of chromatography has found use for the separation of contaminants, for example, the chlorophylls, which tend to be more lipophilic than numerous plant items. This type of chromatography is typically completed in an open-section frame and is less utilized as a component of partition for TLC, which mostly depends on the systems of adsorption and partition.

8.2.7 Ion Exchange Chromatography

This procedure is restricted to compounds carrying a charge (Gibbons and Gray, 1998). Ion exchange chromatography uses stationary phases with positively or negatively charged groups and is particularly suitable for the separation of cations and anions, as well as proteins. Gel permeation chromatography separates substances according to their molecular size. Swollen gels with defined cavities are used as stationary phases. In this form of chromatography, the stationary phase is a polymeric material that is composed of charged groups and movable counter-ions that may interchange with ions present in the mobile phase component (Gibbons and Gray, 1998). Separation occurs due to differences in interaction between the ionic components and the sorbent. In the case of cation exchange, acidic groups, such as –COOH and $–SO_3H$, are constituted in the resin and transfer their protons with the other cations present to form $–CO_2–$, H_3O+, and $–SO_3–$, H_3O+, respectively, at particular pH ranges. In the case of an exchange of anion basic groups, quaternary ammonium moieties (−N+ R3) are constituted in the resin, which are able to trade their cations with anions of the compounds. As in the case of size exclusion chromatography, this type of separation is mainly used in columns but can be applied in thin layers also (Gibbons and Gray, 1998).

The sequence of operation in TLC can be divided into a main operation, a facultative operation, and auxiliary measures. The steps involved in the main operation—sample application, development, detection, evaluation—are indispensable constituents of any kind of chromatography. Facultative measures (prewashing of the layer, pre- and postchromatographic derivatization, preconditioning) permit significant improvements in detection limits, selectivity, and separation and, in many cases, reproducibility. Auxiliary measures consist in sample preparation appropriate for the separation problem at hand and selection of the layer material and solvent prior to sample application; assessment of the separation under UV light, and documentation of the chromatograms are frequently also part of this operation.

8.3 ADSORBENTS USED FOR TLC

The adsorbents used for precoated layers generally consist of irregular particles whose size cannot be described by a single specification of length. Different results are obtained, depending on the method employed to determine the particle size (e.g., sieving analysis, sedimentation, counting under the microscope) (Reich, 1977). Furthermore, the sorbents used in TLC display different distributions based on the size of the individual particles. The specification of a mean particle size is therefore not sufficient to characterize the dimensions of the sorbent; data on the particle size distribution are also required. Particle size distribution values (d_5, d_{10}, d_{50}, d_{90}, d_{95}) are usually determined by conventional particle analysis. The particle size distribution "*d*" values represent mass distributions for defined percentages. The fine particles of the sorbent have a negative effect on the flow properties of precoated layers, and the large particles have an adverse effect on the spreading of the spot. Therefore, a narrow particle size distribution is a decisive quality criterion. In particular, the sorbents employed for HPTLC satisfy these requirements to a high degree. The mean particle size of the sorbent also has a decisive influence on the separation performance of precoated layers. Precoated layers with small mean particle diameters (HPTLC) display a higher separation efficiency than those with a larger mean particle diameter (TLC).

The selectivity of a chromatographic system is determined chiefly by the chemical composition of the sorbent used, and particularly by its surface chemical properties. Its selectivity can be changed by varying the chemical structure of the entire skeleton of the sorbent and by specific chemical modification of its surface. The chromatographic behavior of a sorbent is affected not only by these chemical parameters but also by physical factors. In the case of porous materials, these factors are the specific surface area, the specific pore volume, the mean pore diameter, and the crystallographic structure. The primary parameters discussed in the preceding sections—chemical composition, crystallographic structure, specific surface area, specific pore volume, and pore width—are not the only factors that determine the chromatographic behavior of precoated layers. Secondary parameters, to be discussed later, play a major role in the preparation of the precoated layers. Two important secondary parameters are the mean particle size and the particle size distribution. These two physical parameters are of decisive importance for the packing structure, surface homogeneity, migration behavior, and separation performance (but not for the selectivity) of precoated layers.

The development of commercially available sorbents of constant quality and composition is an important condition for the fruitful application of TLC as a routine method for the quality control and evaluation of herbal drugs. The principal properties of the sorbents and their parameters are summarized in the following sections. Among the many sorbents, silica gel, modified silica gels, alumina, and cellulose have acquired particular significance. About 90% of all separations are performed on silica gel. This material is a porous amorphous powder. Its surface bears Si–OH groups capable of hydrogen bonding among themselves or with polar substances. The greatest number of available Si–OH groups and hence the greatest activity, is found for silica gel activated at 150 °C. During such treatment, it loses absorbed water and bears 4–6 Si–OH groups per 10 nm^2. Modified silica gels are produced on reaction with suitable silanes. In this way, functional groups, such as—BH_2, DIOL, or –CN, can be chemically attached to the surface of the sorbent. Silica gels silanized with alkyl chlorosilanes are of particular chromatographic interest.

8.3.1 Silica Gels

Silica gels are porous and are the most commonly used sorbents in QTLC. These porous systems are characterized by a comprehensive range of data. The skeleton of silica gel sorbents is formed from SiO_4, 4-tetrahedrons and displays an amorphous structure. The SiO_4, 4-tetrahedrons at the surface of the sorbent are saturated by silanol groups (SiOH) or siloxane bridges (Si–O–Si). In adsorption chromatography, the surface silanol groups represent the active centers at which the interactions take place that are required for the retention of the compounds to be separated. These interactions are mainly hydrogen bonding, dipole–dipole interactions, and electrostatic interactions. The density of silanol groups can be assumed to be constant, with a value of approximately 8 for all types of silica gels used in QTLC. As the retention of sample substances in adsorption chromatography is determined largely by the number of accessible silanol groups, the strength of this retention is proportional to the specific surface area of the silica gel used.

The pore width of the sorbents must be larger than the molecular dimensions of the largest substances to be separated in their solvated forms; only then can unimpeded access to the active centers of the internal surface be guaranteed. The pore widths of the silica gels used for precoated layers are between 4 and 20 nm. The angstrom unit of length is often still used. In many cases, these figures are employed in the destination of the type of sorbent. The pore structure parameters mentioned above, including the specific surface area, are not independent of one another. The relationship between them can be described by a mathematical expression defined by Wheeler (1955), which assumes that the pores are cylindrical in shape:

$$D = 4 \times 10^4 V_p / S_{BET}$$

where D is the pore diameter (Å), V_p is the specific pore volume (mL/g), and S_{BET} the specific surface area (m^2/g).

The porous, surface-active silica gels employed by various manufacturers to prepare precoated layers suitable for QTLC have specific surface areas between 200 and 800 m^2/g. The main portion of this area is formed by the surface within the pore system (internal surface area). The specific pore volumes of these silica gels lie within the range 0.5–2.0 mL/g. In addition to the two structural parameters discussed so far (specific surface area and the pore volume), the chromatography result is also determined by the mean pore diameter. This is because the exchange processes between the mobile and stationary phases that lead to the separation of the sample substances take place at the surface or close to the surface of the stationary phase. The length of the pores (determined to some extent by the particle size) and particularly the pore diameter play a decisive role in determining the kinetics of these processes.

In order to obtain reproducible retardation of the sample substances on precoated silica gel layers with a particular solvent, the adsorbent used in adsorption chromatography should be standardized and the state of activity of the silica gel must be controlled. Silica gel adsorbs solvent molecules from the gas phase because of their surface activity. Some of the surface-active centers will be occupied by solvent molecules, especially if water is used; these sites will then no longer be available in their original form for interaction with the sample molecules to be separated. It is therefore advisable to determine the degree of activity of the silica gel (e.g., by adjusting it to a defined relative humidity) and thus influence the strength of retention of a given sample substance with a defined solvent.

The strongest retention can be observed for all sample substances with 0% relative humidity and the weakest with 90% relative humidity. Furthermore, the separation of strongly retarded substances improves as the relative humidity increases, but in the case of substances that are only slightly retarded the separation worsens as the silica gel is deactivated. This clearly shows that not only the use of standardized sorbents, but also control of the influence of the gas phase, is important if reproducible results are to be obtained in QTLC.

8.3.2 Aluminum Oxides

The aluminum oxides (Al_2O_3) used for precoated layers in TLC are preferentially prepared from hydrargillite or γ-alumina. This starting product is thermally dehydrated and converted to aluminum oxide or a mixture of the two that are suitable for chromatographic purposes.

As is the case with silica gel, the hydroxyl groups of the aluminum oxides that are located at the surface are primarily responsible for the retention interactions with sample molecules. Their surface density is approximately 13 mol/m^2 (Combellas and Drochon, 1983). During dehydration, the structural parameters that help to determine the chromatographic separation properties of these sorbents, are fixed. The specific surface areas of porous aluminum oxides employed as precoated layers are between 50 and 350 m^2/g; their specific pore volumes lie within the range of 0.1–0.4 mL/g. The pore diameters derived from these structural parameters are 2–35 nm (Therefore, it is advisable to standardize these surface activities to obtain reproducible chromatographic results).

Surface-active aluminum oxides, such as silica gels, tend to adsorb water molecules from the surrounding atmosphere, leading to a reduction of their activity and their chromatographic retention capacity. This can be done by using a procedure developed by Brockmann and Schodder (1941), which allows classification of the aluminum oxide in question into five levels of activity depending on its water content. (Commercially available precoated aluminum oxide preparations usually have a pH value of about 9.0; however, precoated Al_2O_3 preparations are available that are adjusted to pH 7.0.)

8.3.3 Kieselguhr and Other Similar Compounds

Kieselguhr is a natural product of fossil origin consisting of silicic acid that occurs in deposits in various parts of the world. The chemical composition and physical properties of kieselguhr vary as a result of different biological origins and geological surroundings. Kieselguhrs that are used in the preparation of precoated layers for TLC are composed of SiO_2, Al_2O_3, Fe_2O_3, TiO_2, CaO, MgO, $Na_2O + K_2O$, and $H_2O + CO_2$ at 90%, 2.5%, 1.0%, 0.1%, 0.2%, 0.5%, 1.0%, and 4.7%, respectively. When used for precoated plates, the kieselguhrs must be carefully purified in order to remove the contaminants present as a result of their natural origin. These purification steps lead to changes in the original specific surface area and the pore structure. Purified kieselguhrs that can be employed for precoated plates have specific surface areas of about 1–4 m^2/g, specific pore volumes of about 1–3 mL/g and mean pore widths of 1000–10,000 nm. Purification does not, however, eliminate all the foreign atoms incorporated in the silicon dioxide skeleton. The remaining foreign atoms can affect the chromatographic behavior of the kieselguhr.

In addition to the naturally occurring kieselguhrs, a synthetically prepared inactive silicon dioxide (silica 50,000) with comparable chromatographic properties is used for precoated layers. The highly pure silica 50,000 consists of 100% silicon dioxide and has a specific surface area of approximately 0.5 m^2/g, a specific pore volume of approximately 0.6 mL/g, and a mean pore width of 5000 nm. Both kieselguhr and silica 50,000 are mainly suitable for partition chromatography because of their low specific surface areas. A special application of these inactive sorbents is their use in precoated layers with so-called concentrating zones. These precoated layers are composed of two different layer sections—the actual separating layer and the preceding concentrating zone. The separating layer consists of a surface-active adsorbent (Halpaap and Ripphan, 1997). The concentrating zone is composed of one of the two inactive sorbents and extends for 20–40 mm in the direction of chromatography, depending on the manufacturer.

When such precoated layers are used, the mixture of substances to be separated is applied to the concentrating zone. During development of this layer, the substance spots are transported to the boundary between the concentrating zone and the separating layer by the ascending solvent and are concentrated here in a narrow band. The actual chromatographic separation process begins at this boundary. In this method, chromatography and concentration are performed without interruption with the same solvent. Precoated layers with concentrating zones offer a number of other advantages compared with layers without such zones:

- Improvement of chromatographic separation efficiency, especially if large columns are applied.
- Simple application technique; for example, the starting spot does not have to be precisely positioned in the direction of chromatography.
- Possible decomposition reactions of sensitive substances are avoided (contact of the substances with the surface-active adsorbent takes place only when they are dissolved in the solvent, analogous to wet application in liquid column chromatography).
- Possibility of retardation of interfering accompanying substances (clean-up) in the concentrating zone.

8.3.4 Cellulose

The organic sorbent cellulose is employed quite often for precoated layers in TLC. Cellulose is a naturally occurring polysaccharide with the general formula $(C_6H_{10}O_5)_n$. Natural cellulose in the form of cellobiose units is purified prior to use in TLC and, if required, is subjected to crystallization. The resulting native or microcrystalline celluloses for TLC differ with respect to the morphology of their particles. Native cellulose occurs in the form of long fibers; microcrystalline cellulose exists as rods. In TLC, both types of cellulose are used mainly to separate polar substances in partition chromatographic systems due to their relatively low surface areas (up to approximately $2\,m^2/g$).

8.3.5 Polyamides

Polyamides represent another type of organic sorbent, and they are also used for the preparation of precoated layers for TLC. They are of two types: polyamide 6 and polyamide 11. Polyamide 6 is polycaprolactam and polyamide 11 is polyundecanamide. As polyamides have surface amide groups, they display a particularly high selectivity for substances that can form hydrogen bonds (e.g., phenols, carboxylic acids, amino acids).

8.3.6 Surface-Modified Sorbents

In addition to the unmodified sorbents already discussed, silica gels and cellulose have been used as bases for preparing chemically modified phases in which the surface properties of the precoated layers for TLC are selectively changed.

8.3.7 Hydrophobically Modified Silica Gels

In recent years the hydrophobic, so-called RP precoated preparations in particular have become increasingly important (Siouffi and Wawrzynowics, 1979; Brinkmann and de Vries, 1979). Silica gel with a mean pore width of preferably 6–10 nm is used as a base material for preparing hydrophobically modified precoated layers. Chemical modification is performed by reacting the accessible silanol groups with appropriate alkyl or aryl silanes. In this case, silanol groups are eliminated and new siloxane groups are formed. The chain lengths of the alkyl silanes range from 1 to 18 carbon atoms (Halpaap et al., 1980; Brinkmann and de Vries, 1982). The hydrophobic character of the modified silica gel increases with growing alkyl chain length and with the degree of modification. Consequently, hydrophobic materials of this type are no longer wetted by pure water or solvents that contain a high percentage of water, and it is no longer possible to develop chromatograms on these RP precoated layers with these solvents. The reason for this behavior is that the capillary forces required for development are no longer large enough to overcome the hydrophobic repulsive forces. Solvent systems with a high water content are often used in RP chromatography because of the polarity of the substances to be separated. RP precoated layers with low defined degrees of modification but identical alkyl chain lengths have been developed so that RP-TLC is not limited from the outset (Hauck and Jost, 1983a, b). These RP precoated layers display a hydrophobic, but also a partially hydrophilic, character as a result of the remaining accessible silanol groups; thus, their use with solvents of different polarities is not restricted.

8.3.8 Hydrophilically Modified Silica Gels

A hydrophilically modified precoated layer has also recently been used in TLC in addition to the nonpolar modified forms of silica gel. This material is an aminopropyl modification of silica gel. The selectivity of this functional group is determined by its polar properties and its ability to act as a weakly basic ion exchanger.

8.3.9 Surface-Modified Cellulose

In the case of cellulose precoated layers, it is also possible to realize surface modifications because of the presence of hydroxyl groups in this material. Acetylation of cellulose is one of the most important modifications in this respect. Precoated layers with different degrees of acetylation are available. A second group of surface-modified cellulose precoated layers consists of weakly basic anion exchangers. The functional groups that determine the selectivity are polyethylenimine (PEI), diethyl aminoethyl (DEAE), and ECTEOLA (a reaction product of epichlorohydrin, triethanolamine, and alkali cellulose).

8.3.10 Chemical Characters

- Polar (hydrophilic) phases, also known as “straight phases” or “normal phases.”
- Nonpolar (lipophilic) phases, also known as “reversed phases” (RP).

Between the two lie the medium-polarity phases, usually modified sorbents, such as the CN-, DIOL-, and NH_2-phases. Depending upon the mobile phase system, they sometimes have straight-phase properties and sometimes RP properties. Polar phases are usually combined with nonpolar mobile phase systems, such as chloroform/methanol, whereas RP phases are used with highly aqueous mobile phases. On transition from a straight-phase system to a RP system, the order of elution will clearly be reversed.

In this way, the polar stationary phase silica gel is transformed into a nonpolar phase of reversed polarity, a so-called RP. RP systems with 2, 8, and 18C atoms in the hydrophobic chain are commercially available as RP-2, RP-8, and RP-18 phases. Finally, there are also silica gels with chiral (optically active) functional groups. Such a phase may be able to separate optically active substances through a diastereomeric interaction. Modern natural product and drug synthesis would be inconceivable without careful control of enantiomeric purity: the different enantiomers may have totally different pharmacological activities and therefore have to be examined separately.

8.3.10.1 Grain and Pore Characteristics

The separation efficiency of an adsorbent is determined by its geometric structure. This also involves the particle size and its distribution. The selectivity of an adsorbent on the other hand, depends upon the chemical structure of the material, which will be explained in the following discussions.

(i) Particle size

In principle, the smaller the particles and the narrower the particle size distribution, the better the separation efficiency. The particle size should also be as uniform as possible (narrow particle size distribution). Commercial grade silica gels have particle sizes in the range of 5–40 µm.

(ii) Surface area

In usual practice, large surface areas mean a more intensive interaction between the sample and the stationary phase and, hence, greater adsorption, or stronger retention. Commercial grades of silica gels have surface areas of 400–600 m^2/g.

(iii) Layer parameters

Typical values for the layer thickness of the analytical separation lie at 100–250 µm, around 250 µm for normal TLC and around 200 µm for HPTLC. Plates with layer thickness of 0.5–2 mm are available for preparative separations. Scraping out of distinct zones and elution of the substances permits isolation of the substances after separation. The layer properties are also influenced by the binders used to increase the layer stability and cohesion, such as gypsum or organic binders. Layers with concentrating zones are also commercially available. In the ideal case, the substances in the concentrating zone run with the solvent front and are concentrated to narrow bands in the process (during adsorptive separation processes). Only when the separation layer has been reached does the chromatographic separation process begin. The borderline between the concentrating zone and the chromatographic layer serves as the starting line for chromatography. In this way, the spot size in the direction of chromatography is kept small and the resolution improved.

8.3.10.2 Self-Made Layers

A wide range of ready-made sorbents and layer types are nowadays available for TLC. These products are generally adequate for all purposes, thus relieving the user of any need to coat his own plates. The user should coat his own plates only for applications requiring layers that are not otherwise available. These may be layers containing buffer substances, special reagents, or other binders, or layers comprising mixtures of sorbents. As a rule, $5 \times 20\,cm^2$, $10 \times 20\,cm^2$, or $20 \times 20\,cm^2$ glass plates are coated after thorough prior cleaning (e.g., with scouring powder) and rinsing with distilled water. The plates are usually placed in a row on a frame and coated with a kind of spreading device that dispenses the coating material. This process can also be automated (e.g., using the CAMAG automatic coating device). The layer thickness can vary between 0.1 and 2 mm. For the preparation of the plates, the following points should be considered:

- The surface of the glass plates must be completely free of grease before coating, otherwise the layer will not adhere (after degreasing, the plates should only be handled with clean gloves).
- Coating should be performed rapidly. Owing to the fast hardening process (e.g., just a few minutes in the case of "G" layers), the layer will otherwise not be uniform.
- Coating must be conducted at constant speed.
- During the drying process all drafts must be avoided, otherwise cracks may appear in the layer.
- The manufacturer's instructions should be very carefully followed because any errors will give rise to poor separations and reproducibility.
- The quality and reproducibility of in-house plates are generally inferior to those of ready-made plates. The layers show poorer adherence and the layer thickness is less constant.

8.3.10.3 Precoated TLC Layers

Silica gel 60 is by far the most frequently employed absorbent for both absorption and partition chromatographic separations. Several classes of compounds can be easily separated by adsorption chromatography on silica gel precoated layers, which include aflatoxins, alkaloids, anabolic benzodiazepines, etheric oils, fatty acids, lipids, mycotoxins, pesticides, steroids, sulfonamides, and vitamins.

Several classes of compounds that can be separated by partition chromatography on silica gel precoated layers include aflatoxins, alkaloids, carbohydrates, glycosides, lipids, nucleotides, peptides, pesticides, steroids, sulfonamides, sweeteners, tensids, and tetracyclines.

8.3.10.4 Silica Gel Bonded Phases

Silica gel 60 has 60 Å pore diameter and is the most commonly used type in TLC and HPTLC. Silica gel and other precoated layers usually contain a binder to cause the sorbent to adhere to the glass, plastic, or aluminum support backing. Plates are designated as "G" if gypsum (calcium sulfate) binder is used. Plates with no foreign binder are designated with an "H" and high-purity silica with "HR." SIL G-25 HR plates contain gypsum binder and a very small quantity of an organic, highly polymeric compound. The layer thickness ranges between 0.1 and 0.25 mm for TLC and HPTLC, with preparative layers, designated "P," being thicker. Layers containing an indicator that fluoresces when irradiated with 254 or 366 nm ultraviolet light are designated as "F" or "UV" layers. These layers are used to facilitate detection of compounds that absorb at these wavelengths and give dark zones on a bright background (fluorescence quenching). F254 indicators can give green (zinc silicate) or blue (magnesium tungstate) fluorescence. F366 indicators can be an optical brightener, fluorescein, or a rhodamine dye. Some precoated plates have both indicators to detect compounds that absorb at both wavelengths ($F_{254+366}$ plates). Plates designated with an "s" have a UV indicator that is acid stable (e.g., F_{366s} plates). Hydrophobic RP-TLC, in which the stationary phase is less polar than the mobile phase, was originally carried out on silica gel layers impregnated with a solution of paraffin or silicone oil. Analtech (Newark, DE) sells RP plates with the hydrocarbon liquid phase physically adsorbed onto the silica gel layer surface. Alkylsiloxane-bonded silica gel 60 with dimethyl (RP-2 or C-2), octyl (RP-18 or C-18), octadecyl (RP-18 or C-18), and dodecyl (RP-12 or C-12) functional groups are most widely used for RP-TLC of organic compounds (polar and nonpolar, homologous compounds, and aromatics), weak acids, and bases after ion suppression with buffered mobile phases, and polar ionic compounds using ion pair reagents. The problems of wettability and lack of migration of mobile phases with high proportions of water have been solved by preparing "water-wettable" layers (e.g., RP-18W; EMD Chemicals Inc.) with less exhaustive surface bonding to retain a residual number of silanol groups. The latter layers with a low degree of surface coverage and more residual silanol groups exhibit partially hydrophilic, as well as hydrophobic, character and can be used for RP-TLC and NP-TLC with purely organic, aqueous-organic, and purely aqueous mobile phases. A commercial layer is available under the name chiral plate (CHIR) for separation of enantiomers by the mechanism of ligand exchange. These consist of a glass plate coated with C-18 bonded silica gel and impregnated with the Cu(II) complex of (2*S*,4*R*,20*RS*)-*N*-(20-hydroxydodecyl)-4-hydroxyproline as a chiral selector. Hydrophilic bonded silica gel containing cyano, amino, or diol groups bonded to silica gel through short-chain nonpolar spacers (a trimethylene chain $[-(CH_2)_3-]$ in the case of NH_2 and CN plates) are wetted by all solvents, including aqueous mobile phases, and exhibit multimodal mechanisms. A cyano layer can act as a normal or as a RP, depending on the characteristics of the mobile phase, with properties similar to a low-capacity silica gel and a short-chain alkylsiloxane-bonded layer, respectively. Amino layers are used in NP, RP, and weak basic anion exchange modes. DIOL plates have functional groups in the form of alcoholic hydroxyl residues, while unmodified silica gel has active silanol groups. The vicinal diol groups are bonded to silica with a quite nonpolar alkyl ether spacer group. Several categories of adsorbents, as used commercially, are described in Table 8.1.

8.4 PREPARATION OF THE LAYERS

8.4.1 Activation

There are numerous active sites present on the surface of the sorbent. Substances can be adsorbed at these active sites. The quantity of the adsorbed materials and the stability of the adsorptive binding depend upon the strength (surface energy per unit area) and the number (surface area per unit weight) of the active sites under otherwise constant conditions. The greater these two contributions are, the higher will be the activity of the sorbent and hence the retention power of the stationary phase. High activity thus leads to a shift of the adsorption–desorption equilibrium in the direction of adsorption,

TABLE 8.1 Different Codes Used for Silica Gel as Adsorbent

Code Used	Meaning
40, 60, etc.	Mean pore size in A
C	Channeled (layer divided into bands)
CHIR	Chiral layer for separating enantiomers
CN	Hydrophilic layer with cyano modification
DIOL	Hydrophilic layer with diol modification
F	Containing fluorescent indicator
F254, 366	Fluorescent indicator excitation wavelengths
F254s	Acid stable fluorescent indicator
G	Gypsum
H	Containing no foreign binder
NH_2	Hydrophilic layer with amino modification
P	For preparative work
R	Specially purified
RP	Reversed phase
RP-18	C-18 hydrocarbon chain
RP-8	Reversed phase with C-8 or
Silanized, RP-2	Reversed phase, with dimethylsilyl modification
W	Water-tolerant, wettable layer

leading in practice to shorter migration distances and smaller R_f values. Preloading of the layer with deactivators (generally polar substances) reduces the activity. The deactivation of conventional TLC plates is influenced in particular by water vapor in the air due to adsorption of polar water molecules at the active sites and the resulting reduction of the active surface of the sorbent. Because preparation of the plates and application of the sample generally takes several minutes, the activity of the sorbent after application generally corresponds to the relative humidity of the air in the laboratory. Prior activation of the layer is therefore necessary only in exceptional cases because the activity will, in any case, adapt to the relative humidity of the room or laboratory during sample application. Reproducible chromatographic results can generally only be obtained under reproducible constant conditions. The following parameters need to be remembered for the activation of the plates (Funk et al., 1981).

- High activity is not a precondition of high separation performance.
- The adsorption equilibrium (and hence also the activity) is dependent, like any true equilibrium, on the pressure and the temperature. Changes in temperature and pressure lead to adsorption and release of water from and to the environment.
- During sample application, water vapor present in exhaled air can lead to local changes in activity.
- Reactivation of the layer is generally unnecessary.
- Sometimes it is, however, necessary to activate the layer. In particular in tropical countries it may be necessary if separations are to be repeatable.
- The time required for equilibration (constant activity) is dependent upon the layer thickness and increases therewith.
- In general, the catalytic action of the layer increases with increasing activity. Some solvents can disproportionate or condense in contact with active surfaces (e.g., acetone).
- Reference substances must be chromatographed on the same TLC plate as the sample.
- Air-conditioned rooms with airlocks maintain the effect of activity at an essentially constant level.

8.4.2 Storage, Prior to Cleaning

Sorbents with large surface areas adsorb not only water but also other vapors that may be present in the laboratory air. Layers that have been exposed to the laboratory atmosphere for a prolonged period afford pronounced dirt zones, generally in the area of the solvent front or of β-fronts and fail to give reproducible results. Because the plastic packaging of thin-layer plates is not impermeable to gases, it will not completely protect the plates from environmental contaminants. Storage in plate cassettes protects them from the laboratory air and thus from contamination. Owing to their adsorptive activity, sorbents always contain small quantities of contaminants originating from the environment (e.g., pesticides, plasticizers), which they acquire during the production process. It may therefore be necessary in some cases to preclean the plates. As a rule, the TLC layer can be cleaned by predevelopment with chloroform–methanol (1:1) or with the solvent used for subsequent chromatography; such development should be performed over a longer distance than is planned for a position of the solvent in the subsequent chromatogram. The following points need to be considered:

- No grease should be used for sealing on storage of plates in glass vessels (desiccators).
- Storage over drying agents is generally unnecessary.

8.5 APPLICATION OF SAMPLES

In some cases, for example, in trace analysis or for samples with a complex matrix, samples cannot be applied directly to TLC plates but first have to be subjected to a cleaning and/or enrichment step (clean-up). In this way, a crude sample is prepared from the material to be studied, for example, by extraction, distillation, sublimation, or precipitation (to mention just the most important separation processes). The crude sample is subjected to further clean-up steps before chromatographic separation. Such clean-up steps should ideally lead to group or substance-specific separation of the analyte(s) (Mildau et al., 1984). For the preparation of the samples, see Table 8.2. The following points should be kept in mind.

- Many clean-up steps have to be discovered and optimized empirically.
- Any error in clean-up will persist all the way through to the evaluation of the finished chromatogram.
- The equipment and solvents used during sample preparation must be absolutely clean and pure.
- Sufficient substance should be cleaned-up in order to permit controls (duplicate and replicate determinations).
- After clean-up, the samples should be chromatographed as quickly as possible, particularly in the case of labile substances. Overnight storage should be in a refrigerator and longer storage in a freezer.

TABLE 8.2 Sample Preparation and Application

Operation	Description	Notes
Liquid–liquid extraction	Sample is partitioned between two immiscible solvents in a shaker funnel. The sample matrix is concentrated in one solvent and the analytes in the other	- The sample components should be known in advance
Solid-phase extraction	a) Extraction with columns (prepacked columns containing kieselguhr) The sample (up to 20 mL) is applied directly to the column. Elution with an organic solvent removes all lipophilic substances. The aqueous phase can subsequently be eluted with a polar solvent b) Extraction with Adsorbex columns (prepacked columns containing chemically modified phases that selectively retain substances)	- All conventional extraction procedures can be adapted to this method - Emulsification is avoided - Savings of solvent, material, and time - Eluates are pure - Recoveries and detection limits are better than with conventional extraction - Sample application and extraction with suitable solvents
Special TLC plates	TLC plates with concentration zones focus the applied mixtures to sharp bands and sometimes also serve for sample clean-up by removing interfering substances	- Focusing takes place particularly in adsorption systems
Derivatization	Chemical reactions to modify the chromatographic properties in a manner fitting the problem at hand	- Advantageous when the derivative is easier to handle than the original sample
Column chromatography	Preseparation of a mixture by ion exchange, adsorption, partition, or affinity chromatography in conventional columns	- Further processing depends upon the degree of purity - Direct HPLC/TLC coupling possible

8.5.1 Sample Application

As a rule, the samples applied have been subjected to prior sample preparation. Samples can be applied as spots or as bands. Generally speaking, spot broadening in the direction of development is smaller in the case of band-wise application than for point-wise application. Overloading of the layer, which can lead to spot tailing, can be largely avoided in this way. Samples applied band-wise can usually be better separated and more exactly evaluated. The choice of solvent for the sample also affects the size of the sample zone. Some solvents already chromatograph the substance at the starting point in the form of a ring-shaped chromatogram, while others do not. The sample and the reference substances should be dissolved in the same solvent or solvent mixture. After sample application, the solvent or solvent mixture is completely removed, with an air blower providing a means of accelerating evaporation. It should be noted that asymmetric evaporation of solvent could lead to local changes in concentration in the substance spot. As a rule, sample volumes of 1–5 µL are applied point-wise in TLC and 100–500 µL in HPTLC. The sample concentration generally lies in the range of 0.01%–1.00%. The starting spots for TLC should not exceed a maximum diameter of 5 mm and for HPTLC of about 1–2 mm. Application as several fractions should be avoided wherever possible because the previously applied zone is chromatographed on each subsequent application (this is recognizable from the "dog-bone" shape of the spots in the finished chromatogram). Nevertheless, if it is necessary to perform repeated applications, then the solvent should be evaporated off between each individual application. Sample and reference solutions should contain the same matrix wherever possible in quantitative studies. If necessary, sample matrix should be added in comparable quantities to solutions of the reference substances. The presence of auxiliaries can cause changes in the R_f values of the reference substances.

8.5.2 Preparation of Plates

As a rule, in TLC the starting line is marked at a distance of 1.5 cm (HPTLC: approx. 1 cm) from the bottom edge of the plate. At a distance of some 10–15 cm from the starting line (HPTLC, approx. 5–7 cm), the proposed distance to be reached by the solvent front is marked. The marks should only be made with a soft pencil on the very edge of the plate to avoid any damage to the layer. The starting points are marked at a distance of 1–2 cm (approx. 5 mm in the case of HPTLC) from each other and the edge of the plate along the proposed starting line. Further markings, such as the date, experimental conditions, or designation of the solution applied (spotting pattern), can also be marked between the marking of the solvent front and the top edge of the plate. In quantitative studies, reference standards of known concentration can be applied according to various schemes alongside the analyte tracts, depending upon whether the single standard method, the multiple standard method, or the data pair technique is employed (Pachaly, 1982). Preparative studies are usually performed on $20 \times 20\,cm^2$ plates having a layer thickness of 2 mm. The starting line is marked at a distance of 2–3 cm from the bottom edge of the plates. Up to 200 mg of substance in the sample solution is applied by passing a capillary or a syringe along the edge of a ruler held on the starting line. Multiple applications should be avoided wherever possible.

8.5.3 Application Methods and Equipment

Application is mainly performed with capillaries or syringes. On application with capillaries, the capillary forces of the layer suffice to induce flow of the sample. In the case of syringe application, we are dealing with forced dosage: the sample is pushed out of the syringe by the force of the plunger. The same applies to the spraying technique using the CAMAG Linomat IV. For qualitative investigations, melting point tubes with extended tips are often used as application capillaries. Precise measurement of the volume applied is impossible using this application method. Microcapillaries with defined volumes are preferred for accurate studies. As a rule, capillaries having volumes between 0.5 and 5 µL are used in TLC, and 0.1 and 0.5 µL in HPTLC. Generally, capillaries are used in combination with applicators (Klaus, 1985). The CAMAG microapplicator works on the principle of a micrometer syringe. The desired dosage volume can be selected anywhere between 0.5 and 23 µL by means of the micrometer screw. Using the Nanoapplicator, volumes between 50 and 230 nL can be selected. The sample solution is drawn into the syringe by lever action and applied to the layer in a similar way by lowering the lever. The foot of the holder of the Nanoapplicator on the baseplate (or respectively on the Nanomat I/II/III) ensures that the end of the needle is automatically adjusted during spotting so that it only just touches the surface of the layer without damaging it. The applicator is subsequently removed from the holder and reused after repeated rinsing of the needle. The CAMAG Linomat IV is suitable for point-wise and band-wise sample application. Sample volumes between 1 and 99 µL can be sprayed onto the layer with a predefined nitrogen gas pressure. The contact spotting method is suitable for application of sample volume up to and exceeding 100 µL. In this method, a fluorohydrocarbon polymer film is drawn into recesses in a sample application guide and thus adapts perfectly to the contours of the application device. Sample solutions

are pipetted into the recesses in the film and carefully dried. The concentrated solutions are then brought into contact with the layer. Complete transfer is then accomplished by applying gas pressure to the back of the polymer film. The following parameters should be considered:

- Wherever possible, automatic application devices should be used for quantitative studies.
- On use of capillaries, it is important to ensure that they fill and empty completely.
- Samples of high density can already partly leave the capillary before sample application. Solutions of high viscosity may sometimes only reluctantly or incompletely enter or leave the capillaries.
- Highly volatile solvents undergo partial evaporation before application from the capillary. In these cases, application or dosage from a syringe should be employed.
- The concentration should be selected in such a way that single emptying of the capillary will provide adequate sample to the layer because multiple application can lead to spot deformation during chromatography.
- Even disposable capillaries can be reused after multiple filling and emptying on a filter paper.
- The starting zones must be completely dried prior to development.

8.6 DETERMINATION OF SUITABLE SEPARATION CONDITIONS

In daily practice, recourse can often be made to one's own experience or that of others, and the literature searched to find the solution of many separation problems. A number of general rules and findings useful for determining separation conditions can be considered by "enlightened empiricism" to determine the suitable separation condition. While thinking about the interplay of the factors—mobile phase, stationary phase, and substance investigated—it logically leads to the formation of a triangle; if one apex of the triangle is pointed to a given property of one of the three factors, then the other two follow more or less automatically.

8.6.1 Parameters Influencing Separation in TLC

Many parameters have been found to affect TLC separation, which can be summarized in order of decreasing importance (Geiss, 1987) as follows:

- The stationary phase
- The mobile phase
- Type of chamber
- Prior adsorption of solvent (conditioning)
- Relative humidity
- Activity of stationary phase
- Saturation of chamber and stationary phase with eluent vapors
- Particle size and distribution of grains in the stationary phase
- Spot size on application
- Distance between start zone and level of the eluent in the separating chamber
- Solvent gradients (nonuniform distribution of various mobile phase components along the separation distance)
- Quality fluctuations of the stationary phase
- Velocity, that is, the flow rate of the mobile phase
- Size of the R_f value
- Temperature
- Sample volume
- Binder in the stationary phase
- Nonuniform layer thickness of the stationary phase
- Production of the stationary phase
- Deviation of the layer thickness of the stationary phase
- Convection currents in the vapor space of the separating chamber
- Nature of development (ascending/descending/horizontal direction of migration of the mobile phase)
- Impurities in the mobile phase
- Migration distance
- pH value of the layer
- Solvent for the sample

8.6.2 Selection of Developing Systems

Detailed information of the specific extract producing plant should be accumulated. This will help in the selection of the mobile phase. A full literature search on the following aspects should be considered:

(i) Whether the species has been considered previously.
(ii) What metabolites were detached if considered previously.
(iii) Whether standard TLC techniques are accessible.
(iv) In the event that the science of a species has not been examined, whether there are any data at the nonspecific level.
(v) Chemotaxonomy, or the arrangement of a lifeform according to its chemical constituents, may be helpful in identifying chemically unknown genera, which may be helpful in finding related species, yielding related secondary metabolites (Gibbons and Gray, 1998).

There are several drawbacks of TLC; still because of its various advantages, TLC on silica gel is still the most common method of TLC.

(i) Acidic compounds tend to "tail" on silica because of bonding between acidic groups (e.g., $–CO_2H$, –OH) of the compound and silanol group of the stationary phase. This can be corrected by adding a small amount of acid (e.g., 1% trifluoracetic acid or acetic acid) to the solvent system, which will keep the compound in nonionized form, thus reducing tailing.
(ii) In the case of a basic compound, tailing can be avoided by the addition of a weak base, such as diethylamine or triethylamine.
(iii) Nonpolar compounds, including fatty acids, glycerides, alkanes, and some lower terpenoids, need simple nonpolar solvent systems (e.g., cyclohexane, hexane, pentane, diethyl ether, hexane mixtures) and are not easily detected by UV (due to lack of chromophore) or by spray detection (due to charring of plates caused by reagents, such as vanillin-sulfuric acid).
(iv) Polar compounds, including sugars, glycosides, tannins, polyphenolics, and certain alkaloids, need to be developed in polar solvent systems. During such development, compounds may be adsorbed onto the stationary phase, which is difficult to remove. Mobile phase selection should start with the use of a mono or binary system, for example, 100% $CHCl_3$ of hexane:EtOAc (1:1) as a starting point and then on to the addition of acids or bases to improve chromatography, for example, a ternary system, such as toluene:ethyl acetate:acetic acid (60:38:2) and, as a last resort, a quaternary system, for example, hexane:ethyl acetate:formic acid:water (4:4:1:1).

Apart from the solvent and (in the case of hydrophilic sorbents) the activity of the sorbent, the choice of solvent system or mobile phase exerts a decisive influence on the separation. The solvent dissolves the substances to be separated from the sorbent and transports them across the plate. The more strongly a substance is adsorbed by the sorbent, the greater will be its eluting strength (displacement strength). If the substance has a greater affinity for the solvent than for the sorbent, then it will be eluted closer to the front. A word of warning about possible confusion in terms should be sounded here; the composition of the solvent mixture (often also called the mobile phase) can be, but is not necessarily, identical to the chromatographically effective mobile phase, because the solvent itself is also subjected to the chromatographic process. This becomes evident with multicomponent solvents; so-called "β-fronts" appear, particularly in sandwich chambers.

It has proved expedient to arrange various solvents in order of increasing eluting strength. This "eluotropic series" corresponds essentially to the order of increasing polarity or dielectric constant of the solvent. Of course, such an eluotropic series only strictly applies for a given sorbent. However, the series for silica gel and alumina are almost identical, whereas that for an RP phase has a completely different appearance. The eluotropic series of solvents, in which the solvents are listed in order of increasing elution power, is helpful in the choice of a suitable mobile phase for a particular separation problem. Solvents in the eluotropic series with different polarities have been described in Chapter 6.

8.7 DEVELOPMENT OF CHROMATOGRAM

In TLC, the term development means that a solvent or solvent mixture penetrates the TLC layer, generally under the influence of capillary forces and sometimes also on application of pressure, and transports the substances applied in the direction of forward flow. These substances are separated as a result of the interaction between the sample and the mobile and stationary phase, into individual components. In practice, there are various kinds of development; these are described in the following section. There are three possible ways in which TLC chromatograms can be developed. These are linear development (ascending, horizontal), circular development from the center outward (circular), and circular development toward the center (anticircular).

8.7.1 The Solvent Characteristics

For qualitative studies, it will be adequate to work at purity levels commonly encountered in the solvents used in chemical laboratories. Separation for quantitative evaluation, on the other hand, should be performed with special solvents, which have been subjected to thorough purification for chromatography. It should be observed that some solvents contain stabilizers (e.g., EtOH in $CHCl_3$), which may have a positive or a negative effect on a separation. The solvent composition is best expressed in parts by volume (e.g., 20 + 80, v/v). The components of the solvent mixture should be measured out individually and only then should they be mixed to avoid any possible problems due to volume contraction or dilation. The solvent components should mix in a storage vessel and only then be introduced into the developing chamber. Chambers containing solvent should not be reused for the following reasons.

- Solvent components will evaporate disproportionately according to their volatilities on opening the chamber.
- A chemical reaction can occur between the components of the solvents.
- One or several polar solvent components can be adsorbed preferentially by the layer during development (depletion of one or several solvent components).
- With binary mixtures, and particularly when one of the components is present in only minor amounts, such depletion becomes particularly noticeable.

8.7.2 Chamber Saturation

It has a pronounced influence on the separation. During the course of the separation, solvent evaporates from the plate, mainly in the region of the solvent front. More solvent is required for the front to travel a given distance and the R_f values increase. On the other hand, if the tank is lined with solvent-soaked filter paper, then solvent vapors are soon distributed uniformly throughout the vapor space and the tank is saturated. If a plate is placed in such a tank, and then the dry layer preloaded, less solvent is required for the solvent front to travel the same distance, and the R_f values are lower (Fig. 8.1). The differences in R_f for saturated and unsaturated chambers are only apparent differences. Solvents or solvent mixtures lying between the two extremes in the eluotropic series separate the mixture to a greater or lesser extent.

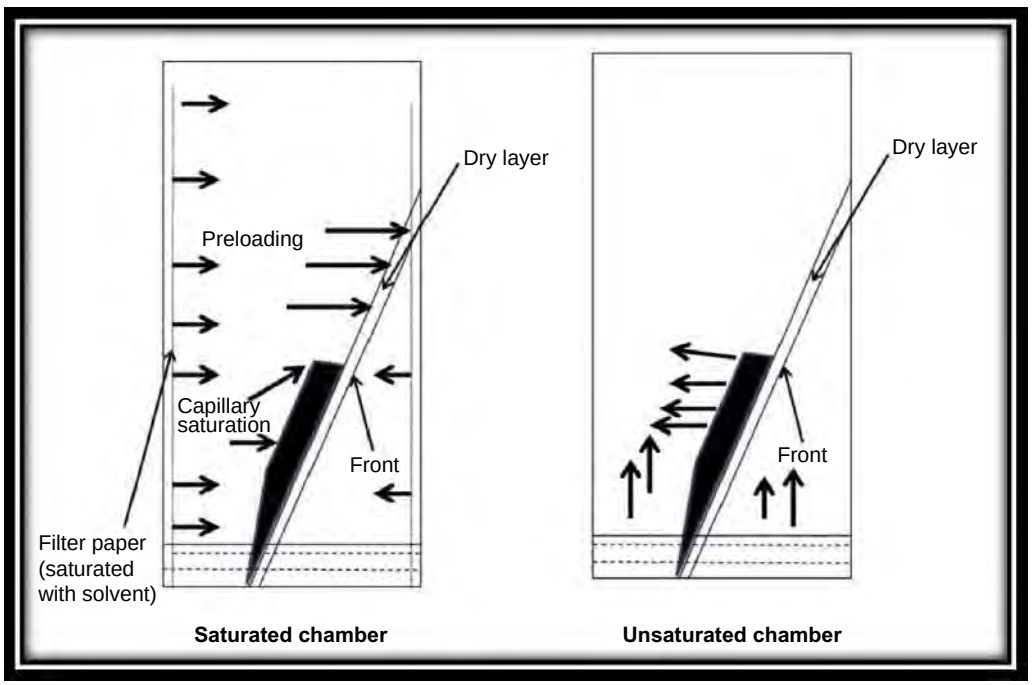

FIG. 8.1 Influence of the vapor phase in the development of chromatograms.

8.7.3 Types of Development

8.7.3.1 Ascending (Linear)

This developing technique is the one encountered most frequently. The TLC plate is introduced into a suitable chamber in such a way that the solvent wets the TLC layer below the starting line. It rises due to the action of capillary forces up to the desired height (10–15 cm for TLC and 3–7 cm for HPTLC) and transports the substance mixture to be separated. The starting areas increase as the spots approach the solvent front. The spots may undergo elliptical distortion during development, particularly in the vicinity of the solvent front. After the solvent front has reached the predetermined height, the plate is removed from the chamber, the solvent front marked precisely (pencil or scratched with a spatula), and the plate is dried.

8.7.3.2 Horizontal (Linear)

Solvent is applied to a horizontally oriented plate via a wick or a capillary slit. Development can be performed from one side or from both sides (Stahl, 1969).

8.7.3.3 Two Dimensional (Linear)

In 2D development, the mixture to be separated is applied to a starting point in a corner of the plate. The plate is placed in a normal developing chamber and first developed once from bottom to top. After drying, the plate is turned through 90 degree and placed in another developing chamber with a different solvent and developed once again. The track of the chromatogram resulting from the first development then becomes the starting line of the second development (Randerath, 1962). 2D development also offers the opportunity of running a standard in each development. However, the standard cannot be developed in two dimensions on the same chromatogram because it would lead to mixing with the sample. One particular feature of 2D-TLC is that different principles or separation mechanisms can be employed for the two development steps (Fried and Sherma, 1986).

Khatib et al. (2010) reported that, in one study, NP silica gel was used for the identification and analysis of compounds in *Vitex trifolia* (L.) having antitracheo spasmolytic activity. The mobile phases used were chloroform–methanol (9:1) 2D development and ethyl acetate–chloroform–methanol (28:28:44) in 3D development. In another study, 2D-TLC was applied to separate 18 pesticides on cyano-bonded silica gel as stationary phase, which can be used in both NP and RP modes of separation, offering a wide range of selectivity. Nonaqueous NP mobile phases (tetrahydrofuran or ethyl acetate in *n*-heptane) and aqueous RPs (a polar solvent [methanol or acetonitrile] in water) were used for subsequent developments (a stationary phase consisting of an NP and an RP on a plate of dual-adsorbent layers containing a narrow zone of silica beside a large zone of RP [octadecyl silica], or vice versa). Petruczynik et al. (2008) opted for 2D-TLC on different stationary phases to separate alkaloids from plant extracts from both types of stationary phases. The most specific systems are utilized for the separation of mixtures of alkaloids in 2D-TLC by the gradient elution technique. In one such study, Hawrył and Waksmundzka-Hajnos (2011) utilized cyano modified silica gel for the separation of phenolics from *Polygonum hydropiper* L. and *Polygonum cuspidatum* L. Organic solvents were used in one direction and aqueous solvents in the other direction. 2D-TLC using RP-18 silica plates was used by Ilic et al. (2011) for the separation of phenols. Ideal separation was obtained when the aqueous mobile phase in the first dimensional development was followed by the nonaqueous mobile phase in the second dimensional development. 2D-TLC can be a distinct process in which the whole TLC plate is scanned in linear mode using a normal slit shape, such as $0.2 \times 8\,mm^2$, with as small as 0.5- or 1-mm steps between scans.

A virtual simulation technique was adopted by Johnson and Nurok (1984) for optimum separation through continuous development of 2D-TLC. Thirty steroids were separated using this approach. It showed good correlation between the calculated and experimental results. Zakaria et al. (1983) separated many different classes of compounds using 2D-TLC. These included lipids (compound that are soluble in organic solvents and much less soluble in water, such as fatty acids and their esters, steroids, phospholipids, and glycolipids), pigments, alkaloids, amino acids, peptides, proteins, carbohydrates, glycopeptides, nucleic acids (and their constituents), environmental pollutants, pesticides, inorganic ions, and miscellaneous compounds. Hawryl et al. (2002) separated phenolic compounds by 2D-TLC on a cyanopropyl-bonded stationary phase utilizing polar solvent in a nonpolar basic solvent (e.g., in hexane) as the mobile phase for NP chromatography and a polar solvent in aqueous phase for RP chromatography (utilization of both organic and aqueous mobile phases in both dimensions for a combination of both types of chromatography in the same stationary phase). Optimum separation was obtained for phenolic compounds of a *Flos Sambuci* extract in the mobile phase of acetone–hexane (6:4) and methanol–water (1:1) in the first and second dimensional run, respectively. Similar satisfactory results were obtained when propan-2-ol–*n*-hexane (4:6) was combined either with tetra-hydrofuran–water (1:1) or with 1,4-dioxane–water (1:1). Hawryl and Soczewinski (2001) reported a NP 2D-TLC separation of flavonoids and phenolic acids from *Betula* sp. leaves. Apart from the flavones

(acacetin, apigenin, astragalin, hyperoside, kaempferol, myricetin, quercetin, quercitrin, and rutin) and flavanones (hesperidin, naringenin, and naringin), three phenolic acids (caffeic acid, chlorogenic acid, and ferulic acid) were also found to be present. Though various other parameters of all compounds were inspected, even 2D-TLC could not make the required separation. Most of the components were distributed on a curve into a collection of four to five partially separated plots. Consequent use of 2-diphenyl boroxy-ethylamine and polyethylene glycol 4000 as the polychromatic spray reagent was highly useful for differentiation and scanning the plate at 365 nm.

Ciesla and Waksmundzka-Hajnos (2009) experimentally proved the advantage of 2D-TLC for medicinal plants. Methodologies were described in detail for performing the most useful 2D-TLC, either by using a single adsorbent or multiple layers of adsorbents, by using graft TLC along with hyphenated methods. At the end, a description of differentiating 2D methods for analysis of the most important phytocompounds was produced. A diol polar-bonded stationary phase was used for 2D-TLC by Hawryl et al. (2012) for estimation of antioxidant phenolic compounds belonging to *Eupatorium cannabinum* extracts, which were analyzed by spraying 2-(diphenylboryoxy)-ethylamine and PEG4000 or DPPH before visualization. Highly polar glycosidic compounds, such as iridoids and triterpene, were separated from saponins by 2D-TLC in the hydrophilic interaction mode in certain verbascum species flowers by Ciesla et al. (2011). TLC silica gel was used as the stationary phase, whereas ethyl acetate–methanol–water–aqueous ammonia (55:35:9:1) and methanol–ethyl acetate–water–formic acid (10:90:26:22) were used as the mobile phase. Several structural analogs of coumarins were separated using 2D-TLC. Structural derivatives of coumarin were nicely separated on attached thin layers comprising either silica with RP-18W or CN-silica with silica CN-silica (Tuzimski, 2004) for which both aqueous and organic phases were used. A complete separation was obtained when both aqueous and organic mobile phases were used. Graft TLC also led to a complete separation. Optimum separation was obtained when silica and octadecylsilica were used as stationary phases for 1D and 2D development. Moreover, cyano-bonded silica gel followed by normal silica gel were used as stationary phases by using solvent system aqueous 30% AcN (acetonitrile) and 35% ethyl acetate in *n*-heptane for 1D and 2D development, respectively (Kalász, 2013).

8.7.3.4 Radial (Circular From the Inside Outward)

The layer is in a horizontal orientation. The substances are applied on a circle around a central point and the solvent applied to the midpoint of this circle. The components migrate outward with the solvent in the form of segments located on concentric rings and are separated in the process. Substances with small R_f values are separated particularly well in comparison with linear development (Vogel et al., 1984).

8.7.3.5 Radial (Circular From the Outside Inward)

The mobile phase is applied around a peripheral arc and migrates toward the center. The starting points are arranged around the outer circles. In comparison with linear development, substances with large R_f values are separated particularly well (Hammer and Kaiser, 1972).

8.7.3.6 Continuous Development (Overrun, Linear, and Circular)

A conventional chamber having a slit in the lid can be used for this kind of development. The solvent must be evaporated continuously outside the chamber. In one variant, the solvent is evaporated continuously by heating. Continuous development always gives superior resolution of the slowly migrating substances compared with normal development because the substances showing such slow migration have a larger separation distance. The price to pay for this superior resolution is a longer analysis time (Stahl, 1969).

8.7.3.7 Multiple Development (Linear, Circular)

In this kind of development, the thin-layer plate is subjected to repeated development with intermediate drying between each step. The solvent front thus travels repeatedly through chromatogram zones, reconcentrating takes place, and deformation of the spots, yielding elliptic shapes or narrow bands, is possible. As a rule, this leads to superior resolutions of substances with R_f values lower than 0.5. Multiple development can be performed with the same solvent or with different solvents of varying polarity, with the individual development steps being performed over separation distances of different length (Perry et al., 1975; Stahl, 1969).

8.7.3.8 AMD (Automatic Multiple Development)

AMD is a fully automated variant of multiple development with solvents of decreasing polarity (sorbents-silica gel). Each consecutive development step is performed over a longer distance than the preceding one. Because the process is started

with a polar solvent, all substances applied first migrate with the front and thus become concentrated to narrow bands. As the "gradient" proceeds, first the most polar and later the nonpolar substances remain in position. All steps can be preselected at will. After each run, the solvent is drawn off under vacuum. The procedure provides excellent resolution and sensitivity. "Gradient development" is the rule here (Burger, 1987). True gradients with continuously varying polarity are possible only in special chambers (e.g., the U-chamber), where solvent feed takes place via a pump. The following parameters should be considered for the development of solvent:

- Temperature variations during development lead to irregular solvent fronts and hence to nonreproducible results.
- The chambers must be protected from one-sided sources of heat (sunlight, drafts).
- Slight fluctuations of room temperature do not generally have a significant effect on the R_f values.

8.7.4 Types of Chambers and Development Techniques

In principle, there are two types of chambers, namely, chambers with a large vapor space (so-called normal chambers) and those with a very small volume of gas phase (sandwich chambers).

The normal chamber made of glass and measuring $21 \times 21 \times 9\,cm^3$ is the one used most commonly in thin-layer chromatographic studies. It is suitable for simultaneous development of at most two TLC plates ($20 \times 20\,cm^2$). In the case of a saturated normal chamber, the clean and dry chamber is lined with filter paper. These strips of filter paper should be cut in such a way that a window remains, allowing observations of the development process. Some 100 mL of the solvent is introduced to a height of 0.5–1 cm in the chamber, which is carefully tilted in order to moisten the filter paper and to equilibrate the chamber with solvent vapors. The lid of the trough should not be greased to ensure that no sealing grease is transferred to the solvent or onto the layer.

After 5–10 min, the chamber has become saturated with vapors and the TLC plate is now introduced in such a way that the solvent does not slop over the starting line and wash the substances away. Any contact between the side of the plates and the filter paper must also be avoided because solvent then enters the layer from the side and the solvent front assumes an upward curvature in this area and the chromatogram cannot be evaluated. After development the plate is removed from the trough and dried. Development in unsaturated troughs without a filter paper lining leads to evaporation of the solvent, above all in the vicinity of the front, into the vapor space of the trough and replacement thereof through the layer. As a result, the solvent throughput for a given development distance increases and hence so do the R_f values.

8.7.4.1 Twin Trough Chamber

The twin trough chamber is divided into two compartments by a ridge on the floor. In normal linear development, the solvent consumption (20 mL for a $20 \times 20\,cm^2$ plate and 4 mL for a $10 \times 10\,cm^2$ plate) is considerably lower than in normal chambers. Hence, the disposal problems are also reduced. The twin trough chamber can also be used for complete preconditioning. To this end, the solvent is placed in the trough, which does not contain the plate. After about 10 min the chamber is tilted to start development. In a variant of this design, the vapor space can be preequilibrated with any desired solvent in which the plate is not immersed. Only when the solvent enters the trough containing the plate does development start.

8.7.4.2 Sandwich Chamber (S-Chamber)

A TLC plate or foil is covered with a glass plate separated at a small distance, for example, using spacers in such a way that only the bottom-most zone of about 2 cm width remains uncovered. The glass cover plate should not be dipped into the solvent. This sandwich can be placed in any chamber. The arrangement described above represents an ideal unsaturated chamber. It can also be transformed into an ideal saturated chamber by using a solvent-soaked TLC plate as cover. The sandwich chamber is used when the sorbent layer is not to be preloaded with solvent vapor or when evaporation of solvent from the layer is to be avoided.

8.7.4.3 Horizontal Chamber (Linear Chamber)

This is a sandwich chamber used for horizontal development of TLC plates. Development can proceed from one or both sides. The TLC plate is placed layer-down at a distance of 0.5 mm from the counter plate. The solvent is poured into the troughs and the two glass strips turned inward. The solvent travels through the resulting capillary slit. Development is stopped automatically when the solvent fronts meet in the middle. A particular advantage of this chamber is its horizontal orientation (the solvent does not have to migrate against the force of gravity).

8.7.4.4 Vario Chambers

Vario chambers serve, above all, for development or optimization of separation conditions. Development is performed horizontally as in the horizontal chamber by the sandwich procedure. In contrast to the horizontal chamber, the Vario KS chamber contains up to eight solvent tanks or conditioning troughs for TLC (up to six in the case of HPTLC).

8.7.4.5 SB/CD Chamber (Regis)

TLC plates can be developed in the ascending mode at five different angles of inclination with the plates protruding to a greater or lesser extent out of the top of the chamber. In this external region, solvent evaporates off and is replenished from below. This amounts to continuous development over a short distance and good separations of substances with low R_f values result.

8.7.4.6 AMD Chamber (CAMAG)

Multiple development of thin-layer chromatograms can be performed fully automatically and thus in highly reproducible fashion in the same direction. The solvent is replaced for each passage and travels just a little farther than in the previous development step. This stepwise multiple development leads to focusing (compression) of the zones in the direction of development. The use of solvent gradients proves to be of particular advantage in that substances with widely differing polarities can be separated in a single chromatogram. A series of further chamber types is also commercially available, for example, the Chrompres chamber, Scilab Mobile R_f chamber, and the HPTLC chamber.

8.7.5 Development of Solvent System

Isocratic development is a technique in which a fixed concentration of mobile phase is used for separation, such as 40:60 (ethyl acetate:hexane). This plate may be kept for subsequent development by keeping the plate in the fixed solvent system until the solvent reaches the top of the plate. This is advantageous as bands with narrow difference of retardation factor may be separated by utilizing nonpolar solvent over a time period. The main drawback of this technique is that labile compounds may degrade on the stationary phase during this long period of time. In development through multiple solvent systems, after development of the plate, the plate is dried and then further development is performed by isocratical or step gradient elution. In the case of step gradient TLC, a nonpolar solvent system (e.g., 20% ethyl acetate in hexane) is used for primary development, dried, and then followed by a relatively polar system (e.g., 20% ethyl acetate in hexane). This technique provides more control over the separation as polarity is increased slowly for optimum separation of closely separating compounds. The choice of whether the system will run isocratically or with a step gradient can be obtained by using a number of analytical plates with the sample and changing the mobile phase ratio. Some of the more frequently used systems for the quality control and separation of herbal drugs are shown in Table 8.3.

8.8 DETECTION OF HERBAL DRUGS BY TLC

After development, the TLC plates are removed from the chamber and dried, and the separated substances detected. This is easiest with colored substances or with substances that can be induced to fluoresce or that absorb UV radiation. Substances that are neither colored nor that absorb UV light nor that fluoresce on exposure to UV light have to be visualized with suitable detection reagents to form colored, fluorescing, or UV-absorbing substances.

Both analytical and preparative TLC require proper visualization and detection for obtaining pure compounds and any error in detection may lead to low recovery of the compound from the stationary phase. Visualization takes place either through a nondestructive process, which allows the compounds to be recovered from the solvent, for example, ultraviolet (UV) detection, or destructive, in which compounds are degraded and unrecoverable from the stationary phase, such as spray detection.

8.8.1 Detection by UV light

This technique relies on the presence of UV-active compounds (indicators) that are mixed into the stationary phase of TLC plates commercially. An example of such a plate is Merck: Alumina, 0.2 mm thick, $20 \times 20\,cm^2$, with a 254 nm UV indicator. Under short-wavelength UV light (254 nm), the indicator, which is usually a manganese-activated zinc silicate, will transmit a pale purple-colored light. Compounds absorbing light at either 254 or 366 nm will show up as dim spots against a light foundation when UV light is exposed onto the plate. Numerous compounds, for example, the fluoro-coumarins, will

TABLE 8.3 Solvent Systems Used for Development of TLC of Some Herbal Drugs (Sorbents Used—Silica Gel 60F254)

Solvent Systems	Usefulness/Note
For drugs containing essential oils	
Acetone–hexane 1:30 and hexane–dichloromethane 1:1.3	*Aloysia gratissima*
Benzene	*Anisi fructus*
Benzene–ethyl acetate (90:10)	Eucalyptus
Benzene–ethyl acetate (95:5)	*Menthae piperitae*
Chloroform	*Curcuma rhizoma* *Melissae folium*
Chloroform–benzene (75:25)	*Absinthii herba*, *Matricariae flos*, *M. piperitae folium*, *Thymi herba*
Chloroform–ethanol–glacial acetic acid (94:5:1)	Separation of cinnamoyl pigments from Curcumae rhizome
Methylene chloride	*Carvi fructus*, *Juniperi fructus*, *Salvia officinalis*
Pet ether	*Pinus longifolia* Roxb, *Eucalyptus citriodora* Hook, *Citrus reticulata* Blanco
Alkaloidal drugs	
Acetone–water–conc. ammonia (90:7:3)	Atropine, hyoscyamine, and other Solanaceaeous alkaloids
Anthracene derivative containing drugs	
Chloroform:acetone:diethylamine (5:4:1)	Imidazole alkaloids (pilocarpine)
Chloroform–diethylamine (90:10)	Cinchona alkaloids, i.e., quinine, quinidine, etc.
Chloroform–methanol	Isoquinoline alkaloids Ipecacuanhae radix
Cyclohexane–chloroform–glacial acetic acid (45:45:10)	Berberine and protoberberine-type alkaloids
Cyclohexane–ether–ammonia (50:50:0.4)	Anthracene, anthraquinone
Dichloromethane:methanol:10% ammonium hydroxide (83:15:2)	Pyridine and piperidine alkaloids (determination of nicotine, nornicotine, anabasine, nicotyrine)
Ethyl acetate–methanol–water (100:13.5:10)	Rauwolfia alkaloids; xanthine derivatives like caffeine, theophylline, etc.; Colchicum alkaloids
Ethyl acetate–methanol–water (100:13.5:10)	Suitable for cardiac glycosides. Bitter principles containing alkaloids
Ethyl acetate–methanol–water (100:17:13)	Suitable for all anthracene derivatives. Slightly different ratio of components prevents the tailing in separation
Light petroleum–ethyl acetate–formic acid (75:25:1)	Anthraquinone aglycons
Methanol: ammonium hydroxide (9:1)	Indole alkaloids (strychnine)
Methanol:chloroform:1% ammonium hydroxide (2:2:1)	Determination of solanum alkaloids (solanidine)
n-Heptane–ethylmethyl ketone–methanol (58:34:8)	Rauwolfia alkaloids
n-Propanol–ethyl acetate–water (40:40:30)	Senna derivatives
n-Propanol–formic acid–water (90:1:9)	*Berberidis cortex*, *Hydrastis rhizoma*, *Colombo radix*, and *Chelidonium*
Toluene–acetone–ethanol–conc. ammonia (40:40:6:2)	Opium; opium alkaloids like morphine, odine, etc.
Toluene–chloroform–ethanol (28.5:57:14.5)	Secale alkaloids
Toluene–ethyl acetate–diethylamine (70:20:10)	Suitable solvent system for majority of alkaloids
Toluene–ethylformiate–formic acid (50:40:10)	Hypericin from *Hypericum perforatum*, *Hypericum hookerianum*, etc.

Continued

TABLE 8.3 Solvent Systems Used for Development of TLC of Some Herbal Drugs (Sorbents Used—Silica Gel 60F254)—cont'd

Solvent Systems	Usefulness/Note
Toluene-methanol (86:14)	*Colchici semen*
Drugs containing bitter principles	
Acetone–chloroform (30:40)	Cnicin
Acetone–chloroform–water (70:30:2)	Amarogentin
Benzene–ethanol–water–acetic acid (200:47:15:1)	Limonin
Chloroform–ethanol (95:5)	Cucurbitacins
Chloroform–methanol (95:5)	Absinthin and quassin
Chloroform–methanol (97:3)	Carnosolic acid, carnosol
Ethyl acetate–dioxan–water (30:10:0.3)	Oleuropein
Ethyl acetate–methanol–water (77:13.5:8)	General system for screening
Iso-octane–isopropanol–formic acid (83.5:16:50.5)	Humulone and lupulone (bitter acids) from *Humulus lupulus*
n-Heptane–isopropanol–formic acid (90:15:0.5)	Bitter acids
n-Propanol–toluene–glacial acetic acid–water (25:20:10:10)	Aucubine from Plantaginis
Coumarin aglycone	
Ethyl acetate	Used for higher R_f value containing coumarin
Ethylacetate:formic acid:glacial acetic acid:water (100:11:11:27)	For polar coumarins
Toluene–ether (1:1, saturated with 10% acetic acid)	It is a universally applicable TLC solvent for coumarin aglycones; it must be freshly prepared. The upper phase is used
For flavonoids	
Benzene–pyridine–formic acid (72:18:10)	Flavonoid aglycones
Chloroform (with chamber saturation)	For petasins in *Petasites folium*
Chloroform–acetone–formic acid (75:16.5:8.5)	Flavonoids
Chloroform–ethyl acetate (60:40)	Flavonoid aglycones of *Orthosiphonis folium*
Ethyl acetate–formic acid–glacial acetic acid–ethylmethyl ketone–water (50:7:3:30:10)	General screening system for the flavonoid glycosides
Ethyl acetate–formic acid–glacial acetic acid–water (100:11:11:27)	General screening system for the flavonoid glycosides
n-Butanol–glacial acetic acid–water (40:10:50) (upper phase)	For the separation of flavonoid glycosides
Toluene–ethylformate–formic acid (50:40:10)	Flavonoid aglycones
Cardiac glycosides	
Chloroform–methanol–water (65:35:10)	For chromatography of Hellchori radix extracts Lower phase to be used
Chloroform–methanol–water (64:50:10)	Separation of all saponin mixtures from plants This is temperature sensitive system and at higher temperatures all zones are shifted to the super R_f range of the chromatogram
Chloroform–methanol–water (70:30:4)	Suitable for the separation of ginsenosides from Ginseng radix
Chloroform–methanol (95:5)	Separation of glycyrrhetic acid
Methylethyl ketone–toluene–water–glacial acetic acid (40:5:3:2.5)	Suitable for the separation of Scilla glycosides

TABLE 8.3 Solvent Systems Used for Development of TLC of Some Herbal Drugs (Sorbents Used—Silica Gel 60F254)—cont'd

Solvent Systems	Usefulness/Note
n-Butanol–glacial acetic acid–water (50:10:40) (upper phase)	General systems for separation of saponins Less temperature sensitive
Ethylacetate–methanol–ethanol–water (81:11:4:8)	Strophanthoside. Addition of ethanol increase of R_f value of strongly polar compounds
Ethyl acetate–methanol–water (100:13.5:10) or (81:11:8)	Generally applicable solvent system for cardiac glycosides
Pungent principles from plants	
Diethyl ether (saturated chamber)	Capsaicin and other related compounds
Toluene–ethyl ether–dioxan (62.5:21.5:16)	General reagent for pungent principals and *Piperis fructus*
Toluene–ethylacetate (70:30)	Piperis, *Capsici fructus*
Isothiocyanates containing drugs	
Ethyl acetate–chloroform–water (30:30:40)	Mustard oils and its thio-urea derivatives
n-Butanol–*n*-propanol–glacial acetic acid–water (30:10:10:10)	Mustard oil glycosides and amino acids in garlic
Cannabis and its derivatives	
Cyclohexane (saturated chamber)	The plate is impregnated with *M*,*N*-dimethylformamide
n-Hexane–diethyl ether (80:20)	Cannabinoids, hashis, etc.
n-Hexane–dioxan (90:10)	Cannabinoids, hashis, etc.
Valepotriates	
n-Hexane–methylethyl ketone (80:20)	Vereric acid, isocaproic acid containing drugs
Toluene–ethyl acetate (75:25)	Acevaltrate and valtrathydrines
Pigment-containing drugs	
Ethyl acetate–isopropanol–water (65:25:10)	Croci stigma
n-Butanol–glacial acetic acid–water (40:10:20)	For anthocyanins
General solvent systems	
Benzene:acetone	Useful for the separation of aromatic products. Care should be taken as benzene is a highly carcinogenic solvent. Substitute toluene for benzene
Benzene:chloroform	Useful for the separation of cinnamic acid derivatives, particularly coumarins
Benzene:diethyl ether	A universal system for relatively nonpolar metabolites. Excellent for terpenes and fatty acids. Care should be taken with Et_2O as explosive mixtures are formed with air
Butanol:acetic acid:water	A polar system for flavonoid and glycosides
Butanol:water:pyridine:toluene	Sugar analysis system. Try 10:6:6:1. Development may take 4 h on a standard $20 \times 20\,cm^2$ plate
$CHC1_3$:acetone	A general system for medium-polarity products
Hexane:ethyl acetate	Universal system can substitute hexane for petroleum spirit or pentane
Toluene:ethyl acetate:acetic acid (TEA)	Vary the composition, e.g., 80:18:2 or 60:38:2 excellent for acidic metabolites

likewise radiate an unmistakable blue or yellow fluorescence under UV light. Long-wavelength light (366 nm) is ordinarily used for compounds that fluoresce, for example, yellow, orange, blue, or red, just as in the case for some of the chlorophylls. The major burden with UV identification is that compounds that do not absorb UV light at 254 or 366 nm will be imperceptible and will require detection by spraying. The main advantage of UV detection is that it is for the most part nondestructive and identification of compounds can be watched promptly through a separation procedure. It ought to be noted, in any case, that UV light can advance free radical responses with certain common natural products (Gibbons and Gray, 1998).

Detection with UV radiation is a fast and straightforward detection procedure for visualizing the separated substances. UV lamps emitting radiation of wavelength 254 nm and/or 366 nm are commercially available. Detection can then be performed in darkened rooms or in cabinets with integrated UV lamps. Substances that absorb radiation at 254 nm can also be detected very easily on layers with the F_{254} fluorescence indicator, which is excited at 254 nm and emits green fluorescence. They reduce the emission of the fluorescence indicator present in the layer and thus appear as dark (dark violet) zones against a fluorescing background (fluorescence reduction or fluorescence quenching). Substances that can be excited to fluoresce under UV light are best detected on layers without a fluorescence indicator. They become visible under UV light as bright shining zones against a dark background. Both procedures do not normally modify or destroy the chemical structure of the substance detected and are therefore very well suited for preparative purposes.

8.8.2 Detection With Spray Reagents/Derivatization

This technique depends on a color response between the compound on the TLC plate and a derivatizing reagent, which is sprayed onto the plate as a fine mist from a spray chamber. When utilizing spray identification in preparative TLC, the majority of the plate ought to be covered and a small portion of the edge (2 cm) is sprayed with reagent. Generally, a scalpel ought to be utilized to score a line 2 cm from the plate edge so that, after spraying, destructive spray reagent does not move into the stationary phase and react with the compounds to be obtained (Gibbons and Gray, 1998).

Derivatization reactions are used whenever individual fractions do not respond to UV radiation or do not fluoresce. In principle, it does not matter very much whether the detection reagent is used before development (prechromatographic derivatization) or after development (postchromatographic derivatization). Prechromatographic derivatization serves not only for visualization, but also for enhancing the selectivity of the separating system for the substances investigated or for transforming labile compounds into stable ones. Postchromatographic derivatization serves above all for visualization of the separated substances or for enhancing the sensitivity (Geiss, 1987).

As a rule, detection reagents are sprayed onto the plate or foil. Some of these reagents are available as ready-to-use products (e.g., Rhodamine B, Ninhydrin, phosphomolybdic acid). However, most of them have to be prepared in the laboratory and applied with suitable spraying devices. Such spraying devices include laboratory sprayers for connection to the compressed air or nitrogen line, and so-called spray guns, consisting of a reservoir or propellant container and a nozzle (Caution: Owing to the detrimental effects of the fluorohydrocarbons in these sprays, their use in the laboratory will probably cease in the foreseeable future).

Spraying should always be performed under a well-ventilated fume hood or some other device ensuring effective removal of the reagent cloud and solvent vapors, which are frequently corrosive or toxic. The chromatogram is first placed at a slight slope. Only when a uniform spray cloud has been formed is the spray jet directed at the plate, which is sprayed in a uniform manner, generally until the layer begins to turn transparent. Any excessive amount of spraying reagent can lead to dissolution of the substance from the inclined plate or to its being washed away. In addition to spraying devices, dipping equipment is also available. Vertical dipping and removal and the residence time in the dipping chamber (several seconds) can be selected and the process automated (Funk et al., 1981). Some reagents can also be admixed to the layer, with the actual derivatization step sometimes only taking place on heating after development. Some reagents can also be applied via the gas phase (e.g., nitrous gases in the case of aromatic compounds) or admixed to the solvent (e.g., Ninhydrin in the case of amino acids) (Touchstone et al., 1982; Bottler et al., 1980).

Not infrequently, the chromatograms have to be placed in a drying cabinet or on a heating plate after application of the detection reagents in order to accelerate the reaction (e.g., 10–15 min at 105–110 °C). After visualization, it makes good sense to immediately mark the spots and to label them (soft pencil) as they may fade or change color. The following points need to be considered:

- Protective glasses and laboratory gloves should always be worn during spraying.
- Derivatization modifies or destroys the chemical structure of the compounds.
- Derivatization is more expensive and more complicated than detection with UV radiation, but in some cases considerably more sensitive.

- Propellant gases in ready-to-use sprays contaminate the environment.
- On dipping, there is a danger that the compounds will be dissolved out by the solvent used (Consequence: change polarity) or that water in the dipping reagent may remove the layer (Consequence: use reagent with organic solvents).
- Substances can diffuse during the dipping process.
- The detection reagent is distributed more uniformly in dipping than on spraying. Dipping reagents prove less of a hazard to personnel and the environment than sprays.
- On heating in a drying cabinet, an unperforated metal tray should be used as a support to ensure uniform heat distribution.

8.9 EVALUATION OF THE TLC PLATES

Once a thin-layer chromatogram has been obtained, it has to be evaluated. There are a variety of methods available for this step, which are suited for various applications. The most important are described below (Jork and Wimmer, 1982; Geiss, 1987).

8.9.1 Qualitative Evaluation

Thin-layer chromatograms are produced with the aim of identifying the individual substances in a mixture and also for testing for purity or for separation of mixtures. They are particularly useful for checking the mixtures used for synthetic reactions or following the course of reactions. The R_f value indicates the position at which a substance is located in the chromatogram. It is appropriate to regard the R_f value as a guide, for it is difficult to obtain exactly reproducible R_f values as a result of the variety of influences operative during chromatography. For purposes of identification, it is necessary to relate the R_f values of the investigated substances and those of reference substances. If the R_f values agree, it is probable, but not certain, that the two spots correspond to the same substance. Reliable identification is only possible by using spectroscopic investigation alongside TLC (e.g., IR spectroscopy, NMR spectroscopy, mass spectrometry, or coupling thereof with TLC).

8.9.2 Semiquantitative Evaluation

Semiquantitative evaluation is required when it is necessary to establish whether analytical values lie significantly above or below preset limiting values or if approximate data are sufficient. In all cases, several concentrations of the substance of interest are chromatographed alongside the sample. Evaluation is conducted either by visual comparison or by measurement of the spot diameters or areas. The obtainable accuracy lies at around ±10%.

8.9.3 Quantitative Evaluation

Both indirect and direct methods are available for quantitative evaluation. In the former case, substances have to be dissolved out of the layer and subjected to further study. In the second case, evaluation is performed directly on the layer (in situ).

8.9.3.1 Indirect Quantitative Evaluation

One method of indirect evaluation (which no longer has any great significance) consists in scraping the substance of interest from the layer after detection and subsequently examining it with the aid of a suitable analytical technique. It should be noted that the substance is diluted by extraction and generally has to be reconcentrated. Here again it is recommended that sample and standards be chromatographed on the same plate. Elution of the substance zones from TLC plates can also be automated (e.g., with the CAMAG Eluchrom) without any need to scrape off the layer. The layer is first removed in a ring around the spots of interest. The elution head is then applied and the spots eluted; six spots can be eluted simultaneously.

8.9.3.2 Direct Quantitative Evaluation

In all of the direct quantitative methods of evaluation, it is of cardinal importance that the same volumes of samples and standards be applied to the same plate. The concentrations should be in roughly the same region and the R_f value should lie between 0.3 and 0.7. The chromatograms are scanned track by track densitometrically and evaluated by comparison of peak heights and peak areas of standards and samples. Measurements are performed in visible light or in the UV region, depending upon the properties of the substances, generally at a wavelength at which the substances to be determined show an absorption or fluorescence maximum.

Quantitative evaluation by the circuitous route of measuring fluorescence quenching should be avoided because substances that lead fluorescence quenching of the UV indicator in UV_{254} will themselves absorb UV irradiation in any case and are better determined directly at the wavelength of maximum absorption. As a rule, light absorption is measured in the reflectance mode (UV radiation is absorbed by glass or silica gel). Colored or fluorescing substances can also be measured in the transmission mode, although this does not normally have any advantage over reflectance.

8.10 BIOAUTOGRAPHY

This method may be used with either spore-forming parasites or microorganisms and can be used to track action through a detachment procedure. It is an extremely sensitive test and gives a precise restriction of dynamic compounds. For the evaluation of antifungal action, the plant pathogen *Cladosporium cucumerinum* can be used, as it is nonpathogenic to people, promptly forms spores, and can be effortlessly developed on TLC plates with the right medium. A straightforward strategy for this purpose is shown in Fig. 8.2. *Aspergillus niger* is another promptly sporulating fungus and might be used as a part of the place of *Cladosporium* sp., yet care must be taken with this organism on account of the danger of aspergillosis. All microorganisms ought to be taken care of aseptically in a laminar stream setup. Controls of antifungal compounds, for example, amphotericin B ought to be utilized each time this assay is done. This assay does not recognize fungicidal and fungistatic metabolites, and additionally measures, for example,

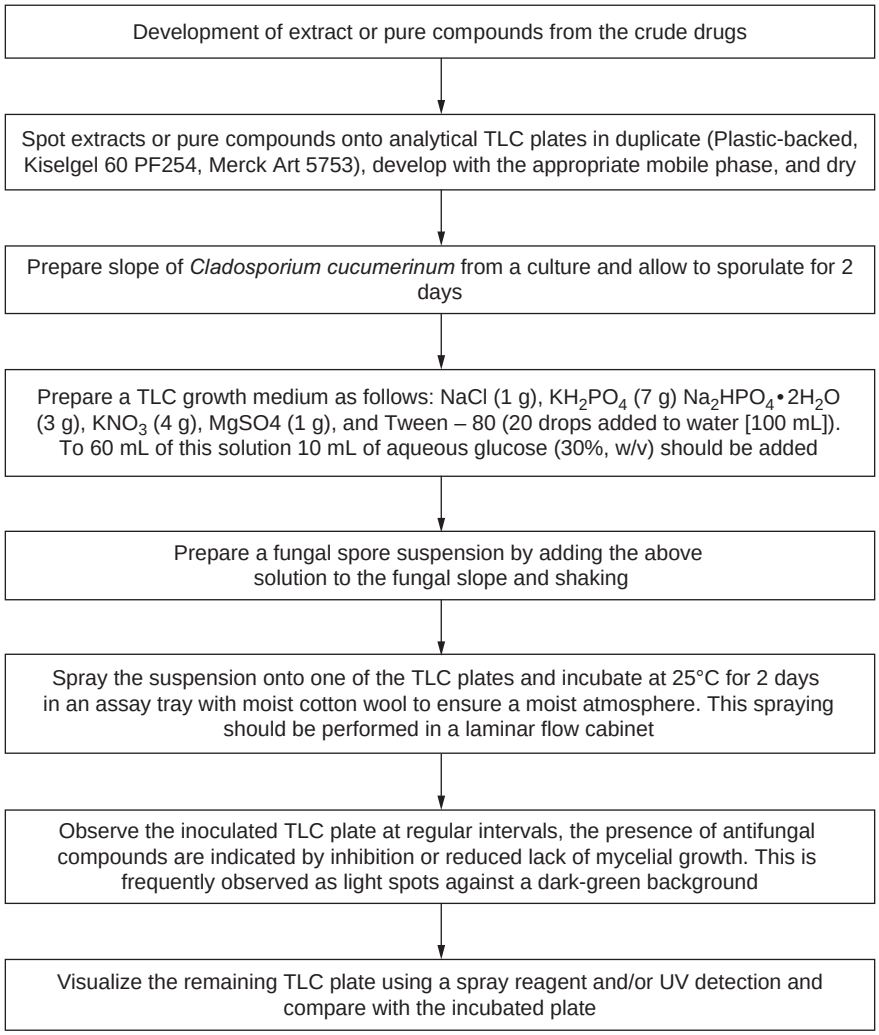

FIG. 8.2 Models for TLC bioautography direct assay.

a liquid broth test, will be expected to quantify minimum inhibitory fixation (MIC) (Cole, 1994). Dellar et al. (1994) separated the antifungal sesquiterpenes aristolen—2-one and prostantherol from two types of Prostanthera (Labiatae). The activity was evaluated and followed through the separation technique by the use of direct bioautography with *Cladosporium cecumerinum* as the target organism.

8.10.1 Overlay Assay by TLC Bioautography

In this form of assay, the extract or pure compound is run on a TLC plate, which is then covered by a medium seeded with the appropriate microorganism. As with direct bioautographic assays, both fungi and bacteria may be investigated. Rahalison et al. (1991) have applied this technique for the evaluation of antimicrobial extracts against the yeast *Candida albicans* and the bacterium *Bacillus subtilis*. A simple overlay assay against *Staphylococcus aureus* has been described in Fig. 8.3. Drug-resistant microorganisms, for example, methicillin-safe *S. aureus*, are required to be cultured in the presence of methicillin (1 mg/L) to limit the danger of loss of resistance. Batista et al. (1995) utilized an overlay strategy in the bioassay-guided fractionation of an acetone extract from the roots of *Plectranthus hereroensis* (Labiatae) to separate the antibacterial diterpene. *S. aureus* was utilized as the test organism. Hamburger and Cordell (1997) have utilized a variation of this examination to explore the action of some basic plant sterols and phenolic compounds. An overlay of nutrient broth having the test organism was spread over the TLC plate and then incubated. Astonishingly, this test was inactive to some cytotoxic compounds, including camptothecin, glaucarubolone, and beta-peltatin, when tried at 5 µg.

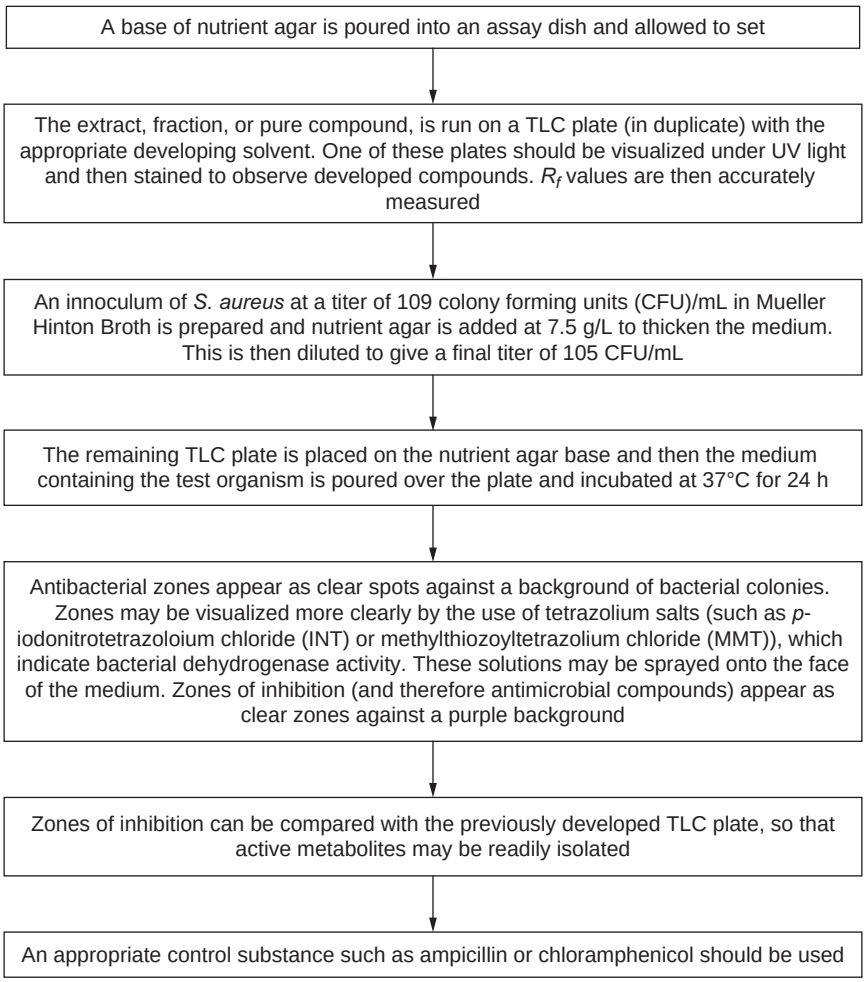

FIG. 8.3 Model for TLC bioautography overlay assay.

8.11 QUANTITATIVE THIN-LAYER CHROMATOGRAPHY (QTLC)

8.11.1 Quality of Sorbents for QTLC

The primary and secondary parameters of the sorbents used for quantitative analysis in TLC must fulfill a series of quality criteria in order to guarantee accuracy and reproducibility. This applies especially to the quantitative in situ evaluation of thin-layer chromatograms. The most important demands made on the quality of a sorbent concern its chemical purity, reproducibility, and standardization of its pore structure, specific surface area, particle size distribution, and the reproducibility of the degree of modification in the case of chemically modified materials.

8.11.2 Demands on the Quality of Precoated Layers for QTLC

Precoated layers for QTLC are supplied on different supports, including glass plates, plastic foils, and aluminum foils. One important criterion for glass plates used as supports for QTLC precoated plates is the accuracy of the measurements and angles of the specified format (e.g., 5×5, 5×10, 10×10, 10×20, $20\times20\,cm^2$). The glass plates should also display no fluorescence; the factory fumes released during the production process should be removed as much as possible before the plate is coated.

Plastic foil supports for precoated layers should, above all, have sufficiently high stability and be resistant to the solvents employed in chromatography. As most plastic foils contain softeners and some low-molecular-weight polymers, which can be dissolved by certain organic solvents, it is advisable to seal the foils with a suitable preliminary covering before they are actually coated.

If aluminum foils are used as supports for precoated layers, care must be taken that their surface is free from the grease employed for drawing during their manufacture; this grease would otherwise cause nonhomogeneous coating. A general quality criterion, which, applies to all of the supports mentioned, is that the layer thickness should be as even and uniform as possible. Precoated layers for QTLC must display sufficient mechanical stability to allow easy handling. This is achieved by incorporating a suitable binder or binding system in the chromatographic layer. The binding agent must satisfy the following requirements:

- The binder should confer an adequate degree of solidity to the separating layer and allow it to adhere to the support.
- The incorporated binder should not, if possible, affect the permeability of the separating layers.
- The binder should not be attacked or eluted by the solvents commonly used in TLC.
- The selectivity of the separating layer should be affected as little as possible by the binder.
- The presence of the binder should not interfere with the detection of the sample substances in the separating layer. This applies both to the detection reagents used and to the detection process in the visible and UV range.
- In addition to a homogeneous packing structure, a uniform layer thickness of the sorbent must be guaranteed if the requirements of QTLC are to be satisfied.

In order to obtain the best possible chromatographic separation performance in QTLC with precoated layers, suitable sorbents and above all an optimal packing structure are required. This means that the particles should be arranged as densely and as homogeneously as possible within the layer. An especially smooth, homogenous layer surface can then be expected. Surfaces of this quality are absolutely necessary for the precoated layers used in QTLC if low detection limits are to be obtained in the in situ evaluation of thin-layer chromatograms. Fluorescence indicators have been incorporated into a series of precoated layers to facilitate the detection of certain sample substances. These indicators must be very homogeneously distributed within the layer, especially for evaluation of thin-layer chromatograms by the so-called fluorescence quenching method. The particle size distribution and the mean particle size of the indicator particles must be coordinated with the particle size of the sorbent used. Like the binders, the fluorescence indicators should not be eluted by the customary solvents.

Precoated layers for QTLC should be as pure as possible. This means that no visible contaminants should be present in the layer and that zones of impurities should not appear in the separation area or close to the solvent front after development.

8.12 APPLICATION OF TLC FOR ASSAY OF STEROIDS, ALKALOIDS, ETC.

8.12.1 Determination of Active Ingredient Content

The main source of analytical problems is related to the quantitative recovery of the active substances. Steroids are used therapeutically in practically every pharmaceutical formulation, including tablets, capsules, ointments, and very frequently,

combinations are administered in which the steroids occur in very different concentrations. In most cases, separation of the active ingredients from each other and from the excipients is not a serious problem because of the different nature of the steroids present in formulation and the selectivity of the color reaction used for the quantitation.

Problems arise when side reactions occur during extraction or elutions, resulting in several byproducts derived from active ingredients that occur in very low concentration compared with others. Problems also arise when a small amount of impurities present in the active substance that is used in higher concentration interfere with the separation.

Regarding quantitation of the separated steroids, the extract is applied to the plate and, after proper development with the required solvent system, different spots are developed, which can be detected by the spray reagents. After detection, the spots are eluted and can be quantified by spectrophotometry, colorimetry, and fluorimetry, among other techniques.

Direct densitometry in the reflectance mode can be used and an assay employing a suitable reaction on the plate is more frequently applied. Sometimes the steroid content of the formulation can be determined after TLC separation by spraying with specific spray reagents and heating. This is followed by measuring the substance in the reflectance mode at 530 nm or in the fluorescence mode using 365 nm for excitation and 560 nm for emission (Szepesi et al., 1979).

8.12.2 Stability Assay of Steroid Preparations

Besides the extraction problems mentioned above, the most critical factors in the determination are the selectivity, efficiency, and sensitivity of the assay method. The decomposition products of steroids occurring in low concentration must be separated and determined in the presence of active ingredients. The impurities originate from the production and the complexity of the formulations, with so many components, and also from the different excipients. When the decomposition products are determined, the accuracy and precision of the method are not very problematic; a relative standard deviation of about ±5% should be acceptable. In determining the active ingredient content, however, this value must be much lower. For this reason, quantitation by in situ densitometry seems to be the only acceptable method for the stability assay of steroid preparations. It should be noted that due to the complexity of the analytical requirements, other chromatographic techniques (primarily HPLC) have superseded TLC methods in this field.

8.12.3 Purity Tests of Steroid Formulated Products

Sometimes the semiquantitative estimation of impurities and decomposition products in steroid formulated products is sufficient to control the quality of the preparation. In such a case, the most important thing is to find the chromatographic conditions that provide the required resolution. For instance, the quality of the formulated products of cardiac glycosides can be investigated by QTLC, using the spot elution technique and color reaction as described by Khafagy and Girgis (1974).

8.12.4 Content Uniformity Tests of Steroid-Containing Tablets and Capsules

QTLC accomplished by in situ densitometry is a very suitable technique for determining the uniformity of the steroid content. In most cases, separation of the active ingredients is not very difficult; neither is the demand on the accuracy and precision of the method very high. The sample preparation, however, requires consideration of the principles mentioned in connection with the assay of steroid formulations. The spot elution technique, which is usually time and labor consuming, is not recommended for this type of analysis.

8.12.5 Problems in QTLC Determinations of Steroids

There are many practical problems that should be considered when steroids are investigated and assayed.

8.12.5.1 Stability of Steroids During Analytical Procedures

Although steroids are considered stable compounds, they can decompose. One source of decomposition may be the sample preparation method, as mentioned in the previous sections. Another source, not mentioned previously, is the oxidative decomposition of steroids adsorbed on the surface of a polar adsorbent, such as silica gel. In this form, some steroids are sensitive to atmospheric oxygen, which may cause problems, especially in separation and analysis in the low concentration range. The decomposition can be suppressed by the application of antioxidants, for example, by using ascorbic acid in the eluent or by pretreating the sorbent with an ethanolic solution of ascorbic acid, which has been explained further by Fragcic and Kniewald (1974).

The D vitamins are the most sensitive compounds among the steroids. They are sensitive to atmospheric oxygen, traces of acids and bases, light, and heat, among others. To avoid decomposition and to obtain reliable, reproducible results, the following precautions are recommended (Bollinger et al., 1965).

- All experiments should be carried out in a cool place, protected from exposure to direct daylight.
- Chromatography should be carried out under nitrogen gas or with an antioxidant dissolved in the eluent.
- Highly purified solvents should be used.
- Because the D vitamins decompose most easily on the surface of silica gel, the sample should not be dried during the spotting and the chromatoplate should be placed in the chamber immediately after sample application.
- After the run, the spots should be located, scraped off, and eluted in the shortest time possible. The duration of UV irradiation at the location should be minimal.

8.12.5.2 Separation Problems

For the separation of steroids, silica gel layers with a variety of eluent systems have been used most widely. As previously mentioned, because of the very different polarities of the steroids investigated, no generally applicable chromatography system can be recommended. The TLC systems most suitable for solving the majority of separation problems within an individual group of steroids (sex hormones, corticosteroids, D vitamins, cholesterols, bile acids, cardiac glycosides, etc.) has been further clarified by the work of different scientists (Görög, 1983; Görög and Szasz, 1978; Heftmann, 1976; Lisboa, 1972; Neher, 1969). Some delicate problems, such as the separation of difficult pairs of steroids, have been mentioned. This section will discuss some methods for improving the selectivity and/or efficiency of separations. When the silica gel alone is not a sufficiently good adsorbent for solving separation problems, the results can be improved by using the following approaches:

1. A multiple development technique, 2D chromatography, and overrun chromatography used on silica.
2. Impregnation of the silica gel with solvents or reagents. For example, this should be used for the separation of cholesterol and its related derivatives. As sterols are the least polar group of steroids, their separation from other groups of steroids and for other lipids is relatively easy. Many systems described in the literature are suitable for this purpose. The separation of structurally closely related sterols is much more difficult. On silica gel, only poor separation efficiency can be achieved. The chromatographic resolution can be improved if silica gel containing silver nitrate is used for the separation. In this case the formation of π-complexes between silver ions and the double bonds of the unsaturated sterols results in satisfactorily separated spots, which has been explained by various workers (Knights, 1977; Claude, 1965; Peerboom and Beekes, 1965; Ikan and Cudzinovski, 1965). Similarly, good results can be achieved when the silica gel layer is impregnated with paraffin or lower hydrocarbons. In the case of cardiac glycosides, impregnation of the silica layer with boric acid or formamide is helpful in solving delicate separation problems (Potter and Barisch, 1972).
3. Chromatography on an adsorbent other than silica gel. Alumina is also a good stationary phase for the separation of sex hormones, corticosteroids, D vitamins, and others. The popularity of RP layers in steroid analysis is not as great as in other fields of pharmaceutical analysis. However, in recent years, some interesting applications have been published, and it is worth mentioning that USP XX prescribes the use of a chemically bonded octadecyl silica layer for purity testing of digoxin, using semiquantitative estimation for the quantitation. In special cases, polyamide layers (Scandrett and Ross, 1976), impregnated cellulose (Tinschert and Trager, 1978), vinyl pyrrolidone layers, and magnesium oxide have been successfully applied (Marcos et al., 1977).
4. *Overpressurized thin-layer chromatography (OPTLC)*: Further improvements in the separation of steroids can be expected with the OPTLC technique, which was introduced a few years ago by Tyihak and coworkers (Mincsovics et al., 1980). The technique is based on a concept similar to that underlying liquid chromatography. The solvents are passed through an ultramicro chamber by an appropriate pump system, and the sorbent layer is completely covered in the chamber with a flexible membrane under external pressure. In this manner, the vapor space over the layer can be excluded, resulting in more reproducible retention, better resolution, and faster development than in classical TLC systems. Linear migration of the solvent front in the ultramicro chamber is ensured by impregnating the sides of the layer and by placing a narrow plastic sheet on the layer behind the starting line.

   In classical TLC the linear flow velocity can be expressed by the following equation:

$$K^{\mathrm{TLC}} = \frac{X^2}{t}$$

where X is the distance of eluent migration (running distance) and t the time required to cover the distance X. This equation shows that the linear flow velocity decreases with increasing running distance, which can cause a loss in separation efficiency. In OPTLC, the linear flow velocity is constant and can be expressed by the following equation:

$$K^{\text{OPTLC}} = \frac{X}{t}$$

In this case, separation efficiency is practically independent of the running distance on high-performance layers with different developed elution. The separation of digitalis glycosides in a generally used standard chamber is compared with that of development in a pressurized ultramicro chamber (PUM chamber) with silica gel TLC and HPTLC plates (Horvath et al., 1982). It can be seen that the chromatographic resolution may be increased considerably by using a PUM chamber and an HPTLC plate. In this case, the optimum migration distance is much longer, and the HETP value is smaller than in the usual HPTLC investigations.

The most important features of the OPTLC technique can be summarized as follows. The method is suitable for fast, efficient, and simultaneous investigation of a large number of samples. The chromatogram can be developed at a higher migration distance in a reasonable running time on microparticulate stationary phases. Solvents of high viscosity can also be used for eluent preparation. The overrunning can be carried out under controlled conditions. A small amount of eluent is required. The higher efficiency and better chromatographic resolution provide a more advantageous spot shape for densitometric evaluation.

8.12.5.3 Detection Problems

Although some steroids have a UV absorbance suitable for their detection, and localization of the spots can be based on the appearance of dark spots in 254 nm UV light if a fluorescent layer is used, most applications still involve methods based on chemical reactions. A variety of spray reagents for the visualization of steroid spots has been described. However, special attention is needed to stabilize the produced color or the induced fluorescence on the plate. Sulfuric acid–methanol seems to be the most applicable reagent. In addition, phosphoric acid, hydrochloric acid, antimony (III) chloride, and others can generally be used to detect steroids on the plate or to determine steroids after spot elution.

Another principle of detection should be utilized for quantitative analysis is to induce fluorescence on the plate. The simplest way is to heat the plate for several hours at about 120 °C in an ammonia atmosphere prepared by in situ heating of ammonium hydrogen carbonate. Although the mechanism of this reaction is unknown, it is considered a simple, generally applicable, and reproducible detection procedure for steroids (Segura and Gotto, 1974). Besides derivatization after the separation of steroids, methods employing derivatives prepared prior to application to the plates have some advantages. The most important one is that errors originating from incomplete distribution of the spray reagent can be eliminated (Graham and Kenner, 1973). In many cases, the stability of the steroids investigated or their chromatographic behavior can also be improved by prior derivatization. A prior derivatization method has been used for separation and detection of keto steroids in 2,4-dinitrophenyl hydrazone form (Treiber, 1976; Knapestein et al., 1968).

A new technique for in situ quantitation involves separation on silica gel-coated quartz rods. The quantitation is performed directly by using a flame ionization detector. This elegant technique was introduced by Japanese workers (Okumura et al., 1975), and it has been used for the determination of conjugated bile acids and cardiac glycosides. The problems of in situ quantitation can be summarized as follows:

- Simultaneous running of an appropriate reference standard is required. No area normalization method can be used for quantitation.
- Errors originating from incomplete distribution of the spray reagent. The variation of layer thickness along the plate influences the results.
- The stability of the product formed by chemical reaction is more or less limited.
- The in situ techniques are naturally less labor intensive than methods based on spot elution. Their high efficiency, selectivity, and sensitivity make these techniques comparable to gas chromatography and high-performance liquid chromatography (HPLC). The advantages of the in situ TLC techniques compared with GLC and HPLC are:
  - Usually no derivatization is necessary.
  - Any type of chemical reagent can be used to increase the selectivity and sensitivity of the steroid determination.
  - Compounds differing widely in polarity can be separated and determined.
  - Scanning the chromatograms requires less time than in GLC and HPLC.
  - A single densitometer can be used for quantitative evaluation of the chromatograms from several methods.

The main disadvantage of these techniques is their lower accuracy and precision, caused predominantly by the imperfections of the sample application procedure and the characteristics of the sorbent layer.

8.12.6 Assay of Alkaloids

8.12.6.1 Introduction

The naturally occurring nitrogen-containing compounds with strong physiological effects, isolated from different plants, were given the name alkaloids by Meissner in 1818 for their basic chemical character. The alkaloids are grouped according to numerous criteria, and the borders of the classes of alkaloids are drawn differently from case to case. In terms of their biosynthesis in plants, the alkaloids are derived from some amino acids or closely related compounds, and they can be classified as alkaloids derived from:

(a) Ornithine and lysine
(b) Tyrosine and phenylalanine
(c) Tryptophan
(d) Anthranilic acid
(e) Histidine
(f) Steroid alkaloids
(g) Xanthine alkaloids
(h) Miscellaneous groups, including the quaternary ammonium salts

The alkaloids and their chemical analysis are most important in the pharmaceutical industry. However, the quantities of alkaloids are investigated more frequently in different herbal drug and food industrial products and in materials of the tobacco industry. The pharmaceutical industry uses >100 alkaloids in different preparations. In this industry the usual aims of quantitative analysis of alkaloids are as follows:

1. Determination of the quantity of alkaloids occurring in fresh or dried plant materials (fermented fluids constitute an exception) for the purpose of plant breeding and/or processing.
2. Investigation of the intermediates and starting materials for the purpose of process control and development.
3. Quality control of pharmaceutical products.
4. Optimization of reaction parameters in the preparation of new semisynthetic or synthetic compounds.
5. Quality control of new experimental materials prior to their pharmacological investigation.

8.12.6.2 General QTLC Methods for Alkaloids

In the chemical analysis of alkaloids, QTLC plays an important role and is an alternative to HPLC. Gas chromatography is used primarily in the field of tobacco alkaloids. The alkaloids, disregarding a few exceptions, are heterocyclic compounds containing tertiary or quaternary nitrogen atoms. Their basicity is usually weak in the case of an N-heteroaromatic ring (pkb = 7–14). However, when the nitrogen atom is part of a saturated ring, the basicity is much stronger, similar to that of the appropriate aliphatic amines. The tertiary and quaternary alkaloids in acidic media can be precipitated with a number of heavy metal ions as complexes, and some of these can be used as visualization reagents for the detection of the compounds, which are generally colorless and frequently do not absorb in the UV.

The visualization reagents commonly used for alkaloids are different varieties of Dragendorff's reagent with H(BiI_4) and the hexa iodoplatinate reagent with $H_2(PtI_6)$ as active ingredients. Only the alkaloids containing tertiary and quaternary nitrogen give positive reactions with these complexes. However, with in situ postchromatographic alkylation, the detection of primary and secondary amines can also be facilitated by using these reagents (Hais and Macek, 1958).

The xanthine alkaloids cannot be detected directly with the complexes, except for those with an amino group in the side chain. However, Hauck (1965) found that these compounds can also be detected when the plate is first sprayed with silver nitrate in 5% sulfuric acid before Dragendorff's reagent is used. A similar effect is obtained by previous spraying with 20% nitric acid and heating (Pellerin et al., 1973).

Different modifications of Dragendorff's reaction for in situ QTLC purposes are customary. Application of this reaction is especially advantageous when a similarly sensitive alternative detection method is not known (Munier, 1953; Thies and Reuther, 1954). With Dragendorff's reagent modified according to Munier (1953), the alkaloids give insoluble precipitates in the reagent solution and the reaction is fast enough not to cause any observable spreading of spots during the detection.

For the detection of alkaloids on TLC plates, different varieties of Dragendorff's reagent are used. These modifications differ from each other in the concentration of the iodobismutate complex, the quality of the solvents, the quality of the acid used, and the treatment subsequently applied. In QTLC practice, Munier's modification (1953) usually seems to be applied, but in special cases, other varieties may also have advantages. Posttreatment with sodium nitrate decolorizes the background; it decomposes the (BiI_4)—complex while I_2 evolves. However, the alkaloid spots also become paler, and in a more-or-less short time, they usually disappear. Care must also be taken because some solvents, for example, dimethyl formamide and formamide, leave residues on the plate that interferes with Dragendorff visualization (Puech et al., 1972). In the case of formamide-impregnated plates, formamide can be decomposed in situ with nitrous acid.

For QTLC purposes in general, and for alkaloids as well, the dipping technique gives better quantitative results than spraying with the reagent (Touchstone and Dobbins, 1978). The tetraiodobismutate reaction gives different sensitivities in the detection of different groups of alkaloids. This reaction can be used especially advantageously for the investigation of certain pharmaceutical products and related compounds belonging to the quaternary ammonium salts, which have no chromophore group to make detection easier. In this group, Dragendorff's reagent permits the detection of as little as 0.1% of contaminants besides the main component.

Another general visualization reagent for the alkaloids is iodine in vapor form or in solution. It can be used for QTLC purposes as well if the plate with the visualized chromatogram is hermetically covered with a glass plate to prevent evaporation of the iodine adsorbed on the spots. In the case of QTLC of quaternary ammonium compounds mentioned above, the iodine reaction gives significantly less reliable results than the iodobismutate reaction.

In conventional TLC there is no effective universal detection method similar to flame ionization detection (FID) used in gas chromatography. In quality control of different industrial preparations, it is frequently important to know that the material examined does not contain any organic impurity above a certain concentration limit. The microrod TLC technique developed by Glunz (1980), has been successfully applied with alkaloids. It can combine the special advantage of TLC, namely that all components chromatographed are necessarily on the chromatogram, with the unique feature of FID of being able to detect practically every organic compound. Unfortunately, it has not been possible to use FID with regular chromatoplates. The microrod technique, on the other hand, has lost some of the advantages of conventional TLC.

Many attempts have been made to find a general detection method for conventional TLC plates. Segura and Gotto (1974) used drastic heating in the presence of ammonium hydrogen carbonate and Bochoff et al. (1976) used ionization to convert a great number of different compounds, among them alkaloids, on the plate to measurable fluorescent derivatives. In other cases, exposure to the vapor of red-fuming nitric acid followed by high temperature treatment gave a good detection method for compounds detectable with difficulty (Zhou et al., 1982). In certain instances, severe reaction conditions are used for the detection of the alkaloids that do not contain a chromophore or fluorophore group. For example, Karlsson and Peter (1978) worked out a fluoro-densitometric method for the detection of Lupinus alkaloids and used heating at 130 °C for as long as 17 h for the detection. A great number of alkaloids naturally absorb in the UV or visible region or fluoresce, and these compounds can easily be detected directly. Some alkaloids, partly because of their easy detection, are even popular as model compounds for demonstrating the effectiveness of new chromatographic techniques. In many cases alkaloids are not directly detectable and can be determined better with special reagents than with general ones. In the investigation of different groups, the alkaloid-specific reagents are well known, and many of them are suitable for QTLC purposes as well. In TLC the postchromatographic reactions can usually be accomplished more easily than in HPLC, so that prechromatographic reactions do not have any great importance in TLC. However, in many instances the transfer of some well-established precolumn reactions into TLC methods is advantageous.

One of the prerequisites of reliable QTLC is suitable separation of the components to be determined. In general, the selection of appropriate solvent systems is somewhat more tedious for alkaloid determinations than for determinations of other organic compounds because of the higher polarity and basic character of alkaloids. The other factor in the efficiency of separation is the chromatographic technique applied. Recent developments in TLC techniques and their separation efficiency have affected the field of alkaloid investigations. In a number of cases, HPTLC plates are used, which give separations that are better or are similar to those with conventional plates in a shorter migration distance and with more reliable quantitative data. Among the other recent technical developments, overpressurized TLC (OPTLC) (Tyihak et al., 1979; Horvath et al., 1982) seems to excel; this is the first forced-flow technique in TLC, and it has the ability to control and optimize the solvent velocity during chromatography. Although OPTLC has its unique advantages, it does not automatically lend itself to transfer to conventional chromatographic systems. A significant improvement in separation with OPTLC occurs only when the other chromatographic conditions, for example, the particle size of the stationary phase, are not suited for conventional developing conditions. Nevertheless, in some areas of the alkaloids, OPTLC has already been used with success. For example, Gulyas and Minesovics (1984) separated several poppy alkaloids by the OPTLC technique. The high separation efficiency can be important, especially for naturally occurring biological materials, such as natural alkaloids,

for which great care is necessary to avoid errors caused by overlapping of the spots to be measured with those of other compounds. A simple method was applied by Karlsson and Peter (1978) in a QTLC procedure for screening of alkaloids of Lupinus species in order to develop a breeding program. To control the efficiency of the chromatographic system, a 2D chromatogram was identical to that in the 1D method. Although this does not constitute conclusive proof, the likelihood of clear separation for the main components is significantly increased. The control method is frequently HPLC; for example, Horvath et al. (1978a, b) used an elution QTLC method for the determination of ergot alkaloids and an HPLC procedure for the control assay.

Many of the alkaloids are optically active compounds, and it is a special problem to investigate the optical isomers in their mixtures. The separation of members of diastereomer pairs does not differ, in principle, from that of any other difficult-to-separate compound pairs. However, the members of enantiomer pairs can be separated only with optically active chromatographic systems. Unfortunately, for most of the separation problems such systems with suitable efficiency have not been available. Another possible method of separation involves a prechromatographic reaction with an optically active reagent that converts the enantiomers into separable diastereomer pairs. Horvath et al. (1978a, b) used another method for the determination of the four optical isomer lysergic acids in their mixtures. They did not attempt a total separation and used a simple, and not optically active, chromatographic system in which the members of the enantiomer pairs do not separate from each other. They supplied the missing analytical information from the data from a multiwavelength optical rotatory dispersion (ORD) measurement. In this case, the chromatographic task simplifies the separation problem to half as many components. The simplification achieved with a combination of chromatographic and ORD data can, in principle, be applied to any number of optical isomer components, provided there are enough differences in the ORD spectra of the diastereomer components (Smith et al., 1973).

8.12.6.3 QTLC Methods of the Alkaloid Groups

Numerous (nearly 1000) QTLC methods dealing with alkaloids may be found in the chemical literature. Most of them were developed, or at least are applicable, for industrial purposes. This is one of the fields, among the industrial applications of liquid chromatography, in which QTLC compares favorably with HPLC in evaluating the quality control of herbal drugs and their preparations. In industrial analytical practice, chemists are often satisfied with quantitative data generated for the purpose of rapid orientation. This allows the use of QTLC procedures, which are not optimized in every respect. It is feasible that the number of methods actually used far exceeds the number of methods published in journals. A substantial portion of the published articles on QTLC still report indirect methods, although more recently the in situ instrumental methods have gained importance. The majority of alkaloids can be determined quantitatively by use of a general reagent, such as Dragendorff's reagent, to name the most important one. In many instances, however, the inherent spectrophotometric or fluorometric character of the compounds or a more specific reagent gives better results.

Because many alkaloids are generally known and easy to determine, they are frequently used as model compounds to demonstrate the effectiveness of technical modifications of TLC. There can scarcely be any new step in QTLC development without its demonstration in the field of alkaloids as well. However, it appears that today the simple reflectance densitometric and "near-side" fluoro-densitometric methods constitute the bulk of applied instrumental QTLC procedures. The novel technical approaches devised to increase the signal-to-noise ratio and provide better resolution and the new detection techniques have still been utilized only in a modest number of cases.

8.12.6.3.1 Alkaloids of Ornithine and Lysine Origin

Pyrrolidine, pyrrolizidine, pyrazole, pyridine, piperidine and quinolizidine alkaloids belong to this group. For the investigation of these alkaloids, several indirect QTLC methods have been developed, most of them for the determination of tropine and cinchona alkaloids. These methods are based on elution of the spots followed by fluorometric or spectrophotometric determination of the alkaloids.

For in situ determinations, Dragendorff's reaction was applied for Leguminosae alkaloids (Faugeras and Paris, 1973), tobacco alkaloids (Jurzysta, 1975), and tropane alkaloids (Wu Chu et al., 1969). The in situ reaction is usually followed by densitometry in the reflectance mode at a wavelength, depending on the alkaloids to be measured and the reaction conditions, in the range 400–530 nm. In the case of Leguminosae alkaloids, Faugeras and Paris (1973) found the transmittance mode to be more sensitive and precise than reflectance. Naturally, the optimal conditions vary from case to case, and depend largely on the instrument parameters.

Karlsson and Peter (1978) determined Lupinus alkaloids by fluoro-densitometry after a drastic in situ reaction to convert the alkaloids into fluorescent compounds. The alkaloids were determined at an excitation wavelength of 360 nm and an emission wavelength of 400 nm. The blue fluorescence of spots is stable for weeks. For the investigation of tobacco

alkaloids, gas chromatography is widely used because of their volatility. However, elution QTLC and densitometry are also used. Massa et al. (1970) used fluorescence quenching for the investigation of pseudotropine alkaloids as well. The tropine alkaloids, which have a condensed pyrrolidine—piperidine skeleton, have only small molar absorptivity at 254 nm. Besides Dragendorff's quantitation they are determined advantageously by conversion of fluorescent compounds by means of an acidic reagent. Messerschmidt (1969) described a method for scopolamine with concentrated sulfuric acid, which was mixed into the mobile phase.

8.12.6.3.2 Alkaloids of Tyrosine and Phenylalanine Origin

Besides detection with general reagents, a number of other detection methods can be used in this group. Seiler and Weichmann (1966) investigated Cactus alkaloids as their densyl derivatives. The quaternary alkaloids of the berberine type are yellow and give strong fluorescence. A fluoro-densitometric method was used by Messerschmidt (1969), with 352 and 353 nm wavelengths for excitation and 530 and 510 nm for emission, respectively. For clear selection of the components, the 2D technique is sometimes necessary (Hattori et al., 1978).

The benzophenanthridine alkaloids can be determined by the elution QTLC method. Balderstone and Dyke (1977) determined sanguinarine in Argemone oil. They reduced the alkaloid to the dihydro compound to allow extraction with organic solvents. After chromatography, the dihydro compound was reconverted by irradiation with long-wavelength UV light. The spots were then eluted with acidic ethanol, and finally the alkaloid was determined spectrophotometrically at 330 nm. Freitag (1980) used a dual-wavelength (285 and 365 nm) chromatographic–spectrophotometric method for the determination of chelidonium alkaloids on TLC and HPTLC plates.

Investigating hydrastic alkaloids, Zwaving and de Jong-Havenga (1972) compared the direct and elution QTLC methods and found good agreement. In both cases, as with many alkaloids, care must be taken to prevent oxidation. Among the bisbensylisoquinoline alkaloids, tetrandrine is known for its tumor-inhibiting effect, and tubocurarine as a muscle relaxant. The aporphine alkaloids, including boldine, can be investigated in plant material and pharmaceutical preparation by means of direct QTLC, detecting the spots either with 0.25% 2,6-dichlor or dibromoquinone chlorimide in methanol or by irradiation with long-wavelength UV light (Fleischmann et al., 1975). Among the Ipecacuanha alkaloids, emetine and cephaeline are frequently investigated in pharmaceutical preparations by QTLC, using spectrophotometry at 283 nm afterward. However, according to Schuyt et al. (1977) spectrofluorometry is more sensitive. For emetine, they used an excitation wavelength of 284 nm and measured the emission at 318 nm. According to Massa et al. (1970), with a preliminary treatment of the plates with iodine, which oxidizes the alkaloids to yellow fluorescent spots, the alkaloids can be determined by direct spectrofluorometry, measuring the fluorescence at 592 nm after excitation at 358 nm. Frei and coworkers (1975) used a prechromatographic reaction with dansyl chloride for the determination of a number of alkaloids in different pharmaceutical preparations containing, besides emetine and cephaeline, ephedrine, morphine, codeine, and noscapine. Of these compounds, noscapine and codeine do not react with dansyl chloride. A strongly fluorescent dansyl derivative is obtained from morphine as well. They measured the fluorescence at 500–510 nm with excitation at 360 nm. The method is suitable for the analysis of alkaloids in the presence of a 10–100-fold excess of other drugs.

For the determination of opium alkaloids, numerous indirect and direct QTLC methods have been developed. The characteristics of the main opium alkaloids differ significantly, so the detection procedures usually differ from component to component. Heusser (1968) used an elution QTLC method for the determination of morphine as nitrosomorphine, measuring the absorbance of the eluate at 470 nm. Fairbairn and El-Masry (1967) separated noscapine and papaverine from the other alkaloids on a thin-layer plate. They eluted spots of noscapine and papaverine and determined the absorption at 313 and 295 nm for noscapine and 251 and 230 nm for papaverine. Poethke and Kinze (1964) separated the principal opium alkaloids by 2D-TLC and determined them spectrophotometrically.

Besides Dragendorff's reaction, several special detection methods are used for the in situ QTLC determination of opium alkaloids. For the determination of morphine in *Papaver* capsules, Paris and Faugeras (1966) found detection with sodium nitrate to be most suitable because of the specificity of this reaction compared with other alkaloids. Massa et al. (1970) compared different detection methods and determined the optimal conditions for each principal opium alkaloid. The fluorescence quenching at 254 nm and the fluorescence method (excitation at 358 nm, emission at 529 nm) were the optimal ones for papaverine and narceine. Nitrosation was best for detection of morphine, and Dragendorff's reaction was the most suitable for codeine.

Hashiba et al. (1978) determined noscapine by fluoro-densitometry after heating the plate for 5 h at 120 °C. Haefelfinger (1976) worked out a fluoro-densitometric method for codeine; after separation the plate was sprayed with a mixture of 65% nitric acid and 96% sulfuric acid (4:1), subsequently heated for 10 min at 110 °C, and then the fluorescence was measured at 578 nm with excitation at 300 nm. Genest and Belec (1967) determined laudanine in opium, using Gibb's reagent for

detection; the spots were measured at 465 nm. Rodder et al. (1971) separated the main opium alkaloids by a 2D method and subsequently measured the absorbance in the reflectance mode. Similar methods were used with codeine and noscapine, Janos et al. (1982) used a RP-TLC plate for the densitometric determination of morphine and quaternary ammonium steroids.

8.12.6.3.3 Alkaloids of Tryptophan Origin

Among the >300 known Strychnos alkaloids, strychnine and brucine have been most extensively investigated. The majority of QTLC methods are indirect ones. Massa et al. (1970) determined numerous alkaloids densitometrically, including strychnine and brucine, using a fluorescence quenching method at 254 nm. Okumura et al. (1975) used an FID detector for the determination of a number of alkaloids, including strychnine and brucine, on sintered silica gel or aluminum oxide rods.

The Rauwolfia alkaloids (the majority of which are β-carboline-type compounds) occur in plants in many variations, so that their clear separation from each other probably cannot be accomplished by conventional TLC methods with a simple solvent system. Court and Habib (1973) and Court and Timmins (1975) investigated about 300 solvent systems, but none of them was suitable for the clear separation of all the Rauwolfia alkaloids. However, they were able to determine the best systems for the separation of reserpine/rescinnamine, ajamalicine/reserpinine, and other difficult cases. They also determined the relationship between structure and retention behavior, which made identification of the components easier. In this group great care must be taken in using QTLC; because these alkaloids are sensitive to light, especially in chloroform and on a dry plate, the analysis must be carried out in the dark or in dimmed light. In this group Dragendorff's reaction modified by Minuer and Macheboeuf (1949) can be used with success, but fluorescence quenching and the measurement of self-fluorescence usually give better results. A number of elution QTLC methods have been used in this field, for example, Potter and Voigt's (1967) method. The method developed by Court and Habib (1973) is suitable for all alkaloids, including those with a β-carboline skeleton. The method is based on complex formation with iodine and subsequent spectrophotometry. With an elution QTLC method, Manara (1967) determined tritium-labeled reserpine by means of a liquid scintillation detector.

The *Rauwolfia* alkaloids can be determined densitometrically. Hartmann and Schnabel (1975) determined reserpine and rescinnamine by densitometry at 295 nm. Fluoro-densitometric methods are suitable for the analysis of plants and other biological materials (Hammer and Kaiser, 1972; Tripp et al., 1975). For the fluoro-densitometric determination of reserpine (with an excitation wave length of 392 nm and emission at 540 nm) fluorescence can be obtained by exposure to acetic acid vapor for 24 h or by spraying the plate with 1% *p*-toluenesulfonic acid in glacial acetic acid to develop the fluorescence immediately. Fluorescence can also be developed by exposing the spots to light for at least 24 h to form fluorescent 3-dehydro derivatives (Frijns, 1971). Katic et al. (1980) determined reserpine and ajmaline in *Rauwolfia vomitoria* by an in situ quantitative HPTLC method. They determined ajmlicine by its UV absorption at 292 nm with 5% RSD and reserpine fluoro-densitometrically with an excitation wavelength of 380 nm and emission at 450 nm with 3% RSD.

Dombrowski et al. (1975) determined the semisynthetic 17-monochloroacetylajmaline and its metabolites by fluoro-densitometry after spraying with 6 M nitric acid and acetic acid and gently heating. For determination of Mytragina and Uncaria alkaloids, both indirect and in situ QTLC methods have been known for some time. Shellard and Alam (1968a, b) and Shellard (1968) compared the densitometric, indirect colorimetric, and spectrophotometric methods and found (in 1968) that the last method proved to be the best one. For densitometry they sprayed the plates with 0.2 M iron (III) chloride in 35% perchloric acid and heated at 120 °C for 1.5 h.

The pharmaceutical importance of Vinca alkaloids is determined by some alkaloids that originally occurred in plants, such as vincamine, vincristine (VCR), and vincaleucoblastine (VLB), and by some semisynthetic and synthetic materials, such as formylleurosine and apovincaminic acid ethyl ester. The binsindol alkaloids isolated from *Catharanthus roseus*, for example, VLB and VCR, are only minor components in the plant material besides the monomer alkaloids. Adequate quantitative determination of these components requires separation in at least two dimensions, using conventional (not forced-flow) TLC techniques. Horvath et al. (1982) developed two different QTLC methods (an indirect spectrophotometric and densitometric method) for the routine analysis of VLB in *C. roseus* plant material. In the elution method, following a partially selective separation in the first dimension, the spots, including VLB (both the investigated and the reference spots), were eluted with a solution containing an internal standard material, VCR (in the first dimension the VLB spot is far from the VCR spot). After concentration, the eluates were chromatographed in another solvent system. The VLB and VCR spots were subsequently eluted again, and the absorbance was determined at 289 nm spectrophotometrically. The special internal method eliminated a number of sources of error and ensured satisfactory reliability. In the densitometric method, the authors employed a 2D chromatographic system on a $20 \times 20\,cm^2$ conventional TLC plate in which the reference spot (VLB) is developed in both dimensions close to the investigated spot but without interfering with it. The results obtained by the two QTLC methods agreed well. However, the direct method is four to five times shorter and requires less manual work.

The direct method was also tried on a $10 \times 10\,cm^2$ HPTLC plate, but in this case the quantitative results were less reliable. In the different elution QTLC methods spectrophotometry usually follows chromatography. Karacsony et al. (1965) used a nonaqueous titrimetric method after the spot elution. In the in situ method, besides direct spectrophotometry, Massa et al. (1970) used Dragendorff's reaction, and Panas et al. (1979) assayed alkaloid-related impurities in vincristine sulfate by a TLC densitometric method, measuring the absorbance at 254 nm.

For the investigation of physostigmine, used mostly in ophthalmic solutions, a number of QTLC methods are available. Rogers and Smith (1973) determined physostigmine after separation from its degradation products. Multiwavelength methods were used to separate the useful signal from that of the contamination. Generally speaking, only higher plants have the ability to biosynthesize alkaloids. The ergot alkaloids are the sole exception; they are produced mainly by fungi (*Claviceps purpurea*), but they can also be found in the higher plant "morning glory." Ergot alkaloids can be produced on rye by cultivation or with fermentation. Elution QTLC methods (Klavehn et al., 1961) for ergot alkaloids usually begin with an extraction step in order to isolate the alkaloids from the plant material or the fermentation broth. The concentrated (and frequently further purified) extract is applied to the TLC plate, and after chromatography, direct spectrophotometry with van Urk reagent and sometimes indirect fluorometric analysis is used (Klavehn et al., 1961). In the densitometric methods, most analysts measure the self-fluorescence of the alkaloids. Different authors have used the following excitation and emission wavelengths: for ergometrine, 358 and 490 nm (Petrova et al., 1972) or 313 and 420 nm (Frijns, 1971); and for ergotamine, 313 and 420 nm or 325 and 445 nm. Frijns (1971) impregnated the developed plates with liquid paraffin in diethyl ether (1:9) to stabilize the fluorescence.

A critical problem in TLC of the lysergic acid derivative ergot alkaloids is separation of the ergotoxine components from each other, especially α- and β-ergocristine and ergocornine. Only two chromatographic systems comply with these requirements:

(i) Using a partition chromatographic system with diisopropyl ether–tetrahydrofuran–toluene–diethylamine (70:15:15:0.1) on precoated silica gel plates impregnated with a mixture of 18 mL formamide, 0.6 mL 25% ammonia, and 81.4 mL acetone (Reichelt, 1976).

(ii) Using a more reproducible adsorption chromatographic system for the same purpose, at the same time solving the separation problem of dihydro ergotoxine alkaloids as well (Szepesi et al., 1979).

In this system, the stationary phase used is silica gel GF_{254} and the mobile phase is acetone–0.1 M ammonium carbonate–ethanol (32.5:67.5:1). The only drawback of this system is that the development is slow; 3 h is necessary for 15 cm migration. In fluoro-densitometric determination of the dihydro ergot alkaloids, the emitted light is not in the visible range; however, the alkaloids can be converted on the plate by UV irradiation (usually 1 h at 254 nm) into the normally fluorescent components. Prosek et al. (1991) used a fluoro-densitometric method for these alkaloids without converting them. They determined the emitted light at 250–320 nm with excitation at 230 nm. Instead of direct densitometry or fluoro-densitometry, for example, Genest (1965), investigating ergot alkaloids in morning glory, sprayed the developed plate with van Urk reagent. After spraying, the plates were stored in a dark place for 20 min for stabilization, sprayed with 1% sodium nitrate, and the absorbances were measured at 570 nm.

8.12.6.3.4 Alkaloids of Anthranilic Acid Origin

The *Cinchona* alkaloids, including those with a quinoline skeleton, belong to this group. Quinine and quinidine are used as drugs and are also important materials in the food industry. Cinchonine and cinchonidine are also frequently investigated alkaloids. Several indirect QTLC methods are known, based on spectrophotometric (Baerwald and Prucha, 1973) determination. The spectrophotometric determinations are carried out at wavelengths in the long-wavelength UV range; in the fluorometric methods, the excitation wavelength is 350 nm and the emission wavelength is 450–456 nm for quinine and quinidine. Studying the optimum conditions for in situ QTLC methods, Massa et al. (1970) found absorption maxima for quinine and quinidine at 330 nm and for cinchonine and cinchonidine at 288 nm, after spraying with ethanol–concentrated sulfuric acid (9:1). The detection limit was about 100 ng. By means of fluorometry, using 1% sulfuric acid in ethanol as a spray reagent, the detection limit is 1 ng for quinine and quinidine at an excitation wavelength of 365 nm and measurement at 450 nm, and 5 ng for cinchonine and cinchonidine with excitation at 313 nm and emission at 410 nm. Roder et al. (1970) used diethyl ether–concentrated sulfuric acid (95:5) as an immersion reagent and measured the fluorescence of quinine and quinidine at 460 nm with excitation at 365 nm. Under these conditions, cinchonine and cinchonidine did not interfere. The latter compound can also be determined fluoro-densitometrically without interference from quinine and quinidine by excitation at 313 nm and measurement of the emission at 390 nm. Ebel and Heroid (1973) used this method for the determination of quinine without complete separation.

8.12.6.3.5 Alkaloids of Histidine Origin

The imidazole alkaloids are used mostly in ophthalmic preparations. Massa et al. (1970) used an in situ chromatographic method with Dragendorff's reagent for the determination of pilocarpine, isopilocarpine, and pilocarpic acid in pharmaceutical preparations.

8.12.6.3.6 Steroidal Alkaloids

Steroidal alkaloids occur mainly in the *Solanum*, *Veratrum*, and *Holarrhena* species. Solanin occurring in potato has been known since 1820. For 20 years indirect spectrophotometric QTLC methods have been used (Valocich and Nad, 1966) for the determination of Solanum glyco alkaloids. For the direct QTLC determination of Solanum alkaloids, Fayez and Saleh (1969) used a 5% solution of phosphomolybdenic acid in ethanol containing 4 mL sulfuric acid per 100 mL. The plates were heated for 10 min at 110 °C. Cadle et al. (1978) used antimony (III) trichloride reagent in chloroform and heating at 150 °C for 4 min for the determination of glycoalkaloids in potatoes. For densitometric purposes Dragendorff's reaction is also suitable in this group. Jellema et al. (1981) used an optical brightener, Blankophor BA 267% (Bayer, Leverrkusen, FRG), for the fluoro-densitometric determination of potato glycoalkaloids by TLC. The excitation wavelength was 300 nm, and the measurement was carried out at 450 nm.

8.12.6.3.7 Xanthine Alkaloids

The xanthine alkaloids cannot be detected directly by Dragendorff's reaction and iodoplatinate reagent except for compounds with an amino group in the side chain. These alkaloids, which include caffeine, theobromine, and theophylline, can be detected spectrophotometrically at their absorption maximum, 272 nm. A number of indirect QTLC methods are used for the determination of caffeine (Fenske, 2007; Treiber, 1986) and theophylline in biological material. Ebel and Heroid (1975a, b, 1976) developed methods for the investigation of caffeine in pharmaceutical preparations. They used phenacetin as an internal standard and 2D-TLC for the separation. Direct densitometric determination of caffeine in plant materials is done by measuring the quenching of UV light at 254 nm on fluorescent plates.

8.12.6.3.8 Miscellaneous Groups

(A) Other alkaloids

Most of the work on TLC of diterpene alkaloids concerns the alkaloid aconitine. For its elution QTLC determination, it is useful to carry out the chromatography on magnesium oxide and subsequently to dissolve the adsorbent with the alkaloid spot because of its strong adsorption to the silica. Massa et al. (1970) used an in situ QTLC method with Dragendorff's reaction and detection at 400 nm.

For the determination of colchicine alkaloids, some indirect QTLC methods are also known in which the components are detected by UV spectrophotometry. Traditionally, some compounds of the amide type have been included among the alkaloids; these are mainly capsaicin and its analogs, which have been investigated primarily in the food industry. These compounds can be detected well with general alkaloid reagents. Suzuki et al. (1980) reported an effective separation of capsaicin and related alkaloids with a RP-HPTLC system. They used ammonia vapor and then 0.1% 2,6-dichloro-quinone-4-chloroimide in methanol for detection, and by in situ densitometric measurement at 610 nm they could determine these components with much better results than were obtained with the easier TLC, GLC, and HPLC methods.

The QTLC determination of quaternary ammonium compounds presents some special problems, in both the separation and the detection of these highly polar compounds. Besides NP chromatography, usually with highly polar solvent systems, partition and ion pair chromatography have been more frequently used for the separation of these compounds than for other compounds (Bonati and Bacchini, 1966; Wartmann-Hafner, 1966). The quaternary ammonium compounds can be readily detected with iodobismutate and iodoplatinate reagents, and for compounds that do not have a chromophore these reactions usually give the best, and sometimes the only suitable, alternative for QTLC determination. Some pharmaceutically important and very effective muscle relaxant steroid compounds belong to this group.

(B) Flavonoids

The seasonal trend of flavonoids in olive (*Olea europaea* L.) leaves was investigated by TLC (Heimler et al., 1996). Five major flavonoids were identified in olive leaves: luteolin, quercitrin, luteolin-7-glucoside, luteolin-40-glucoside, and apigenin. The leaves were crushed and kept for 24 h in 80% methanol or 70% ethanol. The TLC was carried out on two types of stationary phase, silica gel 60F254 for NP-TLC and SIL C18–50 for RP-TLC, respectively. The layers of silica gel were developed with toluene–pyridine–formic acid (100:20:7, v/v), while the layers of silica gel C-18 were developed with methanol–water–acetic acid (50:50:6, v/v). The quantitative determination of these compounds, and of chlorogenic

acid, was carried out for 1 year's time in order to study their dynamics in leaves. Flavonoids were detected as brown spots by UV at 254 nm, or the plates were dipped in a methanolic solution containing 1% ethanolamine diphenylborate. The quantitation of spots was performed by densitometry scanning at 365 nm or at 440 nm after 24 h from dipping the layer in the reagent. Free flavonoids are present in olive leaves, and represent about 35%–40% of the entire flavonoid content of the plant. Determination of flavonoids, flavonoid glycosides, and biflavonoids from *O. europaea* was carried out by TLC (Heimler et al., 1992). The flavonoids, and flavonoid glycosides, were separated by RP-TLC on RP-18 silica gel with methanol–water–acetic acid (50:50:6, v/v), and on silica gel with ethyl acetate–formic acid–water (6:1:1, v/v). The spot visualization was obtained by dipping the plate in a methanolic solution containing 1% ethanolamine diphenylborate and 5% polyethylene glycol 4000, then evaluation under UV at 365 nm. The bioflavonoids were separated by NP-TLC on silica gel with benzene–pyridine–formic acid (100:20:7, v/v) as mobile phase. The spots were detected by spraying the plate with 1% ethanolic $AlCl_3$ and irradiation with UV at 366 nm.

(C) Coumarins

An interesting study refers to 2D planar chromatography separation of a complex mixture of closely related coumarins from the genus Angelica (Harmala et al., 1990). 1D- and 2D-OPLC of 16 coumarins was performed on silica plates with chloroform as mobile phase for the first direction, and 30% ethyl acetate as mobile phase for the second direction. The chromatographic conditions were optimization by Prisma model. The separation was detected by exposing the plates to UV light at 254 and 366 nm. Quantification was performed by densitometry at 310 nm. The transfer of the separation conditions from TLC to 2D-TLC, and further to 2D-OPLC, is also discussed.

(D) Saponins

Saponins are natural substances that form foam-like soap in aqueous solutions. These substances are normally present as mixtures in plants. Chemically, they belong to the category of triterpene glycoside or steroid glycosides. Silica gel is the most used stationary phase for the separation of saponins, together with polar mobile phases. A TLC method is described for the quantitative estimation of saponins in pea (*Pisum sativum* L.) and soya (*Glycine max*) flowers (Curl et al., 1985). The flowers were extracted with methanol in a Soxhlet extractor, the resulting extract evaporated to dryness, and the residuum dissolved in water. The aqueous solution of saponins was purified by flash chromatography on a column containing 3 g of silica gel C-18. The column was washed with water then eluted with methanol until no further color was present in the eluate. The methanol eluate was evaporated to dryness under reduced pressure. TLC was performed on silica gel plates and the plate was developed in *n*-butanol–ethanol–0.880 ammonia (7:2:5, v/v). Detection was realized by spraying the plate with a mixture of anisaldehyde–glacial acetic acid–sulfuric acid (1:100:2, v/v) and heated to 1208 °C for 10–20 min. The characteristic purple–blue colors of steroids and triterpenes were obtained on the plates. Errors caused by changes in the color intensity of the plates due to the heating effects in the densitometer were eliminated by using photocopies. The calibration curve was realized with soyasaponin I as standard, and the regression coefficient was $R^2 = 0.964$; the detection limit was 1 mg of saponin, corresponding to 0.01% (w/w) of saponin in the analyzed flower. GC has confirmed the results obtained by TLC. Quantitative determination of biologically active constituents in medicinal plant crude extracts was obtained by TLC-densitometry of spray-dried aqueous hydroethanolic or ethanolic extract of the following 10 medicinal plants (Vanhaelen and Vanhaelen-Fastre, 1983): *Aesculus hippocastanum* (aescin), *Arctostaphyllos uva-ursi* (arbutin), *Fraxinus excelsior* (fraxin), *Gentiana lutea* (gentiopicrin), *Glycyrrhiza glabra* (glycyrrhizic acid), *Hamamelis virginiana* (gallic acid, tannins), *Hypericum perforatum* (hypericin, pseudohypericin), *O. europaea* (oleuropein), *Salix alba* (salicin), and *Silybum marianum* (silybin). The sample was prepared from the solution of the spray-dried extracts in water–methanol (5:10, v/v) and filtered; the solubility was improved using an ultrasonic bath. TLC plates of silica gel 60 or silica gel 60F254 normal and HPTLC were used for NP-TLC and RP-18 plates for RP-TLC. The solvent system and reagent for visualization are also specified. The spot areas were integrated by TLC-densitometry in UV, vis, or fluorometry. In all cases, CV of these rapid and reliable methods was lower than 3.5% and the recovery coefficients are between 95% and 99%.

8.13 TLC USED FOR SOME BIOACTIVE MATERIALS

8.13.1 Case Study 1

An HPTLC method has been developed for the quantification of 6-gingerol in a mehanolic extract of Zingiber officinale rhizomes. The optimized mobile phase was found to be *n*-hexane; diethyl ether (40:60, v/v). The R_f value of 6-gingerol was found to be 0.40. The proposed HPTLC method for quantitative monitoring of 6-gingerol in ginger may be used for routine quality testing of ginger extracts (Rai et al., 2006). The TLC chromatogram is depicted in Fig. 8.4.

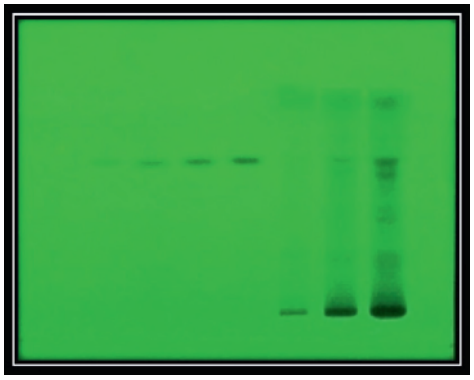

FIG. 8.4 TLC chromatogram of Zingiber officinale rhizomes. Extract (E) and phytomarker (M).

8.13.2 Case Study 2

Triphala formulation has been standardized with HPTLC methods based on fingerprint profiling of individual components in formulation by using gallic acid as a marker compound. Methanol extracts of Triphal, *Emblica officinalis*, Terminalia chebula, and *Terminalia belerica* were used for HPTLC on silica gel plates. The R_f of gallic acid was found to be 0.80 with densitometric scanning at 254 nm and the calibration plot. The gallic acid content in methanol extracts of Triphala with its individual constituents, such as *E. officinalis*, T. chebula, and *T. belerica*, was found to be 14.38, 17.50, 16.60, and 11.92 mg/g. The HPTLC method for quantitative monitoring of gallic acid in Triphala and its constituents can be used for routine quality testing and similar methods can be developed for other herbal formulations (Mukherjee et al., 2008).

8.13.3 Case Study 3

A simple, rapid, and accurate HPTLC method has been developed and validated for the quantification of betulinic acid in a hydro-alcoholic extract of Nelumbo nucifera rhizome. The separation was carried out on a TLC aluminum plate precoated with silica gel and the optimized mobile phase was chloroform: methanol: formic acid (49:1:1, v/v/v). Post chromatographic derivatization was made with anisaldehyde–sulfuric acid reagent and densitometric scanning was performed using a Camag TLC scanner III, at 420 nm. The system was found to produce a sharp peak of betulinic acid at an R_f value of 0.30. The percentage of recovery was found to be 98.36%. This HPTLC method provides a new and powerful standardization method for the quantification of betulinic acid as a biomarker in the N. nucifera extract (Mukherjee et al., 2010).

8.13.4 Case Study 4

A simple, rapid, and accurate HPTLC method has been developed and validated for the quantification of taraxerol in Clitoria ternatea extract. Linear ascending development was carried out in a twin trough glass chamber with a saturated optimized solvent system of hexane and ethyl acetate (80:20, v/v). The HPTLC plate was dried and derivatized with sprayed anisaldehyde reagent. A Camag TLC scanner III was used for spectrodensitometric scanning and analysis at 420 nm. A compact and sharp peak for taraxerol at an R_f value of 0.53 was found. This method for quantitative monitoring of taraxerol in C. ternatea can be used for routine quality testing of C. ternatea extract (Kumar et al., 2008) and the chromatogram is presented in Fig. 8.5.

8.13.5 Case Study 5

A simple and cost-effective 2D-TLC method for the simultaneous determination of 11 standard alkaloids has been developed (Tuzimski et al., 2017): allocryptopine, berberine, boldine, chelidonine, glaucine, papaverine, emetine, columbamine, magnoflorine (M), palmatine (Pal), and coptisine (Cop) in *Thalictrum foetidum* root extract. Separation of the alkaloid mixture was achieved by 2D-TLC using an aqueous mobile phase (RP) in the first dimension (80% methanol + water + 0.05 mL^{-1} diethylamine) and a NP in the second dimension (18% methanol, 18% acetone in 63% diisopropyl ether containing 1% ammonia, v/v) on bilayer multi-K CS5 plates. The composition of the mobile phases was optimized in terms of retention, separation selectivity, spots symmetry, and system efficiency. The procedure was evaluated in terms of natural samples analysis. Magnoflorine and berberine were identified in *T. foetidum* root extract.

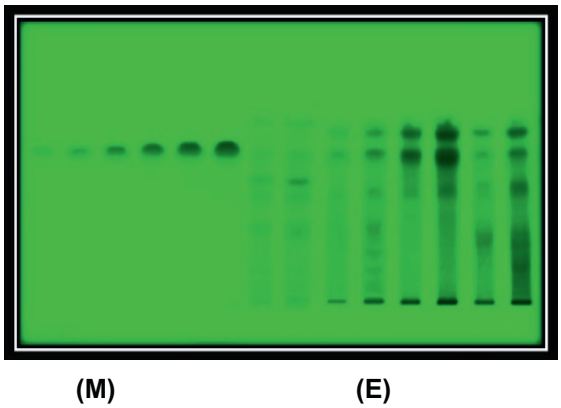

FIG. 8.5 TLC chromatogram of *Clitorea ternatea*. Extract (E) and phytomarker (M).

8.13.6 Case Study 6

Separation of three triterpenic acids (ursolic, oleanolic, and betulinic acid) was achieved on different TLC (silica gel 60) and HPTLC sorbents (silica gel 60, C2 RP, and C18 RP) using several developing solvents based on the nonpolar diluent *n*-hexane and ester (methyl acetate, ethyl acetate, ethyl propionate) as selector. Anisaldehyde and molybdophosphoric acid detection reagents were used. Finally, a simple method on a C18 RP-HPTLC plate was developed using *n*-hexane–ethyl acetate (5:1, v/v) as a developing solvent in a horizontal developing chamber. The method was used for the screening of ursolic, oleanolic, and betulinic acids in different vegetable extracts. Other plant triterpenoids (lupeol, α-amyrin, β-amyrin, cycloartenol, lupenone, friedelin, lupeol acetate, cycloartenol acetate) and phytosterols (β-sitosterol, stigmasterol) did not interfere. TLC–MS was used as a tool for the additional confirmation of the presence of ursolic, oleanolic, and betulinic acids in some of the studied vegetable extracts. Ursolic and oleanolic acids were found in radicchio Leonardo and white-colored radicchio di Castelfranco extracts for the first time, while betulinic acid was not detected in eggplant extract by MS, although it was suggested at first by TLC analysis. Prechromatographic bromination on the HPTLC silica gel 60 plates and subsequent development in toluene–chloroform–diethyl ether–formic acid (20:16:4:0.1, v/v) provided a superior resolution of these compounds (Naumoska et al., 2013).

8.13.7 Case Study 7

Fingerprinting of Eugenia jambolana with gallic acid has been performed. Sample material of 0.5 g was macerated with 10 mL methanol for 12 h and filtered. A quantity of standard gallic acid was dissolved in methanol to produce a solution containing 0.1 mg per mL. A TLC aluminum plate precoated with silica gel 60F254 was used for separation. The mobile phase used was methanol:water:acetic acid (7.6:2.3:0.1). The presence of gallic acid was examined in UV light at 254 nm and TLC showed the presence of gallic acid, as seen in Fig. 8.6.

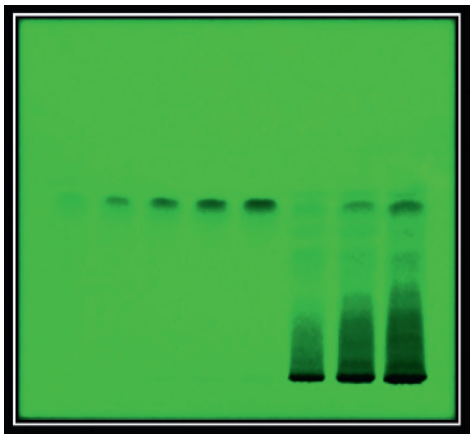

FIG. 8.6 TLC chromatogram of Eugenia jambolana. Extract (E) and phytomarker (M).

8.13.8 Case Study 8

Fingerprinting of Ocimum sanctum with eugenol has been performed. Sample material of 1 g was macerated with 10 mL methanol for 12 h and filtered. A quantity of eugenol was dissolved in methanol to produce a solution containing 0.1 mg/mL. A TLC aluminum plate precoated with silica gel 60F254 was used for separation. The mobile phase used was toluene:ethyl acetate:formic acid (90:10:01). The presence of eugenol was examined in UV light at 254 nm. TLC showed the presence of eugenol and the chromatogram is presented in Fig. 8.7.

8.13.9 Case Study 9

Fingerprinting of Piper longum with piperine has been performed. A sample of 1 g was macerated with 10 mL methanol 12 h and filtered. A quantity of piperine was dissolved in methanol to produce a solution containing 0.1 mg/mL. A TLC aluminum plate precoated with silica gel 60F254 was used for separation. The mobile phase used was toluene:chloroform:ethyl acetate (4:3:3). The presence of piperine was examined in UV light at 254 nm and the TLC chromatogram is shown in Fig. 8.8. TLC showed the presence of piperine.

8.13.10 Case Study 10

Fingerprinting of *Swertia chirata* with ursolic acid has been performed. A sample of 1 g of material was macerated with 10 mL methanol for 12 h and filtered. A quantity of ursolic acid was dissolved in methanol to produce a solution containing 0.1 mg/mL. A TLC aluminum plate precoated with silica gel 60F254 was used for separation. The mobile phase used was toluene:ethyl acetate:formic acid (7:3:0.2). The presence of ursolic acid was examined after derivatizing with *p*-anisaldehyde reagent in visible light. TLC showed the presence of ursolic acid.

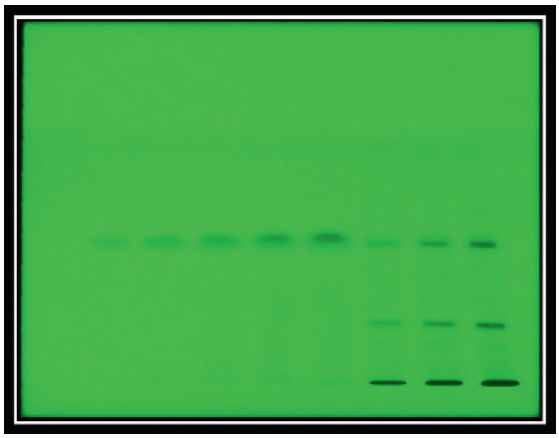

FIG. 8.7 TLC chromatogram of Ocimum sanctum. Extract (E) and phytomarker (M).

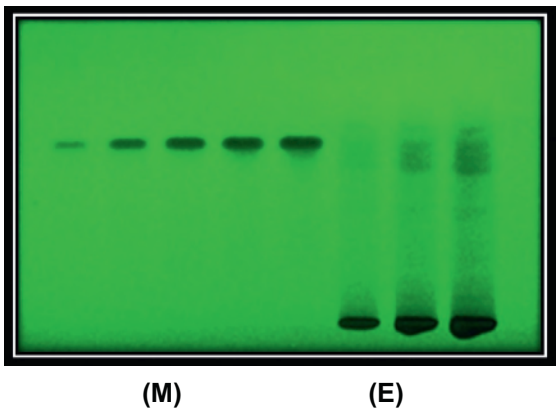

FIG. 8.8 TLC chromatogram of Piper longum. Extract (E) and phytomarker (M).

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Chapter 9

High-Performance Thin-Layer Chromatography (HPTLC) for Analysis of Herbal Drugs

Chapter Outline

9.1 HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY (HPTLC)

Chromatographic techniques help in the separation of compounds. Here, the separation takes place between two phases, one of which is a stationary phase over which the mobile phase migrates in a directional manner. In planar chromatography the sorbent is spread on a planar surface. HPTLC is one sort of planar chromatography and the most advanced form of instrumental TLC, which is widely used as a cost-effective method for rapid analysis of sample mixtures

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(Variyar et al., 2011). HPTLC is an independent instrumental TLC technique in which the sample application, chromatogram development, and detection are independent and widely used to standardize the methodology based on a validated method. The relative independence of sample application, chromatogram development, and detection in time and location makes possible the parallel analysis of many samples on the same plate.

HPTLC is useful in the development of qualitative and quantitative evaluation techniques for the components present in any sample. It includes cutting-edge instruments controlled by a coordinated software programming, which ensures enhanced utility, reliability, and reproducibility of the information produced. HPTLC is a flexible screening procedure with which both qualitative and quantitative analyses can be performed. Instruments can easily be validated and are fully compliant with GMP. The HPTLC technique is widely used in the pharmaceutical industry in process development, identification, quantification of markers, and detection of adulterants in herbal products. It helps in the identification of pesticide content and mycotoxins for quality control of herbs and herbal products. HPTLC can help in repeated detection (scanning) of the chromatogram with the same or different conditions. Consequently, HPTLC has been investigated for simultaneous assay of several components in a multicomponent formulation. With this technique, authentication of various species of plants is possible, as well as an evaluation of the stability and consistency of their preparations from different manufactures. Standardized manufacturing procedures and suitable analytical tools are required to establish the necessary framework for quality control in herbals. HPTLC is the most widely used technique to establish reference fingerprints of herbs, against which raw materials can be evaluated and finished products can be assayed. Various research reports have been available on development of HPTLC method for phytoconstituents in crude drugs or herbal formulations, such as bergenin, catechine, and gallic acid in *Bergenia ciliata* and *Bergenia ligulata* (Kalala and Rajani, 2006). HPTLC has been used as a modern technique for generating and evaluating digital images. HPTLC features highly sensitive scanning densitometry and video technology for rapid chromatogram evaluation and documentation. The HPTLC instruments are computer controlled and can therefore offer improved reproducibility of the analytical result. At the same time, HPTLC is still just as flexible and user-friendly. A detailed flow diagram for HPTLC techniques is shown in Fig. 9.1.

9.1.1 Advantages of HPTLC

The analysis of herbs and herbal preparations is challenging for several reasons. The herbs are extremely complex analytes. Even herbal preparations, such as extracts, contain numerous compounds in concentrations that can cover several orders of magnitude. In many instances, the chemical composition of the herb is not completely known. There are no validated methods available for the analysis of several herbs used in traditional medicine and their formulations. The requirements for a fingerprint analysis can be completely different from those for a quantitative determination of marker or key compounds, although the herbal preparation is the same in both instances. For example, as many components as possible should be separated for fingerprints, but for quantitative determination of marker compounds, it is necessary to fully separate those compounds from all others. Constituents of herbals that belong to very different classes of chemical compounds can often create difficulties in detection. With this in mind, HPTLC can offer many advantages, which are summarized in the following sections.

(i) Flexibility by design and being an offline technique makes HPTLC more flexible

- There is almost no limitation on the composition of the mobile phase. To maximize selectivity of the separation, an enormous choice of stationary and mobile phase combinations is available. The chromatographic conditions can be changed within a few minutes, and the chromatographic chamber requires very little time for equilibration.

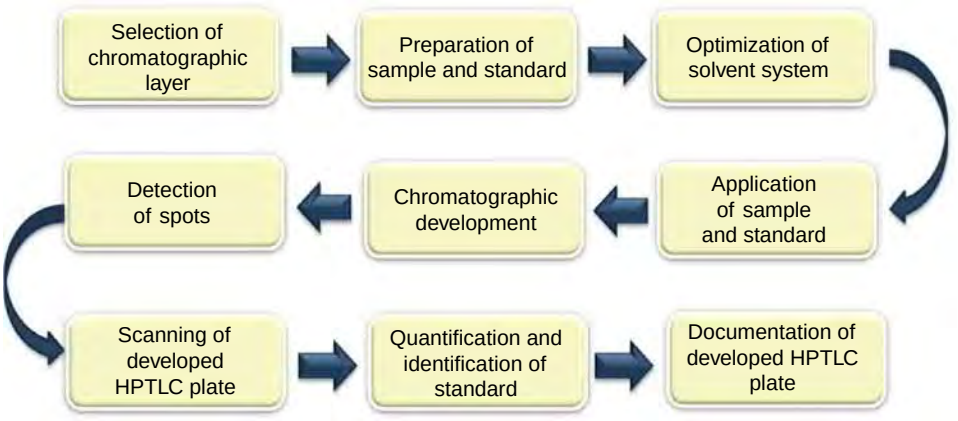


FIG. 9.1 Flow diagram for HPTLC techniques.

- Instruments are dedicated to specific tasks, such as sample application or detection, rather than dedicated to a specific analysis. One densitometer can be used for several chromatographic work stations with different tasks, or one fully automated sample application device can be used subsequently in a large number of completely different analyses.

(ii) Choice of detection

Detection in HPTLC takes place in the absence of the mobile phase and offers, therefore, much greater choice than available with other chromatographic techniques.

- Multiple subsequent detection of the same chromatogram is possible. Besides densitometric evaluation of absorption of fluorescence using visible or ultraviolet light, several hyphenated detection techniques, such as Fourier transform infrared and Raman or mass spectroscopy, are available.
- The TLC plate stores the chromatographic result, that is, the detection can be repeated with constant or different parameters. Portions of the chromatogram can be selectively evaluated, thus allowing optimization of detector sensitivity for specific compounds.
- If postchromatographic derivatization is employed, substance classes in particular can be selectively detected without interference by others.

(iii) Cost and time efficiency

- Multiple samples can be analyzed on one plate. This results in a low analysis cost per sample. Automation in sample application takes 0.5–2.0 min per sample, depending on size, application mode, and number of replicates.
- A typical development of a plate requires about 8 min and consumes 5–10 mL of mobile phase for a $10 \times 10\,cm^2$ or $20 \times 10\,cm^2$ plate in a twin-trough chamber.
- Equilibration time of 15 min is sufficient, and drying times normally do not exceed 10 min. Densitometric evaluation of a plate can be accomplished within 10 min.
- While different samples are to be analyzed using different methods, the flexibility of the system, as discussed above, also becomes a cost-saving advantage.

(iv) User friendliness and result presentation

- By design, TLC is very user friendly and easy to perform, although it requires more manual labor than other chromatographic techniques. Chromatography can easily be monitored and the results can be documented and presented as images, as well as in a numerical form. Especially when dealing with complex analysis, such as herbs and herbal preparations, results can be easily compared.
- Each step of the chromatographic process can be monitored easily and the effects of changes made to the procedure are visible.
- The planar chromatogram can offer two-dimensional information based on migration distance and color of the substance zone (with or without derivatization). At the same time, all samples on one plate can be compared with each other. This is an extremely powerful feature when used in fingerprint comparison.

(v) One-time use of the TLC plate

- Unlike column chromatography, HPTLC does not require postchromatographic clean-up. Before a second sample is analyzed in column chromatography, the column must be reconditioned so that the chromatographic conditions for all samples are alike. HPTLC analyzes samples in parallel, while a given plate is used only once.
- It is possible to investigate some fractions of the sample while others remain on the application position. A part of the TLC plate can be used for sample preparation, such as removal of matrix elements, prechromatographic derivatization, etc.

9.1.2 Main Features of HPTLC

The stationary phase used in this technique is disposable, that is, the HPTLC plates. We can analyze several samples concomitantly through this technique, which results in its high test throughput. It permits more adaptability and ease in sample assessment due to the likelihood of sequential detection by corresponding strategies, postchromatographic derivatization, and quantification. It can be utilized in tests in which analytes do not have a chromophore group, which makes recognition by other strategies troublesome, and can be utilized for analyzing samples with negligible prepurification. TLC and HPTLC are similar to each other in several aspects. HPTLC gives better efficiencies, better mass-exchange properties, and higher working speeds. HPTLC is a modern adaptation of TLC with better and more advanced separation efficiency and detection limits. There are several differences between HPTLC and TLC (Table 9.1).

TABLE 9.1 Major Differences Between TLC and HPTLC

Parameters	TLC	HPTLC
Technique	Manual	Instrumental
Efficiency	Less	High (due to smaller particle size)
Layer	Lab Made/Precoated	Precoated
Mean particle size	10–12 µm	5–6 µm
Layer thickness	250 µm	100 µm
Plate height	30 µm	12 µm
Solid support	Silica Gel, Alumina, Kiesulguhr	Silica Gel—Normal Phase C8 and C18—reverse phase
Sample spotting	Manual Spotting (Capillary/Pipette)	Auto sampler (Syringe)
Sample volume	1–5 µL	0.1–0.5 µL
Shape of sample	Circular (2–4 nm)	Rectangular (6 mm × 1 mm)
Separation	10–15 cm	3–5 cm
Separation time	20–200 min	3–20 min
Sample tracks per plate	≤10	≤36 (72)
Detection limits (absorption)	1–5 pg	100–500 pg
Detection limits (fluorescence)	50–100 pg	5–10 pg
PC connectivity, method storage	No	Yes
Validation, quantitative analysis, spectrum analysis	No	Yes
Analysis time	Slower	Shortage migration distance and the analysis time is greatly reduced
Wavelength range	254 or 366 nm, visible	190 or 800 nm, monochromatic
Scanning	Not possible	Use of UV/visible/fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and scanner is an advanced type of densitometer

For the analysis of herbs and herbal preparations, the HPTLC technique is especially suitable for comparison of samples based on fingerprints and for conveniently performing quantitative determinations based on scanning densitometry. From an analytical perspective, herbs and herbal preparations are particularly difficult to standardize. Pharmacological activity is established in many instances, but there are also many situations in which claimed activity has not yet been scientifically proven. If a plant-based product is introduced to the market, it becomes necessary to define its botanical identity. The starting point must be the botanical authentication of the herb based on its phenotype, possibly the phenotype and some understanding about its natural variability resulting from its geographic location, developmental stage, climate, and so on. The chemical and biochemical composition of the material should be investigated. Fingerprint analysis by HPTLC or HPLC is one of the most powerful tools to link the botanical identity to the chemical constituent profile of the plant. In combination with microscopic investigations, the fingerprint provides the means for a convenient identity check. It can also be used to detect adulterations in raw materials. From the constituent profile, a number of marker compounds can be selected, which might be used to further describe the quality of the herb or the herbal preparation. HPTLC can also be employed for quantitative determination of such marker compounds.

The production of most herbal preparations includes some extraction process. It is essential for quality assurance that this extraction is standardized. The quantity of marker compounds or their relative abundance assayed by HPTLC or HPLC is the principal method of monitoring the production process. When choosing marker compounds for a particular herb or herbal preparation, it is of critical importance that chemically well-characterized standards are available for their quantification. It is often impossible to separate all components of a plant extract completely. Therefore, it must be proven

with an independent method that a given marker compound in the extract is not coeluting with any other substance. HPTLC offers many advantages in this respect based on its great flexibility in detection options. As discussed above, HPTLC works in the off-line mode and many parameters of the chromatographic process can be changed purposefully to affect the result. In addition, different tools, such as plate formats and plate material, application devices and chambers, as well as chromatographic conditions and detection modes, can be employed.

9.2 METHODOLOGY IN HPTLC

The analytical objective should be specified in the light of quantitative or qualitative analysis, or the separation of binary components or multicomponent mixtures, or optimization of the evaluation time before beginning HPTLC. The technique for analyzing drugs in multicomponent dosage forms by HPTLC requires essential information about the nature of the sample, specifically the structure, polarity, volatility, stability, and the solubility parameter. The development of the technique includes extensive trial and error procedures. Generally, the most troublesome issue is where to begin and with what sort of mobile phase. The determination of the stationary phase is very simple, that is, to begin with, silica gel, which is easily available and suits almost all kinds of drugs. The optimization of the mobile phase is performed by utilizing procedures at three levels. The first level includes the utilization of pure solvents and afterward by applying some such solvents that can average separation power for the desired drugs. The second level includes diminishing or expanding solvent strength utilizing hexane or water, respectively. The third level includes the use of mixtures rather than pure solvents from the chosen solvents of the first and second levels, which can additionally be optimized by the utilization of modifiers, such as acids or bases. Analytes are recognized using fluorescence mode or absorbance mode. In any case, if the analytes are definitely not recognized flawlessly then a change of stationary phase or mobile phase or the assistance of pre- or postchromatographic derivatization is needed. Optimization can be said to have begun when a sensible chromatogram is obtained, with only a small variation in mobile phase composition. This leads to a sensible chromatogram, which has all the desired peaks in symmetry and that are very well distinguished. A valuable strategy for this purpose could be to adopt the following parameters as standard:

- dimension of HPTLC plate ($10\times10\,cm^2$ or $20\times10\,cm^2$);
- bands width (5–10 mm in length) using the spray-on technique;
- twin-trough or flat-bottom chamber;
- solvent front should be 50–60 mm;
- derivatization can be performed; and
- documentation and evaluation will be performed by scanning densitometry.

As with TLC, HPTLC utilizes plates composed of small particles with a narrow size distribution. Accordingly, homogenous layers with a smooth surface can be acquired. HPTLC utilizes smaller plates (10×10^2 or $10\times20\,cm^2$) with very short migrating distance (generally 6 cm) and analysis time (7–20 min). HPTLC plates give enhanced resolution, enhanced detection sensitivity, and enhanced in situ measurements. They can be utilized for densitometric quantitative examinations. Normal phase adsorption TLC on silica gel with a mobile phase with relatively less polarity, for example, chloroform–methanol, has been utilized for over 90% of the reported analyses of pharmaceuticals. Hydrophobic C-18, C-8, and C-2, as well as phenyl-substituted silica gel stationary phases and hydrocarbon-incorporated silica gel plates, have been created with a relatively polar aqueous phase, for example, methanol–water or dioxane–water, and are utilized for reversed-phase TLC. Other precoated layers that are utilized incorporate aluminum oxide, magnesium silicate, magnesium oxide, polyamide, cellulose, kieselguhr, particle exchangers, and polar-altered silica gel layers that contain fortified amino, cyano, diol, and thiol groups. Optical isomer separations that are carried out on a chiral stationary phase produced from C-18 substituted silica gel incorporated with a Cu (II) salt and an optically active enantiomerically unadulterated hydroxyproline derivative, on a silica stationary phase incorporated with a chiral molecule, such as brucine, on molecularly engraved polymers of α-agonists, or on cellulose with mobile phases, including chiral molecules, for example, cyclodextrins, have been reported for the most part for amino acids and their derivatives. Mixtures of stationary phases have been utilized to develop layers with special selectivity. HPTLC plates need to be stored under suitable conditions. Before utilization, plates should be examined under white and UV light to identify damage and contaminants in the stationary phase.

9.2.1 Instrumentation

9.2.1.1 LINOMAT

Semiautomatic application of samples for qualitative and quantitative investigations is offered by the Linomat. The instrument is appropriate for regular use, having medium sample throughput. As opposed to the Automatic TLC Sampler (ATS), changing the sample for the Linomat requires the continuous attention of the operator. The spray-on technique associated

with the LINOMAT tests consists of spraying onto the stationary phase as narrow bands. This system permits larger volumes to be applied than by contact transfer, such as spotting. During spray application, the solvent present in the sample evaporates fully, thus reducing the sample in the form of thin bands. Starting zones applied as thin bands guarantee the highest resolution achievable with any other thin-layer chromatographic system. For qualitative and quantitative HPTLC and, in addition, to ensure preparative separation, spray-on application is a necessary step.

9.2.1.2 Automatic TLC Sampler (ATS)

Automatic application of the sample increases the productivity of the HPTLC laboratory. The prerequisites for an instrument meeting this need, that is, precision, robustness during regular use, and convenient handling, are completely met by the automatic TLC sampler. The ATS provides completely automatic sample application for qualitative and quantitative analyses. It is ideal for regular use, having a high sample recovery rate in mass investigation. Samples are either developed as spots through contact transfer (0.1–5 µL) or as bands or rectangles (0.5 to >50 µL) using the spray-on technique. Starting zones applied as narrow bands provides the optimum separation achievable with a specific chromatographic system. Application in the form of rectangles permits exact application of required volumes without harming the layer.

9.2.2 Selection of Mobile Phase

The choice of mobile phase depends on the adsorbent material used as the stationary phase and the physicochemical properties of the analyte. Common mobile phases utilized according to the various selectivity properties are diethyl ether, methylene chloride, and chloroform mixed individually or along with hexane as a strength-modifying solvent for normal-phase TLC and methanol, acetonitrile, and tetrahydrofuran blended with water for adjusting the strength in reversed-phase TLC. Separations involving ion-pairing on C-18 layers are carried out with a mobile phase, such as methanol–0.1 M acetic acid derivation cradle (pH 3.5), containing 25 mM sodium pentanesulfonate (15.5:4.5). Exact volumetric estimations of the components of the mobile phase must be carried out individually and absolutely in a satisfactory volumetric apparatus and shaken to guarantee appropriate blending of the content. Volumes of up to 1 mL are estimated with an appropriate micropipette. Volumes of up to 20 mL are estimated with a graduated volumetric pipette of reasonable size. Volumes larger than 20 mL are estimated with a graduated cylinder of reasonable size.

9.2.3 Preparation of HPTLC Plate

Readiness of HPTLC plate precovered layers: TLC plates can be made in any lab with a reasonable mechanical assembly. However, such layers do not stick well to the glass support. Precoated plates that utilize small amounts of very-high-molecular-weight polymer as binder surmount most of the problems of a homemade layer. Precoated layers are notably abrasion resistant, extremely uniform in layer thickness, reproducible, preactivated, and ready to use. They are present with glass or aluminum or polyester backing. Aluminum-foil plates are more affordable to purchase and can be cut, thus making them easier to carry, transport, or mail. Glass plates are best for obtaining the highest quality result. Generally, layers containing a fluorescent marker F254 are utilized. This allows the samples to be visualized in a UV cabinet very easily, swiftly, and in a nondestructive way (Mukherjee, 2002).

9.2.4 Sample Preparation and Application

9.2.4.1 Sample Application in HPTLC

The sample in thin-layer chromatography (TLC) is manually applied with capillaries for basic investigations. Test volumes of 0.5–5 µL can be placed as spots onto ordinary layers without drying in between. HPTLC layers require up to 1 µL per spot. More laborious qualitative, quantitative, and preparative analyses or separations can be performed by instruments by band-wise application of the samples using the spray-on technique. In particular, HPTLC makes use of the advantages of increases in separation power and reproducibility, accessible by exact positioning and volume dose.

9.2.4.2 Sample Application in the Form of Bands or Rectangles

Sample application as bands or rectangles enables the application of fundamentally larger volumes. Any zone widening that would ordinarily be caused by chromatography amid application by contact spotting can be avoided. In extraordinary cases, for example, during trace analysis, huge sample volumes or samples with high matrix content can be sprayed in the

form of rectangles, which, preceding chromatography, are concentrated into thin bands, enabling a short development step with a solvent of high elution strength. The Linomat, as a single machine, is the perfect instrument for sample application in instrumental and preparative TLC. The product-controlled variant allows modification up to HPTLC.

9.2.4.3 Development of Plate

A look at the mechanical assembly of TLC shows that critical advances have been made with scanners; however, chromatography itself is mainly performed in "scaled down" paper chromatography (Berkham, 1988). The AMD system (automatic multiple development) is now available for automatic development, making it possible to reduce errors in chromatography. They range from simple, one-dimensional developments to stepwise development techniques. A control unit ensures that human error is reduced to a minimum. Developments are constantly being made:

- rising with fixed plate situation;
- absence of chamber saturation in classical sense; and
- with just 8 mL mobile phase per run.

The development distance depends on the time control. The chamber is not opened until all the chromatographic procedures have been finished (even in the case of multiple or gradient development). The HPTLC plate is dried in between the various developments (in vacuum) and freed from solvent on completion of development. The possible contamination of the workplace by mobile phase vapors is thus avoided.

9.2.4.4 Processes in the Developing Chamber

The classical way of developing a chromatogram is to place the plate in a chamber, which contains a sufficient amount of developing solvent. The lower end of the plate should be immersed several millimeters. Driven by capillary action, the developing solvent moves up the layer until the desired running distance is reached and chromatography is stopped. The following considerations primarily concern silica gel, which is used as a stationary phase and for developments; this can be described as adsorption chromatography.

- Among the components of the developing solvent and its vapor, equilibrium will eventually be established, known as chamber saturation. Depending upon the vapor pressure of the individual components, the gas phase composition can vary significantly from that of the developing solvent system.
- The still-dried part of the stationary phase adsorbs molecules from the vapor phase. This procedure, known as adsorptive saturation, is additionally approaching an equilibrium in which the polar components will be pulled back from the gas phase and stacked onto the surface of the stationary phase.
- At the same time, the part of the stationary phase already wetted with the mobile phase interacts with the gas phase. In this manner, particularly the less polar parts of the solvent system are discharged into the gas phase. This procedure is governed more by adsorption forces than vapor pressure.
- During the course of migration, the components of the mobile phase can be isolated by the stationary phase under specific conditions, causing the development of secondary fronts.

9.2.4.5 Important Aspects to be Considered in the Development Process

Except for individual pure solvents, migrating solvent and mobile phase are not the same. Their ratio changes as chromatography progresses. The terms migrating solvent and mobile phase are frequently used as equivalent words. In the true sense, just the liquid in the chamber ought to be called the developing solvent, while the liquid traveling through the layer constitutes the mobile phase. Just the composition of the developing solvent when it is put into the chamber is unequivocally known. Amid chromatography, parts of the developing solvent, which have been stacked onto the dry layer from the gas phase, are pushed in front of the true, yet imperceptible, solvent front. Special cases are extremely polar components, for example, water, methanol, acids, or bases. This results in R_f values being lower in saturated chambers and especially on preconditioned layers, than in unsaturated chambers and sandwich chambers. Note that because of possible demixing of the solvents and possible beta fronts, development in a sandwich chamber or in an unsaturated horizontal developing chamber works best with single-component solvents or multicomponent solvents that act like single component solvents. TLC generally progresses in an equilibrium between stationary, mobile, and gas phases. Consequently, it is extremely hard to effectively portray the conditions in a developing chamber. Reproducible chromatographic results can only be expected to be normal when all parameters are kept as steady as possible. The chamber shape, along with saturation, plays an important role in this regard (Mukherjee, 2002).

9.2.4.5.1 Influence of Temperature on TLC Analysis

Under a constant relative humidity (RH), R_f values tend to be higher when developing samples at a higher temperature and vice versa. However, the fluctuation of R_f values generally does not exceed the range of ± 0.02 if the change of developing temperature falls within the range of $\pm 5°C$. As a result, the influence of temperature on the chromatographic behaviors of the target components is not significant. However, if the fluctuation and developing temperature reach a high level of change, TLC performance will be influenced to varying degrees. First, the boiling point, vapor pressure, evaporation number, and relative density of each organic solvent in the mobile phase may differ, generating different effects on the extent of evaporation, which in turn results in changes in the spatial distribution of each solvent vapor in the developing chamber, inevitably leading to alterations in the developing behaviors of the components to be separated. Second, temperature changes will surely change the proportion of water in the organic portion of a two-phase mobile phase in either storage or development, resulting in altered polarity of the mobile phase and impaired performance of TLC analysis.

9.2.4.5.2 Influence of Coating Materials and Plates on the Performance of TLC

As mentioned above, certain differences may be exhibited in the reproducibility and chromatographic behaviors for TLC plates. There are different granular sizes, properties of silica gel, and adhesive employed in TLC plates (Peishan and Yuzhen, 1987). Therefore, the specifications of TLC plates should be clarified beforehand.

9.2.5 Retardation Factor

The location of any solute spot in TLC depends on its retardation factor R_f. It is an essential value expressed as.

$$R_f = \text{Distance traveled by the analyte / distance traveled by the solvent front.}$$

It is sometimes more advantageous to report as $R_f \times 100$. The reproducibility of the R_f value depends upon numerous factors, for example, nature of the sorbent, humidity, layer thickness, migrating distance, and surrounding temperature. Overloading of the sample typically brings about a slight increase in the R_f value. System errors influence this basic TLC parameter when the correct position of the solvent front cannot be found. The loss of mobile phase or the "piling up" of mobile phase components previously present lowers the value. These R_f values are therefore not genuine and cannot be utilized to ascertain k, the capacity factor. The correct values are acquired when no gradient occurs along the separation path, no loss of mobile phase occurs, the right position of the solvent front can be measured without error, and by excluding any impacts that occur because of prevaporization. An HPTLC chamber for acquiring genuine R_f values has no gas phase, does not show a temperature variation, the layer is in an even horizontal position, and it is completely symmetrical. This is the only effective method for preventing any change in phase ratios. In other methodologies, complex hardware is utilized to repair and manage numerous factors that affect the R_f value. The ideal technique for getting provisional identification of a substance is to spot the substance along with a series of reference compounds on the same chromatogram. By this process, the mobilities of all compounds are compared in the same conditions, and a match in R_f value between a sample and a standard is proof of the identity of the sample. The trial conditions ought to be picked so the compound to be distinguished moves to a point near the middle of the layer ($R_f = 0.5$) and resolution between spotted standards is optimum. If R_f values on silica gel are higher than desired, the polarity of the mobile phase is reduced. For very low R_f values, the polar segment of the mobile phase is increased. If the spot from the sample does not match the standard, they are either not the same or their mobilities have been influenced by contaminating material present in the sample. A correlation of the R_f values between round and straight HPTLC was proposed by Geiss and is stated as R_f direct = (R_f round). This relation was found to be totally correct when the starting point is precisely in the center of the circular chromatogram. In the event that the substance is applied at a distance from the center point, the chromatogram will look like a run in linear TLC (Variyar et al., 2011; Mukherjee, 2002).

9.2.6 Detection and Densitometric Scanning

Qualitative sample identification by the scanning densitometer is performed by recording spectra of the analyzed components. The sensitivity of the scanner depends on the quality of the electronic and optical components of the instrument. The signal increases very slightly as the slit width is increased. As the slit height approaches the diameter of the spot, the signal increases. Signal distortion will result at high scan rates if the electronic time constant of the instrument and recording device are too slow.

9.2.6.1 Derivatization and Detection

A developed chromatogram can be seen directly as a colorful image if the analytes appear as color bands/spots in daylight. However, most of the components in crude drugs are not visible under white light, needing a derivatization agent to enhance visibility. For example, volatile oils in many crude drugs are only visible on the TLC plate when a chemical reagent, such as vanillin-sulfuric acid solution, is added. Applications of the derivation agent include the spraying method and immersion. Pressurized aerosol bottles containing reagent solutions can spray a fine uniform mist onto the surface of the plate. This spraying method is most commonly used. The immersion method requires a special glass immersion tank. The developed thin-layer plate is placed into the immersion tank steadily and vertically for one or more seconds. The residual agents on the back of the glass plate need to be wiped clean when it is removed from the immersion tank. In some cases, the plate may require heating for derivatization for bands/spot visualization in daylight or under ultraviolet light. The ultraviolet lamp cabinet for observing TLC images is equipped with two lamps, one of long wavelength (366 nm) and one of short wavelength (254 nm). The former is used for observation of fluorescent chromatograms, while the latter is usually used to observe fluorescence quenching dark bands/spots under a fluorescent background on a silica GF_{254} plate. The power of the fluorescent lamp and the filter specifications should be considered when selecting an ultraviolet lamp.

The optical density of the individual spots on the TLC plate determines the resolution of the compounds isolated therein. Densitometry helps in measuring the concentrations of the compounds developed in individual plates. The sample quantities are measured by comparison to a standard curve from reference materials chromatographed in parallel under similar conditions. A data assessment using the regular techniques for scanning is made by estimating the optical density of the transmitted light as a function of the concentration of the sample or standard applied on the silica gel. With the evaluation of the optical density as a function of concentration, the detection device came to be called a densitometer. A scanning densitometer is a more advanced workstation for evaluation of TLC/HPTLC and electrophoresis objects by measuring the absorbance (optical density) or fluorescence. This advanced workstation measures using either the reflectance or transmittance mode by absorbance or fluorescence and is called a "scanner" (Mukherjee, 2002).

The performance of a scanning densitometer depends on several parameters, including component resolution, dynamic signal range, and sample detectability (the S/N ratio, etc.). The experimental variables that affect these parameters include the slit dimensions governing the size of the beam, the scan rate, the total electronic time constant of the instrument, and the recording device. There are several densitometric scanners that have been used in HPTLC and they are discussed below.

9.2.6.2 Opto-Mechanical Scanning

Almost all densitometers in operation today perform scanning by opto-mechanical means. The support table with the chromatogram is displaced by a corresponding amount while the beam is stationary. Scanning is achieved by moving the support table of the chromatogram in the appropriate pattern. It can be displaced in a continuous motion or in discrete steps generated by a stepper motor. The stepper motor drive seems to yield superior performance. For point scanning, the illuminating beam is given a circular or approximately quadratic cross section. Slit scanning works with a rectangular illuminating aperture with adjustable width. The adjustment has to ensure that the illuminating slit covers the scanned track at all times, but does not extend over its boundaries.

9.2.6.3 Electronic Scanning

Line sensors use electronic scanning in one direction and mechanical displacement in the other. Their main advantage is high spatial resolution. In other respects, their performance is a hybrid between conventional and mechano-optical scanning versus purely electronic scanning systems. The advantage of this scanning is the speed with which the data acquisition is possible. One scan cycle usually lasts for 20 ms.

9.2.6.4 Laser Scanning

When extremely high resolution is required, laser scanning is the method of choice. It is more prominently useful in gel-electrophoresis in which high resolution is required. The scanning motion of the beam is almost always implemented by "opto-mechanical" means. The high resolution is achieved by a very small diameter scanning beam, which is made possible by the coherent nature of the laser light. One of the major shortcomings of lasers in densitometry is their fixed wavelength. Lasers with variable wavelength are available, but are extremely expensive.

9.2.6.5 Detection Methods Used in Scanning Densitometry

9.2.6.5.1.1 Single-Beam Mode The single-beam mode is capable of producing excellent quantitative results, but spurious background noise resulting from fluctuations in the source output, nonhomogeneity in the distribution of extraneous absorbed impurities, and irregularities on the plate surface can be troublesome. An incident beam of monochromatic radiation is projected onto the plate at a 90 degree angle. The reflected light is measured by a photomultiplier at an angle of 45 degree. For fluorescence measurements, a cut-off filter is inserted between the sample and the photomultiplier tube (PMT). In the normal mode the excitation wavelength, and in the reverse-beam mode the emission wavelength, can be selected by the monochromator.

9.2.6.5.1.2 Double-Beam Mode Background disturbances can be compensated for to some extent by double-beam operation. The two beams can either be separated in time at the same point on the plate or separated in space and recorded simultaneously by two detectors. The design of this instrument can be classified as double beam in space with variable wavelength capability. The ratio between the signals of the two photomultipliers is recorded. This instrument operates in a transmission–reflectance mode and can measure absorption, fluorescence, and fluorescence quenching. At the beginning of the scan, the electric output signals are set at a ratio of 1:1, which is equivalent to 100% transmission or reflectance. As the two beams impinge on different areas of the plate, small irregularities in the plate surface and undesired background contributions from the impurities in the sorbent layer may still pose a problem.

9.2.6.5.1.3 Single-Beam Dual-Wavelength Mode Fluctuations caused by the scattering at a light absorbing wavelength (γ_1) are compensated for by subtracting the fluctuations at different wavelengths (γ_2) at which the spot exhibits no adsorption but experiences the same scatter. The two beams are modified by a chopper and recombined into a single beam to provide different signals at the detector. As the scatter coefficient is, to some extent, dependent on the wavelength, the back-ground correction is better when (γ_1) and (γ_2) are as nearly identical as possible. This requirement is often difficult to meet as absorption spectra are usually broad and the two wavelengths at which the absorption occurs in one, have no absorption in the other.

9.2.6.6 Type of Scanning Mechanisms

9.2.6.6.1.1 Slit Scanning/Linear Scan Densitometric chromatogram evaluation is made in the reflectance mode with a light beam in the form of a slit, selectable in length. The length and the width of the slit that is selectable is moved over the sample zones that are to be evaluated. In order to avoid systematic errors, the scanning is done in the direction or against the direction of chromatography. Scanning at right angles is never recommended as systematic errors are introduced in the detection. The light that is diffusely reflected is measured by the photosensor and the difference between the optical system from the sample-free background and that from a sample zone is correlated with the amount of the respective fraction of calibrated standards chromatographed on the same plates. The detection limits for scanning by fluorescence are typically 100–1000 times lower than for scanning by absorbance. Therefore, it is always wise to choose the fluorescence mode when substances with inherent fluorescence are to be measured. In the scanning slit mode, the apparatus construction is less complicated and the apparatus noise is averaged over a slit width.

The densitometer response is comparable when all the spots are of the same size and diameter and are positioned in a reproducible part of the scanning slit. A major obstacle to slit scanning is the nonlinearity of the tracks. There are advantages in using the slit-scanning vs the flying-spot method. One inherent drawback of scanning with a light spot that traverses the separation zones in a zigzag or meandering path is that the S/N ratio, which determines scanning quality and detection limits, decreases with the size of the illuminated areas. For this reason alone, the light spot cannot be reduced as would be necessary or desirable to exploit the spatial resolution of an HPTLC chromatogram. Owing to the light scattering in the sorbent layer, the smallest possible light spot that can be produced is more than 0.2 mm in diameter even when the irradiating beam is much narrower. When the chromatogram is scanned with a narrow slit, a high speed can be selected without the need for any concessions on data reduction. This makes slit scanning superior to meander scanning.

9.2.6.6.1.2 Meander/Flying-Spot Scanning Here the light spot is moved in a meandering way over the sample zones with a swing corresponding to the length of the slit. There are more disadvantages than advantages. The signal-to-noise ratio and the spatial resolution is lower when the meander scan mechanism is used. The principle of flying-spot scanning is that the monochromatic light beam from the diffraction grating is rapidly moved from side to side by the spiral slit of the rotating disk. If the area seen by the photodetector is larger than the diffusion-limited area of constant concentration, for ex-

ample, when conventional slit scanning is employed, special steps must be taken to ensure that a response is produced that is linearly dependent upon the total light flux incident upon the photodetector. No such special precautions are needed for the "Flying-Spot Method/Arrangement." The advantage of flying-spot scanning is that it improves contrast and amplitude resolution. This advantage applies to all modes, including fluorescence. Different aspects for development and detection in HPTLC are explained further in Fig. 9.2.

9.2.6.7 In Situ Quantitative Evaluation

9.2.6.7.1 Baseline Compensation

The baseline method was first used in QTLC. The baseline technique can be realized in dual-beam operation (comparison of a scan of a blank part of the plate with the part used for separation) and a single-beam version for electronic smoothing. After chromatography the same lane is scanned under identical conditions. The difference between the two signals provides an electronically smoothened chromatogram. Another method employs scanning of the same chromatogram in the reflectance and transmission mode. Both chromatograms are stored and digitized.

9.2.6.7.2 Computer-Controlled Multiwavelength Evaluation

Multiwavelength (MW) evaluation requires a computerized TLC scanner with stepper motors in the *x*- and *y*-direction to move the plate under computer control and a computer-controlled monochromator to record the spectra or to scan the spectra at a preselected wavelength. This MW evaluation can be done in the absorbance (reflectance) mode or fluorescence mode. In each mode, the detection wavelengths are preselected by the operator and the first scan is made at the shortest wavelength. All raw data are stored for each scan. All reflectance or fluorescence measuring curves are first baseline corrected and the peaks integrated. Detection, identification, and quantification of the separated spots are possible with this feature.

9.2.6.7.3 Photodiode Array Scanning

The concept of the photodiode array technique in QTLC is very useful in detection of components. Videocon-type instruments have been introduced to obtain special information about the separated spots. This type of instrument has two advantages compared with a photodiode array system. The first concerns the resolution in space and the second the illumination

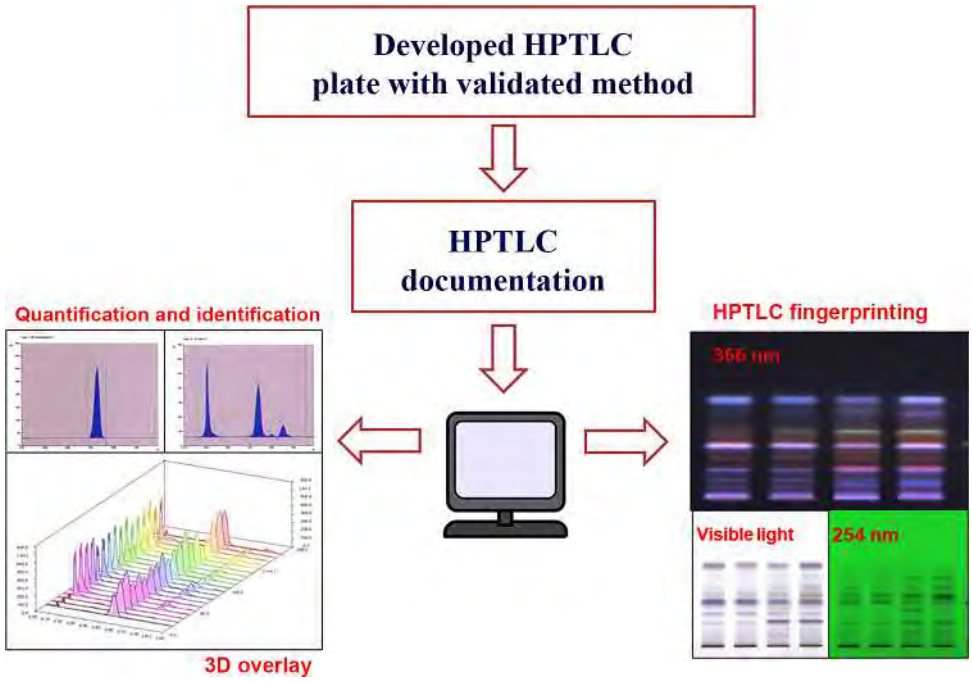


FIG. 9.2 Development and documentation of HPTLC.

of the plate. The linear photodiode array TLC scanner has the advantage that there is no need to position the spot in the center of the light beam. The integration can be performed in the direction of development by summing up the photodiode data to achieve an enhanced S/N ratio. A 3D plot of the separated dyestuff mixture demonstrates the applicability of the photodiode array scanner in QTLC.

9.2.6.8 Parameters of the Scanner Affecting the Performance of Detection

9.2.6.8.1.1 Slit Width of the Monochromator The slit width should be 20 nm for all normal measurements and 5 nm for those having a detailed structure and for scanning specific applications in the multiwavelength mode in which high selectivity is necessary. The slit widths control the amount of light that is available for scanning and resolution of data collection.

9.2.6.8.1.2 Macro and Microparameters for the Slit Dimensions

- The dimensions of the slit are halved if the switch is made from macro to micro. The spot should not cover the adjacent tracks.
- The length of the slit should:
  - Completely cover a substance applied spot-wise/band-wise.
  - For band-wise application, it should cover 70%–75% of the area of the applied band.
  - If samples are applied as spots, the slit length should be 10% larger than the largest spot in the track.

9.3 HPTLC VALIDATION PROCEDURE

Validation should not be seen separately from the development of a method. It begins from a plainly characterized analytical goal, method selection, optimization, and development, known as prevalidation considerations before coming to the elaboration of a validation protocol. This is the beginning stage of the actual validation. After performing each analysis portrayed in the validation protocol, the data obtained are assessed and matched with the acceptance criteria. In the case of all criteria being met, the method can be viewed as valid. In a less formal way, some validation data might be taken from experiments that were performed previously as part of the method development. The important parameters for method validation in HPTLC are shown in Fig. 9.3.

This approach is generally acknowledged for validation of qualitative HPTLC techniques for identification in routine use. It is possible that the validation technique in some circumstances may require a few changes in the standard validation protocol. Such changes may incorporate limitations with regard to humidity, waiting times, precision, and so on. The validation protocol is a key instrument for organizing, controlling, and recording the validation processes, relying on the quality management system. The following components must be incorporated.

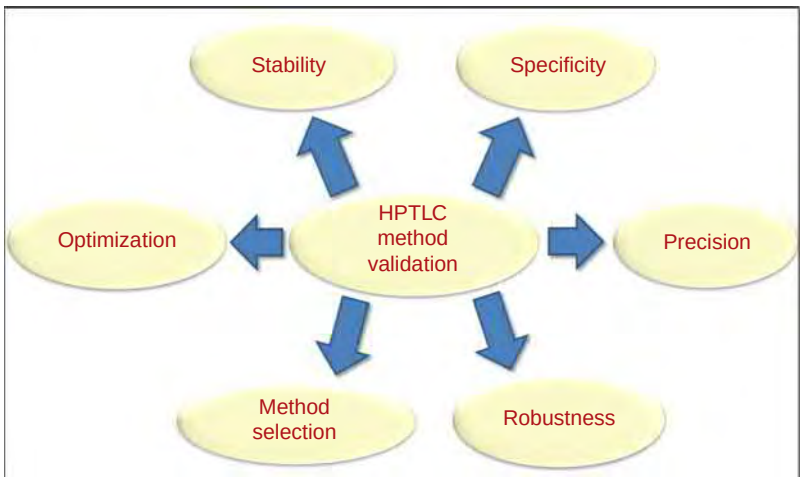


FIG. 9.3 Parameters for method validation in HPTLC.

9.3.1 Selectivity

The ability of the analytical technique developed is to recognize an analyte quantitatively in the presence of other components that are anticipated to be present in the sample matrix. The results are communicated as a resolution. In the event that normal contamination is present, it ought to be chromatographed alongside the analyte to check the system suitability, retention factor, tailing factor, and resolution.

9.3.2 Sensitivity

Sensitivity is the ability of the technique to acquire test results inside a given range in direct proportion to the concentration of analyte in the sample–calibration curve for the analyte.

9.3.3 Precision

Precision gives an indication of random error. Its outcome ought to be expressed as a relative standard deviation (RSD) or coefficient of variation (COV). Precession is seen in terms of replication: precision under the same conditions, same expert, same mechanical assembly, a short time interval, and similar reagents using the same sample; *estimation of peak area*: RSD ought not be more than 1% based on measuring the same sample seven times; *peak position*: RSD ought not be more than 2% based on repositioning the instrument seven times after every measurement; *test application*: an equal volume applied as seven spots and RSD ought not be more than 3% under diverse conditions, such as different analyte, different laboratory, and different days and reagents from different sources utilizing the same sample. RSD ought not be more than 10% within laboratory reproducibility.

9.3.4 Accuracy

The accuracy of an examination is dictated by the systematic error involved. It is defined as how well the real value and the mean analytical value obtained by applying the test method at different time intervals agree. The accuracy is satisfactory if the similarity between the true value and the mean estimated does not surpass the RSD values obtained for repeatability of the method. This parameter is essential for formulated pharmaceutical dosage forms as it provides data about the recovery of the analyte from the test sample and the impact of the matrix. If the recovery rate is observed to be 100%, it means that the proposed analytical technique is free from constant and proportional systematic error. A blank matrix and known impurities must be present to test the accuracy of the strategy.

9.3.5 Ruggedness

This is one of the most critical parameters for validation of an HPTLC technique. Tests are typically prescribed for the ruggedness of an HPTLC method:

- *Sample preparation*, which includes composition, quantity of solvent, pH, shaking time, temperature, and number of extractions.
- *Sample application*, including volume applied, spot shape and size, band, and spot stability.
- Chromatographic conditions, which include chamber saturation, eluent composition, eluent volume, temperature, humidity, and development distance.
- Spot visualization, including post chromatographic derivatization, spraying, dipping, reaction temperature and time, quantitative evaluation, drying of plates, detection, and wavelength.

Once the analytical strategy is developed, it ought to be performed autonomously by three experts who are well acquainted with the functional parts of the strategy, analyzing the same sample under the same trial conditions to check the reproducibility of the strategy.

9.3.6 Limit of Detection

The smallest amount of analyte that can be recognized is not more than 10% of the individual impurity limit. If this is not possible, then the amount of analyte to be applied should be increased. The limit of detection (LOD) is expressed as the ratio of signal to noise. An average of the areas of 15 noise peaks and their absolute SD values are measured. The LOD is the amount of applied sample producing a peak area that is equivalent to the total of the mean blank area and three times the standard deviation.

9.3.7 Stability

The analyte ought not to decay amid advancement of the chromatogram and ought to be stable in solution and on the stationary phase, for no less than 30 and 15 min, respectively. The intensity of the spot on the chromatogram ought to be steady for no less than 60 min during optimization of the extraction/decontamination method and one must remember the chemical properties and purity of the extraction solvent. Reactions between solvents and their contaminants may create additional spots/peaks, hence prompting false test results. Another important factor is the pH of the aqueous phase used for extraction/filtration, which may prompt hydrolysis, oxidation, and isomerization. The complete evacuation of the organic solvent should be avoided.

9.4 USE OF HPTLC FOR STANDARDIZATION OF HERBS AND EXTRACTS

Densitometric evaluation through HPTLC is a very useful method for standardization of medicinal plants and other natural products, especially those used in different systems of medicine. It has been approved as an authenticated method of analysis in several pharmacopeias, including the USP and the IP. Results from a number of medicinal plants studied in the laboratory have shown that, much of the time, TLC-densitometry is a more advantageous strategy than HPLC or GLC. The prepurification steps fundamental to the use of HPLC and GLC are impressively streamlined or can be avoided. In addition, the chromatograms acquired amid quantitative investigation are helpful, both for extract characterization as an overall fingerprint, and for the identification of the possible adulteration or degradation of the drugs.

Because the plant kingdom is an inexhaustible source of biologically active compounds, a portion of our most valuable drugs are obtained from plants. Also, a significant number of their constituents have been used as models for the synthesis of other therapeutic agents. Because of the purification and pharmacological screening techniques, most medicinal plants used in the past have made their way into current medicine as purified substances as opposed to older galenical preparations of crude extracts, although an increased enthusiasm for these extracts is now being observed. The wide assortment of natural substances that are defined and deposited by plants requires profoundly complex techniques for their separation, purification, identification, and quantitative determination. The bioactive constituents of plants are not generally clearly defined or are generally considered to be a complex mixture of numerous plant constituents. Furthermore, the active constituents of plants are mixed with several other components, which generally hinder determinations. As the substances identified are of natural origin, a high fluctuation of content is normal; thus, the strategies proposed must be adequately adaptable and particular to consider such a vast number of varieties (Mukherjee, 2002).

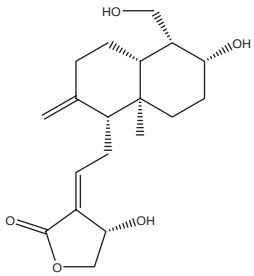
Quantitative TLC, which generally requires less sample preparation, provides a valuable contrasting option to the GLC and HPLC techniques. Another feature of TLC-densitometry is the utilization of chromatograms as total fingerprints of crude extracts. Therefore, the likelihood of distinguishing possible degradation or adulteration of the drug is higher by HPTLC. Many postchromatographic reagents may be used to give compound-specific or class-specific colors or fluorescences for enhancing specificity and selectivity. Components engaged with the precision of the TLC-densitometry strategy have been well documented in various books and publications dealing with this subject. Most TLC scanners are fit for measuring absorbance, reflectance, fluorescence, and fluorescence quenching, and give the spectra of individual spots in situ. On account of exceptionally complex mixtures, this method of sample application greatly enhances the resolution and accuracy of quantification by eliminating interference with the constituents of interest. Additionally, the distribution of the compound inside the band is more uniform and of the sample application, which is considered less often sometimes. The first deals with separation between the underlying spot or band and the surface of the mobile phase. This parameter includes the reproducibility of the position of the sample from the lower edge of the plate and the level of the mobile phase in the tank. The second factor includes the nature of the solvent utilized for the sample application. In the best conditions, it ought not support the migration of the constituents around the spot or the band amid application, and must be at any rate less polar than the mobile phase with a specific end goal to acquire a preconcentration impact of the constituents in the solid phase and a dispersion inside this phase that is as small as could be expected under the circumstances. Such an impact can be represented by the chromatography of quinine, the coefficient of variation (COV) in the densitometric estimations of this alkaloid is more prominent when the polarity of the solvent utilized for the application increases (Mukherjee, 2002).

TLC has advanced as a vital analytical tool with the development of a range of alkyl-bonded phases and of small-sized adsorbent particles. Improvements in resolution, sensitivity, and reproducibility are the fundamental attributes of HPTLC. However, in some cases it has been observed that the reproducibility of the densitometric measurements is higher when ordinary plates are used. It is likewise intriguing to take note that estimations performed in the migration direction provide

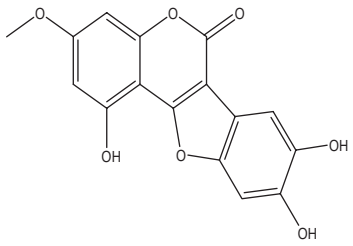
lower coefficients of variation than perpendicular measurements. Notwithstanding the conventional development conditions, including a single development with the mobile phase, various new strategies have been developed, for example, circular and anticircular methods (Vanhaelen and Vanhaelen-Fastré, 1988). There are several scientific reports available on the marker profiling and standardization of herbal medicine through HPTLC methods. Several methods have been developed and validated for various parameters, including precision, accuracy, LOD, LOQ, ruggedness, and robustness. HPTLC plays an important role in the characterization and quantification of marker compounds for the development and standardization of herbal medicines. To strengthen this statement, some examples have been provided of some medicinal plant species in the subsequent sections.

9.4.1 Simultaneous Measurement of Andrographolide and Wedelolactone

Andrographolide (1) and wedelolactone (2) are bioactive components of *Andrographis paniculata* and *Eclipta alba*, respectively. The concentrates of these plants are utilized as a part of numerous customary hepatoprotective formulations. An attempt has been made to build up an accurate, precise, and specific HPTLC technique to measure simultaneously both these chemical markers in different dosage forms, for example, tablets and syrups. Precoated Silica gel 60F254 plates with toluene: $(CH_3)_2CO$: formic corrosive (9:6:1) as a solvent system and a detection wavelength of 254 nm were utilized. The technique was validated in terms of linearity, accuracy, precision, and specificity. The calibration curve was observed to be linear in the range of 200 and 400 ng/spot for andrographolide and 100–200 ng/spot for wedelolactone. The LOD and the limit of quantification for andrographolide were found to be 26.16 and 79.28 ng/spot, respectively, and for wedelolactone 5.06 and 15.32 ng/spot, respectively (Patel and Prajapati, 2008).



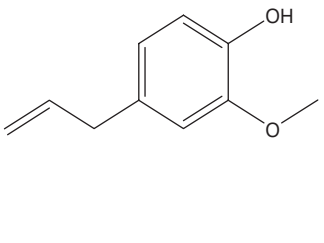
Andrographolide (**1**)



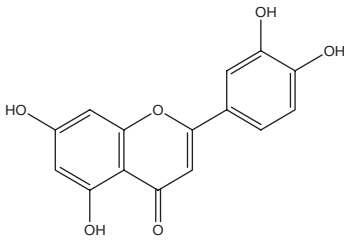
Wedelolactone (**2**)

9.4.2 Quantification of Eugenol, Luteoline, Ursolic Acid, and Oleanolic Acid in Black and Green Varieties of *Ocimum sanctum*

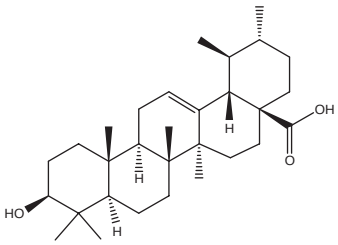
O. sanctum (Lamiaceae) is a well-known plant used as a part of Ayurveda, popularly known as Tulsi or Holy Basil. In traditional medicine, the plant is utilized in cardiopathy, blood disorders, leucoderma, asthma, bronchitis, genitourinary disorders, and skin diseases. It has been demonstrated that of the four compounds, eugenol (3) and ursolic acid (4) were in free form, although luteolin (5) and oleanolic acid (6) were distinguished only after hydrolysis. Thus, eugenol and ursolic acid were evaluated from methanolic extracts and the samples were hydrolyzed to acquire the aglycons of luteolin and oleanolic acid. The optimized mobile phase separated all the marker compounds with the accompanying R_f value as: eugenol, 0.77; luteolin, 0.27; ursolic acid, 0.50; and oleanolic acid, 0.56. Other compounds in the same extract did not interfere with the process. Concurrent evaluation of all the four markers was impossible despite the fact that they were resolved in the same solvent system for various reasons. Eugenol and ursolic acid are available in free form, yet eugenol is recognized under UV light (λ_{max} 280 nm) without derivatization, though ursolic acid can be distinguished simply after derivatization with anisaldehyde–sulfuric acid reagent (λ_{max} 530 nm). Luteoline and oleanolic acid are available in bound form; however, luteolin is distinguished under UV light (λ_{max} 350 nm) without derivatization, while ursolic acid can be identified simply after derivatization with anisaldehyde–sulfuric acid reagent (λ_{max} 530 nm). The plates were examined at the particular wavelength of the four markers for evaluation. All the four were observable after derivatization and this characteristic can be utilized for the TLC fingerprinting process, in which the extracts of the samples can be cochromatographed with markers and viewed after derivatization. This strategy is useful in recognizing the two varieties of *O. sanctum*, Black and green variety (Anandjiwala et al., 2006).



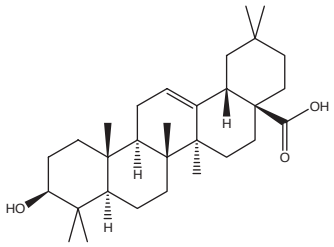
Eugenol (**3**)



Luteoline (**4**)



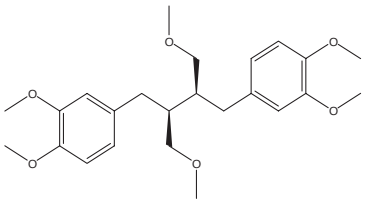
Ursolic acid (**5**)



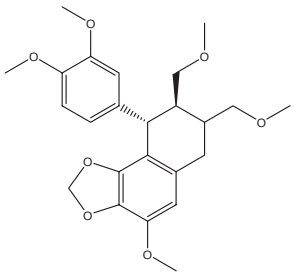
Oleanolic acid (**6**)

9.4.3 Estimation of Phyllanthin and Hypophyllanthin

Phyllanthin and hypophyllanthin are very important phytopharmaceuticals derived from *Phyllanthus amarus*. An HPTLC analysis was performed for measurement of these constituents. Preactivated silica gel 60F254 plates were used. The plate was allowed to develop to a distance of 10 cm utilizing a mobile phase of hexane:acetone:ethyl acetic acid (74:12:8) under identical laboratory conditions. Amid the HPTLC investigation of the variation of phyllanthin (7) and hypophyllanthin (8) in samples of the phyllanthus species gathered from various geological sources, none of the samples was found to contain higher concentrations of hypophyllanthin when compared with phyllanthin. This is due to the fact that the higher concentration of hypophyllanthin reported was because of different lignans present at the same R_f as that of hypophyllanthin. In this manner several mobile phases of various concentrations were used. The optimized mobile phase was hexane:acetone:ethyl acetic acetate (24:12:8, v/v/v), which gave good resolution of phyllanthin and hypophyllanthin from other closely related lignans. The reagent used for color development gave an intense blue color at R_f values of 0.24 and 0.29, respectively. The scanning of the TLC plates was performed at 580 nm in the absorbance reflectance mode (Tripathy et al., 2006).



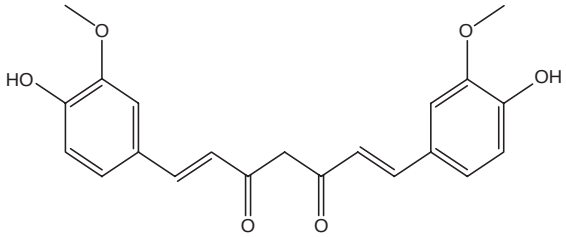
Phyllanthin (**7**)



Hypophyllanthin (**8**)

9.4.4 Determination of Curcumin

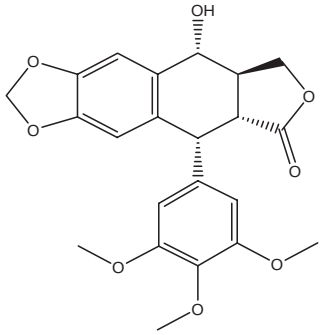
The rhizomes of the plant present in the genus Curcuma have been traditionally used as a coloring agent in food, cosmetics, and textiles. *Curcuma amada* Roxb., generally known as mango ginger, is one of the species with rhizomes having the trademark smell of raw mango. A basic HPTLC technique for a quick examination of the major curcuminoids in *Curcuma longa* and *C. amada* was performed. The strategy was found reasonable for a quick screening of the plant material for a genotypic assessment and can be performed with no uncommon special sample pretreatment. Chromatography was performed on preactivated silica gel plates 60F254. Samples and standards were applied on the plates as bands of 6-mm width. The TLC plates were developed with mobile-phase chloroform:methanol (95:5) for a height of around 8 cm. Peaks of curcumin (9), demethoxy curcumin, and bis-demethoxy curcumin were found at R_f 0.69, 0.44, and 0.29, respectively (Gupta et al., 1999).



Curcumin (**9**)

9.4.5 Determination of Podophyllotoxin

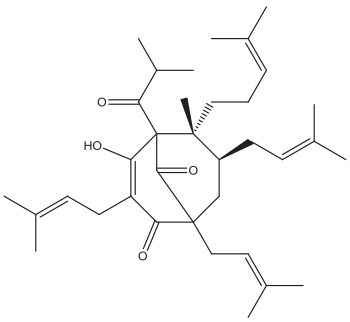
Podophyllum hexandrum (Berberidaceae) is an herbaceous, rhizomatous type of species with immense medicinal importance. The rhizomes of *P. hexandrum* yielded cytotoxic lignin podophyllotoxin (10) and gum, because of which podophyllum has antitumor activity. Besides, vital medications used as a part of the treatment schedule of testicular and small-cell lung tumor, specifically etoposide and teniposide, are developed by semisynthesis from the plant lignin podophyllotoxin. HPTLC investigation was performed by applying bands of width 6 mm on RP18 F254TLC plates and developed utilizing the mobile phase acetonitrile:water (50:50, v/v). Densitometric scanning was performed in the absorbance—reflectance mode at 217 nm. The location for podophyllotoxin was obtained by comparing the R_f values and spectra of the sample with those of the standard podophyllotoxin (Mishra et al., 2005).



Podophyllotoxin (**10**)

9.4.6 Determination of Hyperforin in *Hypericum perforatum*

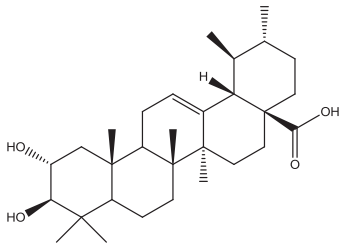
H. perforatum (St. John's Wort) has been broadly used as an antiinflammatory and healing agent in traditional medicine. Hypericin, flavonoids, and hyperforin (11) are the constituents responsible for the antidepressant activity of the plant. A quantity of 6 mg extract of *H. perforatum* was accurately weighed and extracted with methanol by vortexing. The extract was concentrated and the final volume made up to 10 mL with methanol. Two milligrams of standard hyperforin was dissolved in 10 mL of methanol and a calibration curve from 0.2 to 2 mg was developed and checked for reproducibility, linearity, and for validating the method. Silica gel 60F254 plates were utilized with petroleum ether:ethyl acetate (90:10, v/v) as a mobile phase. Scanning of the plates was performed at 290 nm for measurement (Tiwari et al., 2008).



Hyperforin (**11**)

9.4.7 Determination of Corosolic Acid

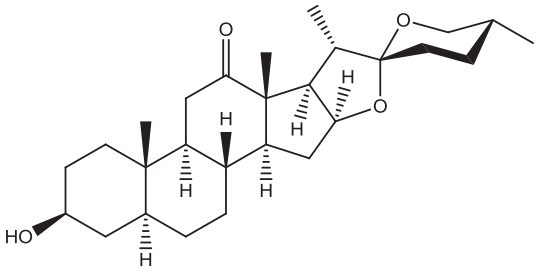
Lagerstroemia speciosa (Lythraceae), commonly known as "Banaba," is an ornamental plant and is generally used for the prevention and treatment of diabetes. Corosolic acid (12), the bioactive marker compound of Banaba extracts showed potential antidiabetic properties. To evaluate the amount of corosolic acid in *L. speciosa* leave extracts, aliquots of 10 mL were utilized for HPTLC and plates were developed to a distance of 8 cm in a chloroform:methanol (9:1, v/v) mobile phase. The plates were scanned at 20 nm (Vijaykumar et al., 2006).



Corosolic acid (**12**)

9.4.8 Determination of Hecogenin from *Agave americana*

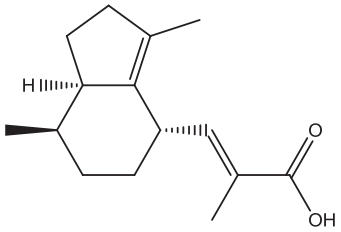
A TLC densitometric strategy for the measurement of hecogenin from the leaves of *A. americana* was performed utilizing HPTLC. The strategy was validated for precision, repeatability, and accuracy. The method was found to be precise with RSD of 0.78 (intraday) and 0.82 (interday) for various concentrations of hecogenin (13). The amount of hecogenin in various samples was evaluated by the reported technique and was observed to be in the range of 0.05%–0.14% (w/w) in the samples investigated. The accuracy of the method was verified by conducting recovery studies at three different levels for hecogenin and the average percentage recovery was 98.98%, 101.92%, and 103.33%, respectively. The TLC densitometry technique created for the evaluation of hecogenin was observed to be simple, precise, specific, sensitive, and accurate and can be utilized as a part of regular quality control (Ghogari and Rajani, 2006).



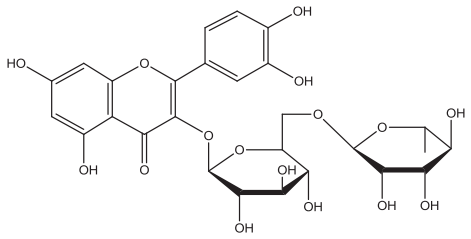
Hecogenin (**13**)

9.4.9 Quantification of Valerenic Acid in *Valeriana jatamansi* and *Valeriana officinalis*

A straightforward, fast, inexpensive, and accurate high-performance thin-layer chromatographic method has been reported for the measurement of valerenic acid in *V. jatamansi* and *V. officinalis*, which is one of the stable compounds of *V. officinalis* and is assigned as a key marker compound. Valerenic acid (14) makes a significant contribution to the narcotic and spasmolytic action of the essential oil and extract of *V. officinalis*. Separation and quantification was accomplished by HPTLC utilizing a ternary mobile phase of hexane:ethyl acetate:acetic acid (80:20:0.5, v/v) on precoated silica gel 60F254 aluminum plates and densitometric scanning was performed after derivatization with anisaldehyde–sulfuric acid reagent at 700 nm, in absorption reflectance mode. The calibration curves were found to be linear in the range 25–500 ng (Singh et al., 2006).



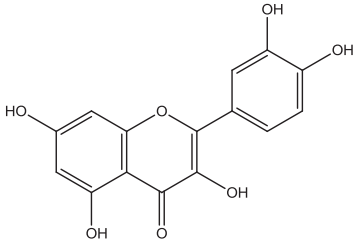
Valerenic acid (**14**)



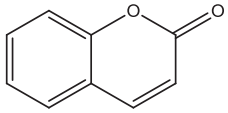
Rutin (**15**)

9.4.10 Analysis of Quercetin, Rutin, and Coumaric Acid in Flowers of *Rhododendron*

An analysis of quercetin, rutin, and coumaric acid in the flowers of *Rhododendron arboreum* using HPTLC was developed for simultaneous quantitative estimation of three biologically active phenolic compounds, namely, quercetin (16), rutin, and coumaric acid (17) in flowers of *R. arboreum* using HPTLC. The separation was acquired on TLC aluminum plates precoated with silica gel RP-18 F254S. Optimum separation was accomplished in the mobile phase of methanol–water–formic acid (40:57:3, v/v/v) and densitometric determination of these compounds was carried out at 280 nm in reflectance/absorbance mode. The accuracy of the method was verified by a recovery study conducted at two different levels with an average recovery of 99.90%, 99.02%, and 99.16% for quercetin, rutin, and coumaric acid, respectively (Swaroop et al., 2005).



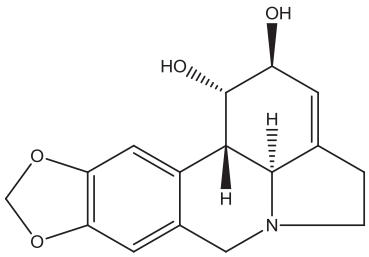
Quercetin (**16**)



Coumaric acid (**17**)

9.4.11 Determination of Lycorine in Amaryllidaceae Plant Extracts

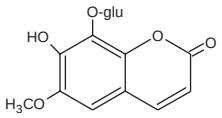
Lycorine (18) is the most common amaryllidaceae alkaloid and has a wide range of bioactivities, including antiviral, cytotoxic, antimalarial, and antiinflammatory. The mobile phase was comprised of chloroform:methanol (9:1, v/v). An ascending development of the plates was performed. The plates were developed to a height of 7 cm from the origin. The run time was 11 min. After development, the plates were dried in air for 5 min. Densitometric scanning was done on a TLC scanner in the reflectance–fluorescence mode at 368 nm (Abou-Donia et al., 2007).



Lycorine (**18**)

9.4.12 Quantification of Fraxin in *Fraxinus excelsior*

Sometimes the separation of phytoconstituents by HPLC and HPTLC may cause some confusion for the analysis of the exact components present in them. Leaf extracts from *F. excelsior* are especially used for the treatment of rheumatic diseases and may owe their ethnopharmacological reputation to some diuretic and antiinflammatory properties. For HPTLC separation, similar conditions were employed to those used in HPLC. Migration was performed at room temperature on precoated HPTLC plates (RP 18 WF 254 s; $10 \times 20\,cm^2$). Plates were developed to a distance of 7 cm (from the concentrating zone) with the following solvent system: phosphoric acid (0.4%, v/v) and acetonitrile (60:40, v/v) in an unsaturated chamber. After development, the plate was dried for 60 min in a stream of cold air.

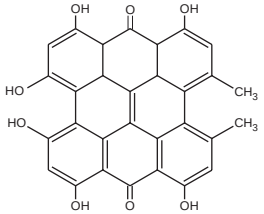


Fraxin (**19**)

A chromatographic system previously described for the quantitative evaluation of fraxin (**19**) was tested using HPTLC silica gel and as mobile phase: ethylacetate–2-butanone–water–formic acid (5:3:2:1, v/v/v/v). The "Partridge phase" was also tested on these plates: butanol–water–acetic acid (40:50:10, v/v/v). Unfortunately, the separation was incomplete and the quantitative results were higher than those obtained by HPLC. For this reason, it was decided to use eluent conditions similar to those for HPTLC and this gave results comparable to those for HPLC. Acetic acid (10%, v/v) was also tested as a mobile phase on HPTLC RP 18 plates (Mukherjee, 2002).

9.4.13 Quantification of Hypericin in *Valena N* Extract with St. John's Wort

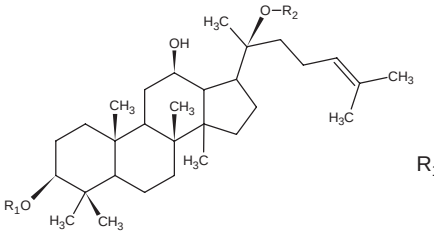
Hypericin (**20**) is a secondary metabolite produced by St. John's Wort. It shows a photo-dynamic effect, which can cause skin irritation. Nevertheless, as a dilution (1:1000), it is used for tonic purposes (improves cell respiration) and is recommended for developmental disturbance, delayed convalescence, anemia, and diabetes. The amount of hypericin content is strongly dependent on the source from which the plant is derived. This results in a certain range of expected or permitted amount of hypericin. To ensure the amount of hypericin, and to detect possible adulteration, hypericin has to be monitored. After application, the plates were developed in a horizontal developing chamber (HDC) of $20 \times 10\,cm^2$ with ethyl acetate–formic acid–water 20:2:1; running distance 50–70 mm. After proper development, the plates were scanned for the presence of hypericin in the extract (CAMAG, 1996).



Hypericin (**20**)

9.4.14 Quantification of Commercial Ginseng Medicine

The roots of Ginseng have held the esteem of the Chinese as a "cure-all" medicinal herb for thousands of years. It occurs nowadays in single- or multicomponent pills, tablets, capsules, oral liquid, and even cosmetics, in addition to the crude drug itself. Commercial Ginseng is classified into white ginseng (dried naturally) and red ginseng (steam-processed) (*Panax ginseng*, family: Araliaceae), produced mainly in China and Korea (it can therefore be called "Asian ginseng"). American ginseng (*P. quinquifolium*) is exported from the eastern United States and Canada via Hong Kong. Notoginseng (Sachi) (*P. notoginseng*) is a native of southwest China. A booming market in Asian Ginseng, American Ginseng, and various kinds of their preparations in recent years has presented analysts a task for quality control using an effective, rapid, and economic analysis method. As a routine drug control, TLC/HPTLC undoubtedly meets the requirements and the fingerprint differentiation further shows the potential of TLC from the viewpoint of methodology.



Ginsenoside (**21**)

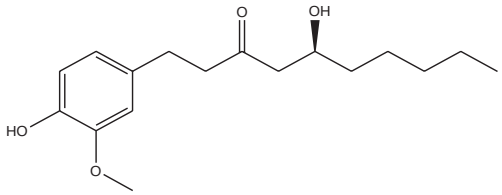
R_1, R_2 = Glucose Units

HPTLC measurements of Asian ginseng, American ginseng, Notoginseng (Sanchi), and some of their preparations have been reported by Xie and Yan (1987a, b). The feasibility of HPTLC fingerprint identification has revealed reliable experimental data and reproducible chromatograms. It has been reported that upon analysis of more than hundreds of specimens of commercial radix Asian Ginseng (*Panax ginseng*) and American Ginseng (*P. quinquifolium*), as a whole, the HPTLC patterns of Ginseng are always simpler than those of Asian Ginseng. The fluorescence intensity of the main ginsenosides spots is much stronger than the minor saponin spots. In contrast with American Ginseng, the minor ginsenosides in Asian Ginseng (red ginseng in particular) are easier to observe and the patterns are therefore more complicated. To optimize the condition of HPTLC of ginsenosides (**21**) it has been reported that the solvent system, chloroform–ethyl acetate–methanol–water (15/40/22/10), left to stand overnight at 8–10°C (lower phase), has a higher resolution, better reproducibility of R_f values and more impact spots by comparison with the solvent systems established by the previous investigators and in common use. Detection and scanning in fluorescence mode after visualization with 5% sulfuric acid/EtOH reagent by dipping technique improved and enhanced the sensitivity over that seen in absorbance mode, which is the most commonly used (Xie and Yan, 1987a). A sample pretreatment through an adsorption clean-up step via a small basic alumina column followed by 1-butanol extraction, instead of only a butanol-extraction step, made the chromatogram clearer, with less background contamination, and reduced the trailing of some ginsenosides spots. The experimental data demonstrated that the RH has a significant influence on the chromatographic behavior of ginsenosides. The optimum RH for preequilibration of the precoated HPTLC plate (Merck) is 42%–47% and the optimum temperature of development is 25–28°C (Xie and Yan, 1987a).

9.4.15 Determination of 6-Gingerol in *Zingiber officinale*

Z. officinale (Fam. Zingiberaceae), commonly known as ginger, is very widely used in foods as a spice globally. It has been used as an important ingredient in the Chinese, Ayurvedic, and Tibb-Unani systems of medicine. Ginger contains a number of different pungent and active ingredients. The major pungent compounds found in ginger are the gingerols. 6-Gingerol (**22**) is one of the major bioactive principles of ginger and has been shown to have a number of pharmacological activities, such as antipyretic, antitussive, hypotensive, cardiotonic, antiplatelet, antiangiogenic, antiinflammatory, analgesic, cytotoxic, apoptotic, antitumor, anticancer, antioxidant, antihepatotoxic, antifungal, and antiemetic activities. An HPTLC method has been developed for the quantification of 6-gingerol in methanolic extract of *Z. officinale* rhizomes. The optimized mobile phase was found to be *n*-hexane; diethyl ether (40:60, v/v). The R_f value of 6-gingerol was found to be 0.40. The calibration plot was linear in the range of 250–1200 ng of 6-gingerol and the correlation coefficient was 0.9997, which indicates the good linear dependence of peak area on concentration. The quantity of 6-gingerol

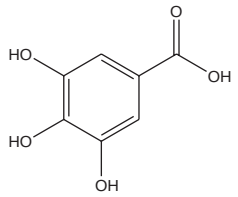
was found to be 60.44 mg/g of plant extract. The proposed HPTLC method for quantitative monitoring of 6-gingerol in ginger may be used for routine quality testing of ginger extracts (Rai et al., 2006).



6-Gingerol (**22**)

9.4.16 Determination of Gallic Acid in "Triphala"

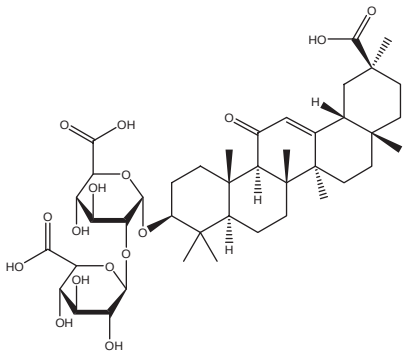
"Triphala" is a well-known polyherbal formulation from Ayurveda in India. It is a Rasayana drug that has been used in the Indian System of Medicine (ISM) for many years. This is a well-known formulation made in combination with an equal amount of the fruit of *Terminalia chebula*, *Terminalia bellirica*, and *Emblica officinalis* based on the observation of the Ayurvedic Formulary of India (AFI). Triphala is most commonly used as a laxative, detoxifying agent, and rejuvenator. The individual herbs of the triphala formulation have several potential therapeutic activities. The Triphala formulation has been standardized with HPTLC methods based on fingerprint profiling of individual components in the formulation by using gallic acid (**23**) as a marker compound. Methanol extracts of Triphala, containing, *E. officinalis*, *T. chebula*, and *T. bellirica*, were used for HPTLC on silica gel plates. The R_f of gallic acid was found to be 0.80 with densitometric scanning at 254 nm and the calibration plot was linear in the range of 400–1800 ng of gallic acid. The correlation coefficient, 0.999, was indicative of good linear dependence of peak area on concentration. The gallic acid content in methanol extracts of Triphala, with its individual constituents, *E. officinalis*, *T. chebula*, and *T. bellirica,* was found to be 14.38, 17.50, 16.60, and 11.92 mg/g. The HPTLC method for quantitative monitoring of gallic acid in Triphala and its constituents can be used for routine quality testing and similar methods can be developed for other herbal formulations (Mukherjee et al., 2008).



Gallic acid (**23**)

9.4.17 Determination Glycyrrhizin in *Glycyrrhiza glabra*

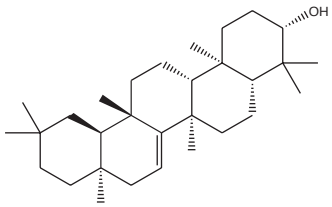
G. glabra (Fam. Fabaceae) consists of peeled or unpeeled roots and stolons and is commonly known as licorice. It has several therapeutic benefits and is mostly used as a demulcent, expectorant, antiallergy, antiinflammatory, spasmolytic, mild laxative, antistress, antidepressive, antiulcer, liver protector, and antidiabetic. It is also used to treat bronchitis, dry cough, respiratory infections, genitourinary diseases, urinary tract infections, abdominal pain, gastric and duodenal ulcers, and mouth ulcers. A simple HPTLC densitometric method for the quantification of glycyrrhizin (**24**) from the *G. glabra* was developed. The method was validated for precision, repeatability, and accuracy. The optimized mobile phase was chloroform:methanol:water (65:36:7.5, v/v/v). Extract and standard were dissolved in 70% methanol and applied on a precoated HPTLC plate. After development, the plate was scanned at 254 nm to develop a chromatogram, then the quantity of glycyrrhizin was determined in the extract. The method was validated in terms of specificity, linearity, precision, LOD, and LOQ. The amount of glycyrrhizin in the extract was found to be 9.1% (w/w). The developed method provides a rapid and cost-effective quality measure for *G. glabra* hydroalcoholic extract (Gantait et al., 2010a, b).



Glycyrrhizin (**24**)

9.4.18 Determination of Taraxerol From *Coccinia grandis*

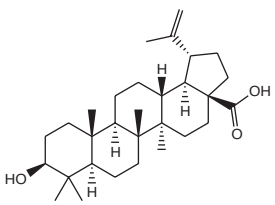
C. grandis (Fam. Cucurbitaceae) is mostly used in the Indian system of medicine for various skin diseases, bronchial catarrh, and bronchitis. It has been traditionally used as a carminative, antipyretic, galactagogue, antiemetic, antispasmodic, and expectorant. A simple, rapid, cost-effective, and accurate high-performance thin-layer chromatographic method has been developed for the quantification of taraxerol in *C. grandis*. Chromatograms of taraxerol (**25**) standard and *C. grandis* extract were developed on silica gel with optimized mobile phase hexane:ethyl acetate (9:1, v/v). HPTLC plates were scanned at 540 nm after spraying with anisaldehyde–sulfuric acid reagent and subsequent heating. The developed method was validated for accuracy, precision, and specificity. The amount of taraxerol in the *C. grandis* extract was found to be 0.1% (w/w) (Gantait et al., 2010a, b).



Taraxerol (**25**)

9.4.19 Determination of Betulinic Acid in *Nelumbo nucifera*

A simple, rapid, and accurate HPTLC method has been developed and validated for the quantification of betulinic acid (**26**) in a hydro-alcoholic extract of *N. nucifera* rhizome. The separation was carried out on a TLC aluminum plate precoated with silica gel and the optimized mobile phase was chloroform:methanol:formic acid (49:1:1, v/v/v). Post chromatographic derivatization was made with anisaldehyde–sulfuric acid reagent and densitometric scanning was performed using a Camag TLC scanner III, at 420 nm. The system was found to produce a sharp peak of betulinic acid at an R_f value of 0.30. The percentage of recovery was found to be 98.36%. This HPTLC method provides a new and powerful standardization method for the quantification of betulinic acid as a biomarker in *N. nucifera* extract (Mukherjee et al., 2010).



Betulinic acid (**26**)

9.4.20 Determination of Taraxerol in *Clitoria ternatea*

A simple, rapid, and accurate HPTLC method has been developed and validated for the quantification of taraxerol (**25**) in *C. ternatea* extract. Linear ascending development was carried out in a twin-trough glass chamber with saturated optimized solvent system hexane and ethyl acetate (80:20, v/v). The HPTLC plate was dried and derivatized with sprayed-on anisaldehyde reagent. A Camag TLC scanner III was used for spectrodensitometric scanning and analysis at 420 nm. A compact and sharp peak for taraxerol appeared at an R_f value of 0.53. The calibration plot was linear in the range of 100–1200 ng of taraxerol with a correlation coefficient of 0.9961. The concentration of taraxerol in a hydroalcoholic extract of *C. ternatea* root was found to be 12.4 mg/g (w/w). The method was validated for accuracy and precision and recovery studies were performed. This method for quantitative monitoring of taraxerol in *C. ternatea* can be used for routine quality testing of this plant and its extract used in different formulations (Kumar et al., 2008).

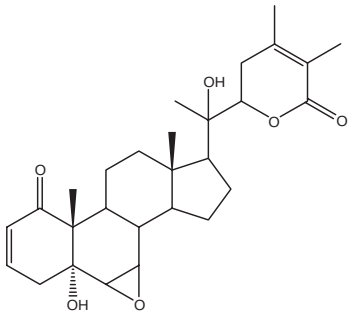
HPTLC standardization parameters for some herbal drugs have been summarized in Table 9.2, with respect to the solvent, mobile phase, and marker compounds in individual HPTLC method development.

9.5 CASE STUDIES OF HPTLC WITH SOME SPECIFIC MEDICINAL PLANTS

This section provides some case studies of HPTLC analysis with specific medicinal plant extracts, which has been performed in my laboratory at Jadavpur University, Kolkata, India. I would like to thank all my research team members and acknowledge their contributions for developing the HPTLC profiles of those medicinal plants in this section. I would also like to acknowledge the support from Anchrom Enterprises, Mulund (East), Mumbai for maintaining the CAMAG HPTLC instrument setup at my laboratory.

9.5.1 *Withania somnifera*

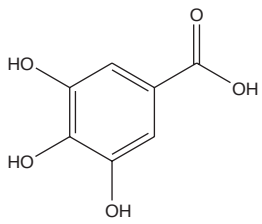
HPTLC fingerprint analysis of *W. somnifera* root extract is shown in Table 9.3 and Figs. 9.4 and 9.5.



Withanolide A (27)

9.5.2 *Eugenia jambolana*

HPTLC fingerprint analysis of *E. jambolana* seed extract is shown in Table 9.4 and Figs. 9.6 and 9.7.



Gallic acid (28)

TABLE 9.2 HPTLC Standardization of Some Important Herbal Medicines

Plant Name (Family)	Part Used	Extraction With Solvent	Mobile Phase	Marker Compound	Reference
Acacia salicina, *Acacia laeta*, *Acacia hamulosa*, and *Acacia tortilis* (Fabaceae)	Leaves	Ethanol	Acetonitrile:water (6:4, v/v)	Rutin	Alam et al. (2017)
Acalypha fruticosa (Euphorbiaceae)	Aerial part	Chloroform, methanol	Chloroform, methanol, and glacial acetic acid (17:3:0.5, v/v/v)	2-Methyl-5,7-dihydroxychro mone 5-*O*-b-D-glucopyranoside	Al-Taweel et al. (2017)
Aerva lanata (Amaranthaceae)	Aerial part	Ethanol	Chloroform:methanol:water (70:30:4, v/v/v)		Zhao et al. (2015)
Aloe barbadensis (Asphodelaceae)	Leaves	Ethanol	Ethyl formate:methanol:water (100:14.5:10, v/v/v)	Aloin	Coran et al. (2011)
Aloe ferox (Asphodelaceae)	Leaves	Ethanol	Ethyl formate:methanol:water (100:14.5:10, v/v/v)	Aloin	Coran et al. (2011)
Aloe vera (Asphodelaceae)	Gel	Water	*n*-Butanol:*n*-propanol:glacial acetic acid:water (30:10:10:10, v/v/v/v)	Aloeverose	Lobo et al. (2010)
Aloe vera (Xanthorrhoeaceae)	Leaf skin	Methanol	Ethyl acetate:methanol:water (100:16.5:13.5)	Barbaloin	Pandey et al. (2016a)
Andrographis paniculata (Acanthaceae)	Dried leaves	Methanol	Chloroform:methanol (8:2)	Andrographolide	Akowuah et al. (2006)
Asparagus racemosus (Asparagaceae), *Withania somnifera* (Solanaceae), *Vitex negundo* (Lamiaceae), *Plumbago zeylanica* (Plumbaginaceae), *Butea monosperma* (Legumes), and *Tephrosia purpurea* (Legumes)	Roots	Soxhlet extract with water, methanol:water (1:1, v:v) mixture, methanol, and ethyl acetate	Ethyl acetate:methanol:formic acid:water (20:2.5:0.5:2, v/v)	Quercetin, rutin, luteolin, and vitexin	Nile and Park (2014)
Astragalus spinosus five different samples (Fabaceae)	Aerial part, root	Successively extracted with light petroleum followed by methylene chloride, ethyl acetate, and finally *n*-butanol	Chloroform:methanol (17:2, v/v)	Astragalosides I, II, and IV	Shawky and Selim (2017)
Bryophyllum pinnatum (Crassulaceae)	Aerial parts (leaf and stem)	Petroleum ether	Chloroform:ethanol (9.8:0.2, v/v)	Stigmasterol	Kamboj and Saluja (2017)

Continued

TABLE 9.2 HPTLC Standardization of Some Important Herbal Medicines—cont'd

Plant Name (Family)	Part Used	Extraction With Solvent	Mobile Phase	Marker Compound	Reference
Calendula officinalis (Asteraceae)	Flower	Soxhlet extract with ethanol	Gradient mobile phase used for HPTLC hexane:ethyl acetate:water (0:80:20; 66:34:0; 100:0:0)	Rutin, chlorogenic acid, caffeic acid, and faradiol	Loescher et al. (2014)
Calendula officinalis (Asteraceae)	Aerial parts	Methanol	Ethyl acetate:formic acid:glacial acetic acid:water (100:11:11:26, v/v/v/v)	Rutin	Munoz et al. (2011)
Caulophyllum thalictroides (Berberidaceae)	Roots and rhizomes	Methanol	Chloroform:methanol:water (65:35:10.5, v/v/v)	Magnoflorine	Avula et al. (2011)
Citrullus lanatus (Cucurbitaceae)	Seed	Ethanol	Ethyl acetate:butanone:formic acid:water (5:3:1:1, v/v)	Quercetin	Varghese et al. (2013)
Citrus limetta, *Citrus sinensis*, and *Citrus paradise* (Rutaceae)	Peel	Methanol	Ethyl acetate:methanol:water (15:3:2, v/v)	Hesperidin	Alam et al. (2014)
Clitoria ternatea	Root	Hydroalcoholic	Hexane and ethyl acetate (80:20, v/v)	Taraxerol	Kumar et al. (2008)
Clitoria ternatea (Fabaceae)	Root	Methanol	Hexane and ethyl acetate (80:20, v/v)	Taraxerol	Kumar et al. (2008)
Coccinia grandis (Cucurbitaceae)	Leaf	Methanol	Hexane:ethyl acetate (9:1, v/v)	Taraxerol	Gantait et al. (2010a, b)
Crocus sativus L. (Iridaceae)	Grounded dried stigma	Ethanol	*n*-Butanol:water:acetic acid (4:1:1)	Crocin, picrocrocin, and crocetin	Kabiri et al. (2017)
Curcuma longa (Zingiberaceae), *Silybum marianum* (Daisy)	Plant material	Water, ethanol	Toluene:ethyl acetate:formic acid (9:6:0.4)	Curcumin, silibinin	Taha et al. (2015)
Cyperus rotundus (Cyperaceae)	Rhizomes	Acetone	Hexane–ethyl acetate (30:70, v/v)	Solavetivone, aristolone, and nootkatone	Rani and Padmakumari (2012)
Diplazium esculentum (Athyriaceae)	Fern	Ethanol	Ethyl acetate:formic acid:glacial acetic acid:water (10:0.5:0.5:1.3, v/v)	Quercetin	Das and Paul (2013)
Eleutherococcus giraldii, *Eleutherococcus lasiogyne* (Araliaceae)	Root	Ethanol		Eleutherosides B, E, and E1	Załuski et al. (2016)
Ficus religiosa L. (Amaryllidaceae)	Fruit	Methanol	Toluene:methanol (9:1, v/v)	Stigmasterol, Lupeol	Rathee et al. (2015)

Flueggea virosa (Euphorbiaceae)	Aerial parts	Methanol	Acetonitrile:water (4:6, v/v)	*Ent*-phyllanthidine, Rutin	Siddiqui et al. (2017)
Forty-four Ophiorrhiza accessions belonging to nine species (Rubiaceae)	Whole plant	Soxhlet extract with methanol	EtOAc:$CHCl_3$:MeOH (5.0:4.5:0.5, v/v) EtOAc:$CHCl_3$:MeOH (4.5:5.0:0.5, v/v)	Camptothecin	Rajan et al. (2013)
Galinsoga ciliate, Galinsoga parviflora (Daisy)	Whole plant	Water, ethanol	Ethyl acetate:acetic acid:formic acid:water (100:11:11:26), ethyl acetate:methanol:formic acid–water (50:3:4:6) ethyl acetate:methyl ethyl ketone:formic acid:water (30:9:3:3)	Patulitrin, quercimeritrin, quercitagetrin, caffeic, chlorogenic acids	Bazylko et al. (2015)
Glycyrrhiza glabra (Fabaceae)	Roots	Methanol	Chloroform:methanol:water (65:36:7.5, v/v)	Glycyrrhizin	Gantait et al. (2010a, b)
Harpagophytum procumbens (Pedialaceae)	Root	CO_2/ethanol (25%, w/w)	Dichloromethane:methanol (4:1, v/v)	Harpagoside	Gunther and Schmidt (2005)
Harpagophytum procumbens (Pedaliaceae)	Roots	Methanol	Dichloromethane:methanol:acetic acid (79:20:1, v/v/v)	Harpagoside	Wagner et al. (2008)
Ipomoea batatas (Convolvulaceae)	Fresh roots	Water	Ethyl acetate:methanol:acetic acid:formic acid:water (27:2:2:2:2, v/v/v/v/v)	Chlorogenic acid, 3,4-, 4,5-, and 3,5- dicaffeoylquinic acids (diCQAs)	Lebot et al. (2016)
Kava (*Piper methysticum*) (Piperaceae)	Root samples of 88 different accessions	Acetone	Hexane:dioxane (8:2, v/v)	Methysticin, dihydromethysticin, kavain, dihydrokavain, yangonin, desmethoxygangonin, flavokavins A, B, and C	Lebot et al. (2014)
Madhuca longifolia (sapotaceae)	Leaves	Ethanol	Toluene:ethyl acetate:formic acid (5:4:1, v/v)	Quercetin	Annalakshmi et al. (2013)
Mangifera indica (Anacardiaceae)	Peel and pulp	Ethanol:acetone (7:3, v/v)	Toluene:EtOAC:MeOH (7:2:1, v/v)	Mangiferin	Jyotshna et al. (2015)
Matricaria recutita (Asteraceae)	Flowers	Aqueous	Ethyl acetate:formic acid:acetic acid:water (30:1.5:1.5:3)	Apigenin 7-*O*-glucoside, luteolin 7-*O*-glucoside	Guzelmeric et al. (2015)
Nelumbo nucifera (Nymphaeaceae)	Rhizome	Hydroalcoholic	Chloroform:methanol:formic acid (49:1:1, v/v)	Betulinic acid	Mukherjee et al. (2010)
Nelumbo nucifera (Nelumbonaceae)	Rhizome	Methanol	Chloroform:methanol:formic acid (49:1:1, v/v/v)	Betulinic acid	Mukherjee et al. (2010)
Passiflora foetida Linn. (Passifloraceae)	Leaf	Ethanol	Ethyl acetate:methanol:water:formic acid (50:2:3:6, v/v)	Vitexin	Shuayprom et al. (2016)
Plantago major (Plantaginaceae)	Aerial parts	Methanol	Toluene:acetone:formic acid (78:22:0.15, v/v/v)	Ursolic acid and oleanolic acid	Kartini et al. (2014)
Podophyllum hexandrum (Berberidaceae)	Root	Methanol	Dichloromethane:methanol:formic acid (9.5:0.5:0.5, v/v/v)	Podophyllotoxin etoposide	Kamal et al. (2017)

Continued

TABLE 9.2 HPTLC Standardization of Some Important Herbal Medicines—cont'd

Plant Name (Family)	Part Used	Extraction With Solvent	Mobile Phase	Marker Compound	Reference
Putranjiva roxburghii (Euphorbiaceae)	Leaf, bark	Chloroform	Toluene:chloroform (9:1, v/v)	Friedelin	Abhimanyu et al. (2017)
Rauvolfia serpentina L. *Rauvolfia tetraphylla* L. (Apocynaceae)	Root	Methanol	Toluene:ethylacetate:formic acid (7:2:1)	Reserpine, Ajmalicine	Pandey et al. (2016a)
Rauvolfia species (*R. hookeri*, *R. micrantha*, *R. serpentina*, *R. tetraphylla*, *R. verticillata*, and *R. vomitoria*) (Apocynaceae)	Root	10% ammoniacal chloroform	Hexane:acetone:methanol (6:3.5:0.5, v/v)	Reserpine	Bindu et al. (2014)
Rauwolfia tetraphylla (Apocynaceae)	Leaves	Methanol	Hexane:ethylacetate:methanol (50:40:10, v/v/v)	Reserpiline, yohimbine, isoreserpiline, 10-methoxy tetrahydroalstonine	Gupta et al. (2012)
Rhododendron arboreum and *Rhododendron campanulatum* (Ericaceae)	Leaves and flowers	Methanol	Methanol:5% formic acid in water (50:50, v/v)	Epicatechin, syringic acid	Sharma et al. (2010)
Rosa hybrid (Rosaceae)	Wood, shoots, early buds, buds before flowering, flower, leaves	EtOH:H_2O (90:10, v/v)	Acetonitrile:water:formic acid (50:50:5)	Epicatechin gallate, kaempferol-3-*O*-rutinoside, quercetin-3-oglucoside	Riffault et al. (2014)
Rosmarinus officinalis (Lamiaceae)	Aerial parts	Methanol	Toluene:ethyl formiate:formic acid (95:3:2, v/v/v)	Rosmarinic acid	Munoz et al. (2011)
Ruellia tuberose (Acanthaceae)	Root	Methanol	Chloroform:toluene:ethyl acetate (6:3:1, v/v/v)	Syringin	Chothani et al. (2012)
Salicis cortex (Salicaceae)	Barks	Methanol	Chloroform:ethanol:formic acid (50:40:6, v/v/v)	Catechin	Pobłocka-Olech and Krauze-Baranowska (2008)
Salix purpurea (Salicaceae)	Barks	Methanol	Ethyl acetate:methanol:water (77:15:8, v/v/v)	Salicin	Wagner et al. (2008)
Semecarpus anacardium (Anacardiaceae)	Seeds	Methanol	Toluene:acetone:acetic acid (7.5:2:0.5, v/v)	Tetrahydroamentoflavone	Aravind et al. (2008)
Sisymbrium irio (Brassicaceae)	Aerial parts	Ethanol	Chloroform:methanol (16:4)	B-sitosterol glucoside	Al-Massarani et al. (2017)
Stereospermum chelonoides (Bignoniaceae)	Root	Methanol	Toluene:ethyl acetate:acetic acid:formic acid (10:10:0.2:0.2, v/v)	*p*-Coumaric acid	Sumanth et al. (2013)

Terminalia chebula, *Terminalia bellirica*, and *Emblica officinalis* (Triphla formulation)	Fruit	Methanol	Toluene:glacial acetic acid:formic acid (1:2:0.1, v/v)	Gallic acid	Mukherjee et al. (2008)
Thymus vulgaris (Lamiaceae)	Whoe plant	Methanol	Methyl formate:hexane:formic acid (4:6:0.1)	Luteolin	Bazylko and Strzelecka (2007)
Thymus vulgarisi (Lamiaceae)	Aerial parts	Methanol	Toluene:ethyl acetate (97:3, v/v)	Thymol	Munoz et al. (2011)
Tinospora cordifolia (Menispermaceae)	Stem	Hydroalcoholic	Chloroform:ethyl acetate:methanol (13:8:0.3, v/v)	*N*-formylannonain, 11-hydroxymustakone, Yangambin	Bala et al. (2015)
Wattakaka volubilis (Asclepiadaceae)	Leaves	Methanol	Toluene:methanol (9:1, v/v)	Oleanolic acid	Gopal et al. (2014)
Zingiber officinale (Zingiberaceae)	Rhizomes	Methanol	*n*-Hexane:diethyl ether (40:60, v/v)	6-Gingerol	Rai et al. (2006)

TABLE 9.3 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Withania somnifera* Root Extract

Sample preparation	Take 1 g of sample material and add 10 mL methanol and macerate it for 12 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of Withanolide A in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard and test solution leaving 6 mm between the bands
Mobile phase	Chloroform and methanol (9:1), 20 mL
Chamber for development	Twin-trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	0.6% (w/w)

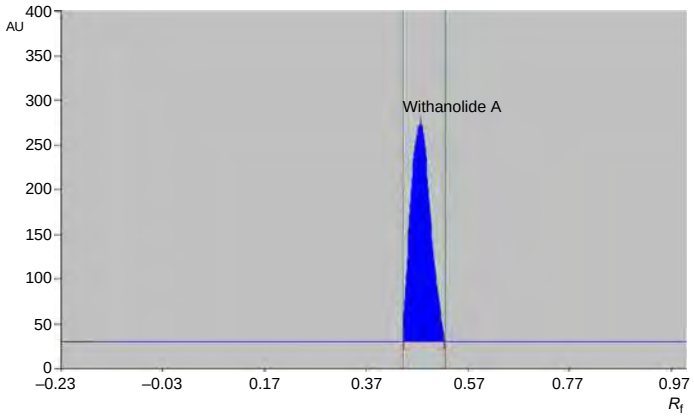


FIG. 9.4 Typical HPTLC chromatogram of standard withanolide A.

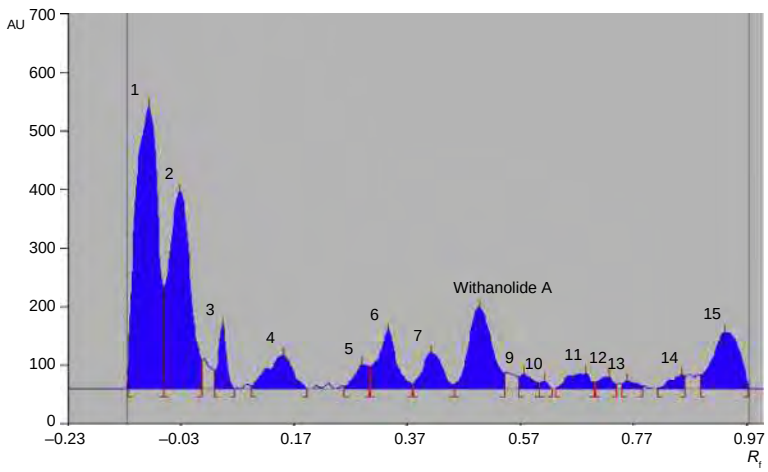


FIG. 9.5 Typical chromatogram of *Withania somnifera* extract.

TABLE 9.4 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Eugenia jambolana* Seed Extract

Sample preparation	Take 0.5 g of sample material and add 10 mL methanol and macerate it for 12 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of Gallic acid in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard and test solution, leaving 6 mm between the bands
Mobile phase	Methanol:water:acetic acid (7.6:2.3:0.1), 20 mL
Chamber for development	Twin-trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	0.61% (w/w)

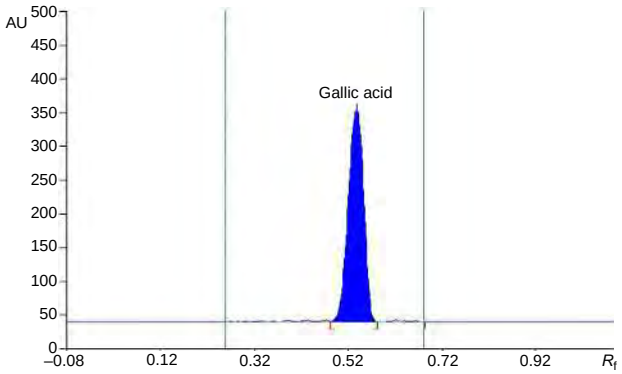


FIG. 9.6 Chromatogram of standard gallic acid.

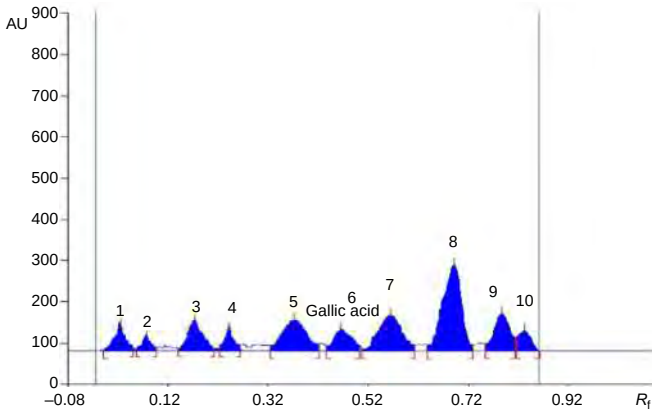
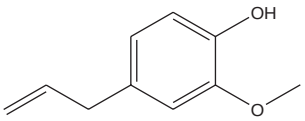


FIG. 9.7 Chromatogram of *E. jambolana* extract.

9.5.3 *Ocimum sanctum*

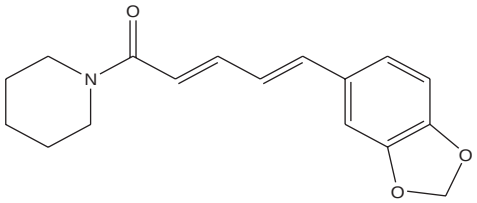
HPTLC fingerprint analysis of *O. sanctum* leaf extract is shown in Table 9.5 and Figs. 9.8 and 9.9.



Eugenol (29)

9.5.4 *Piper longum*

HPTLC fingerprint analysis of *P. longum* fruit extract is shown in Table 9.6 and Figs. 9.10 and 9.11.



Piperine (30)

TABLE 9.5 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Ocimum sanctum* Leaf Extract

Sample preparation	Take 1 g of sample material and add 10 mL methanol and macerate it for 12 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of Eugenol in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard, and test solution, leaving 6 mm between the bands
Mobile phase	Toluene:ethyl acetate:formic acid (90:10:01), 20 mL
Chamber for development	Twin-trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	0.13% (w/w)

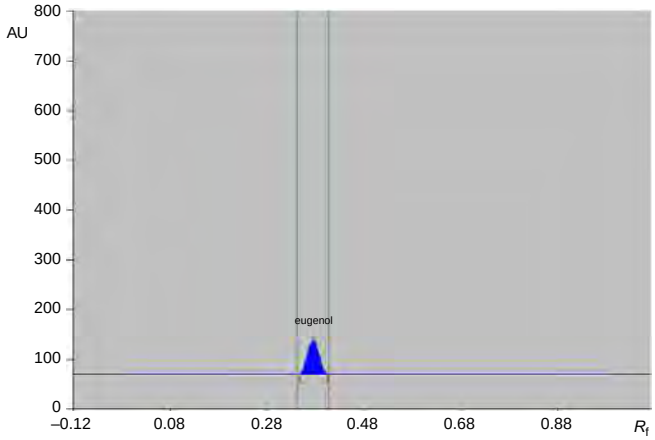


FIG. 9.8 Typical chromatogram of Eugenol.

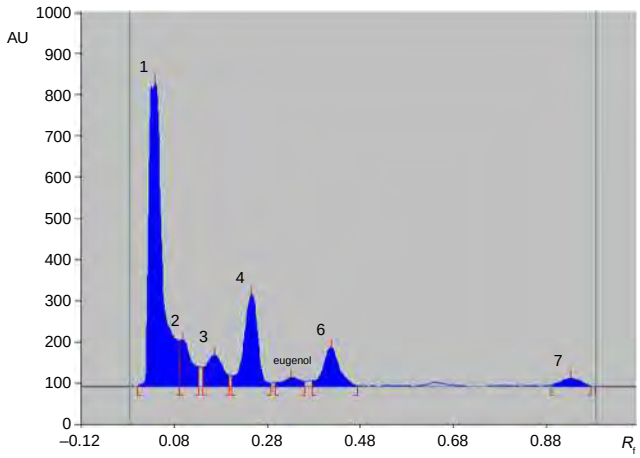


FIG. 9.9 Chromatogram of *Ocimum sanctum* extract.

TABLE 9.6 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Piper longum* Fruit Extract

Sample preparation	Take 0.5 g of sample material and add 10 mL methanol and macerate it for 12 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of Piperine in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard, and test solution, leaving 6 mm between the bands
Mobile phase	Toluene:chloroform:ethyl acetate (4:3:3), 20 mL
Chamber for development	Twin-trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	2.64% (w/w)

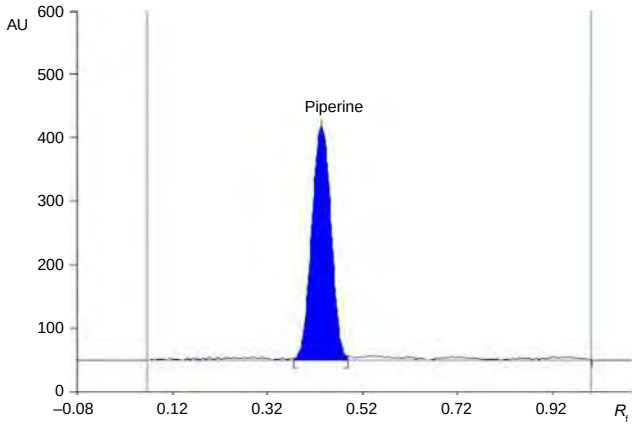


FIG. 9.10 Typical chromatogram of standard Piperine.

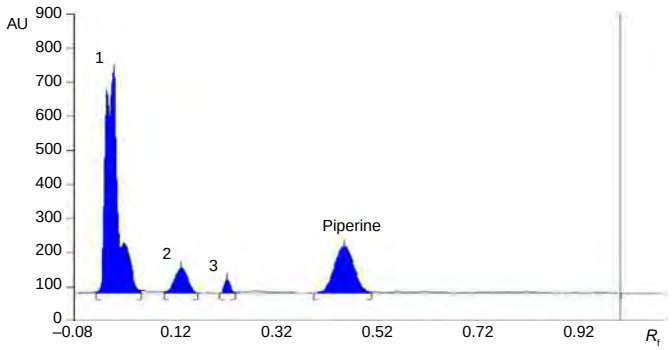
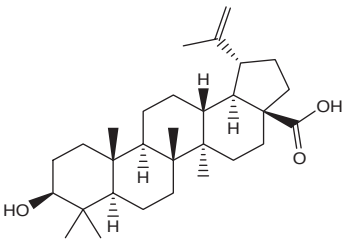


FIG. 9.11 Typical chromatogram of *Piper longum* extract.

9.5.5 *Nelumbo nucifera*

HPTLC fingerprint analysis of *N. nucifera* rhizome fruit extract is shown in Table 9.7 and Figs. 9.12 and 9.13.



Betulinic acid (31)

9.5.6 *Andrographis paniculata*

HPTLC fingerprint analysis of *A. paniculata* leaf extract is shown in Table 9.8 and Figs. 9.14 and 9.15.

TABLE 9.7 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Nelumbo nucifera* Rhizome Extract

Sample preparation	Take 1 g of sample material and add 10 mL methanol and macerate it for 6 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of betulinic acid in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard, and test solution, leaving 6 mm between the bands
Mobile phase	Chloroform:methanol:formic acid (49:1:1), 20 mL
Chamber for development	Twin-trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	0.34% (w/w)

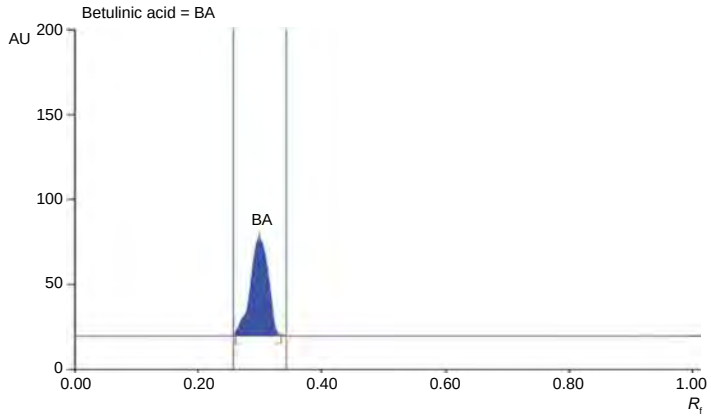


FIG. 9.12 HPTLC chromatogram of betulinic acid.

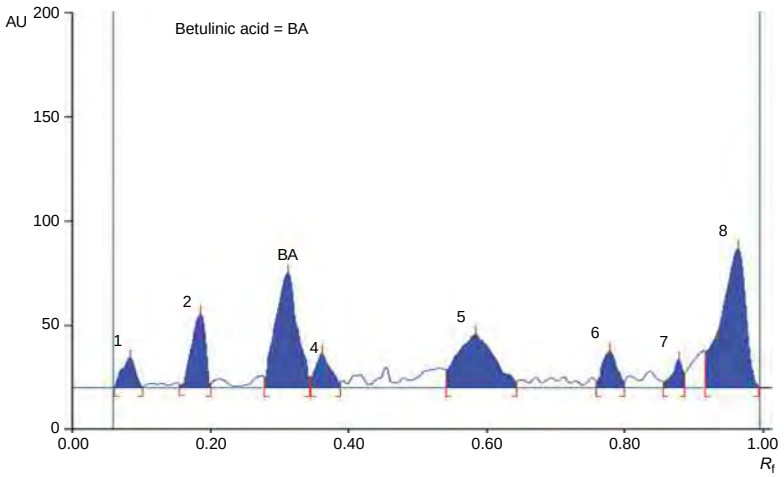


FIG. 9.13 HPTLC chromatogram of *Nelumbo nucifera* extract.

TABLE 9.8 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Andrographis paniculata* Leaf Extract

Sample preparation	Take 1 g of sample material and add 10 mL methanol and macerate it for 6 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of andrographolide in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard, and test solution, leaving 6 mm between the bands
Mobile phase	Ethyl acetate:toluene:methanol (80:10:15), 20 mL
Chamber for development	Twin-trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	3.21% (w/w)

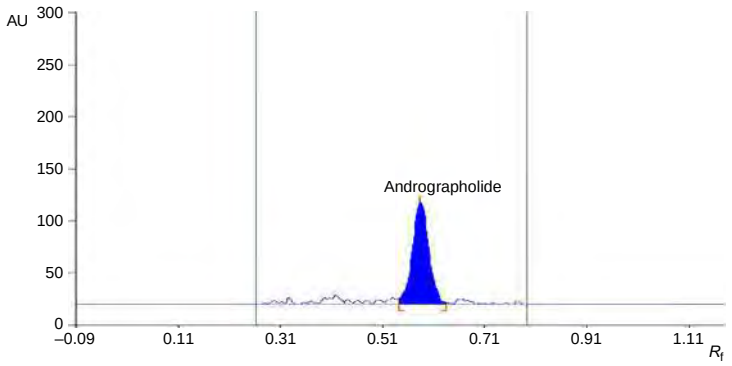


FIG. 9.14 Typical HPTLC chromatogram of andrographolide.

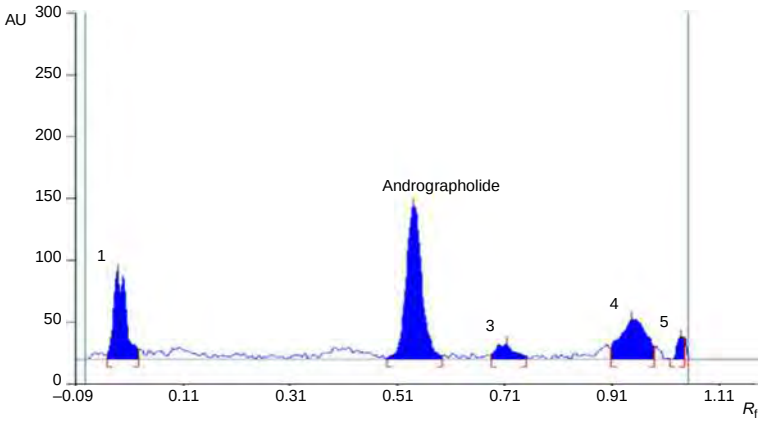
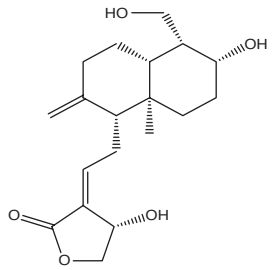


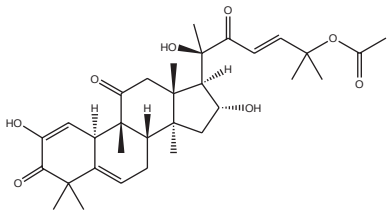
FIG. 9.15 Typical HPTLC chromatogram of *Andrographis paniculata* extract.



Andrographolide (32)

9.5.7 *Cucurbita pepo*

HPTLC fingerprint analysis of *C. pepo* fruit extract is shown in Table 9.9 and Figs. 9.16 and 9.17.



Cucurbitacin E (33)

TABLE 9.9 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Cucurbita pepo* Fruit Extract

Sample preparation	Take 2 g of sample material and add 20 mL methanol and macerate it for 6 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of Cucurbitacin E in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard, and test solution, leaving 6 mm between the bands
Mobile phase	Petroleum ether:ethyl acetate (7:3), 20 mL
Chamber for development	Twin-trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	0.18% (w/w)

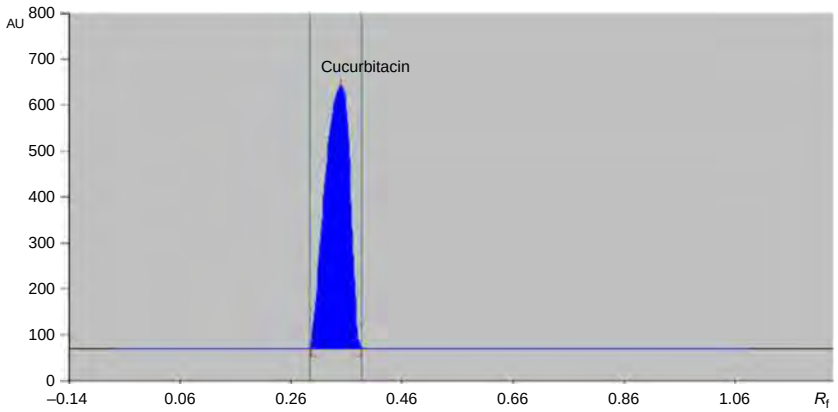


FIG. 9.16 Typical HPTLC chromatogram of standard Cucurbitacin E.

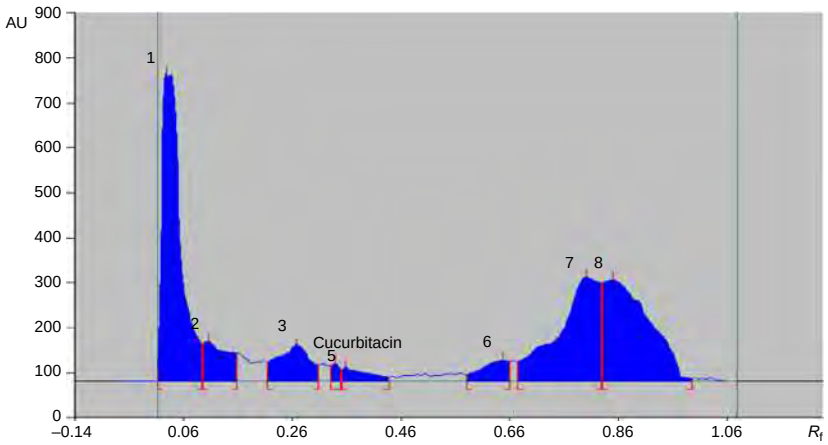
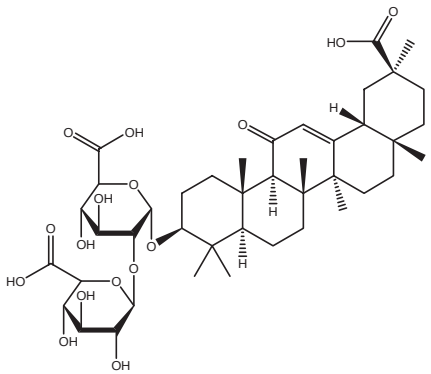


FIG. 9.17 Typical chromatogram *of Cucurbita pepo extract*.

9.5.8 *Glycyrrhiza glabra*

HPTLC fingerprint analysis of *G. glabra* root extract is shown in Table 9.10 and Figs. 9.18 and 9.19.



Glycyrrhizin (34)

TABLE 9.10 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Glycyrrhiza glabra* Root Extract

Sample preparation	Take 1 g of sample material and add 10 mL methanol and macerate it for 12 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of glycyrrhizin in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard, and test solution, leaving 6 mm between the bands
Mobile phase	*n*-Butanol:acetic acid:water (12:3:4), 20 mL
Chamber for development	Twin trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	1.78% (w/w)

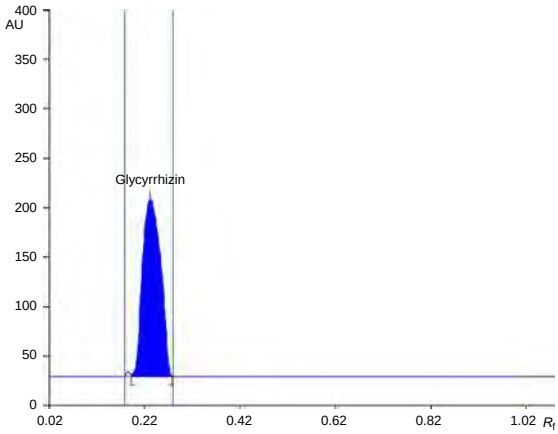


FIG. 9.18 Typical HPTLC chromatogram of glycyrrhizin.

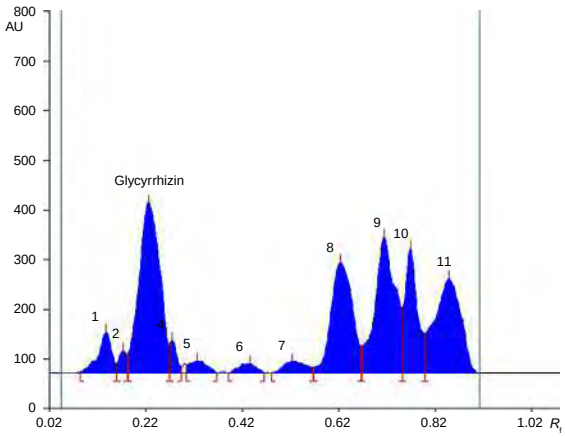
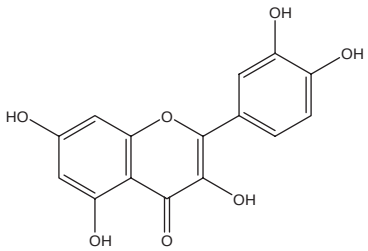


FIG. 9.19 Typical chromatogram of *Glycyrrhiza glabra* extract.

9.5.9 *Messua ferra*

HPTLC fingerprint analysis of *M. ferra* root extract is shown in Table 9.11 and Figs. 9.20 and 9.21.



Quercetin (35)

TABLE 9.11 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Messua ferra* Root Extract

Sample preparation	Take 1 g of sample material and add 10 mL methanol and macerate it for 12 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of Quercetin in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard, and test solution, leaving 6 mm between the bands
Mobile phase	*n*-Butanol:acetic acid:water (12:3:4), 20 mL
Chamber for development	Twin-trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	2.10% (w/w)

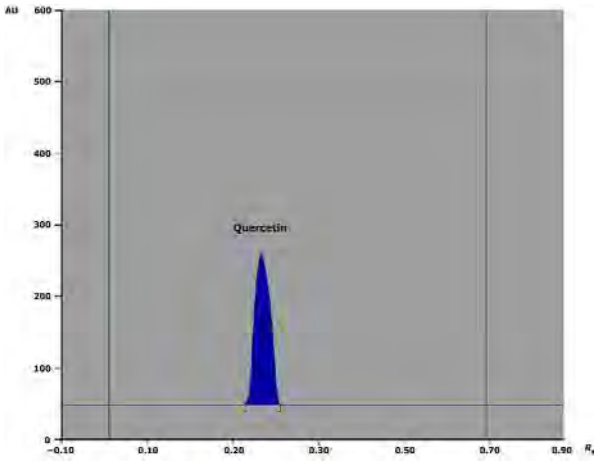


FIG. 9.20 Typical HPTLC chromatogram of Standard Quercetin.

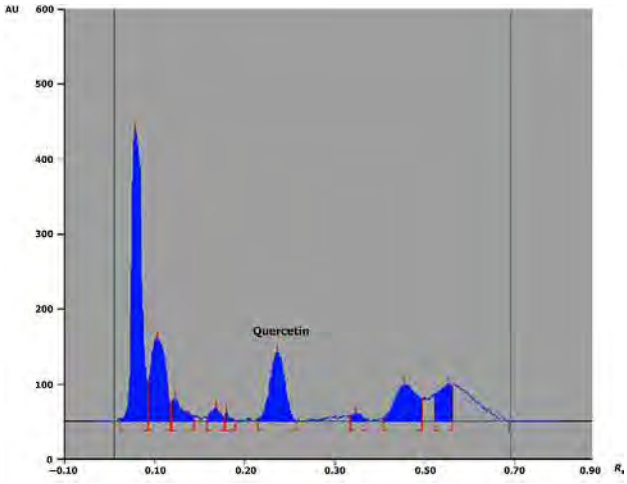
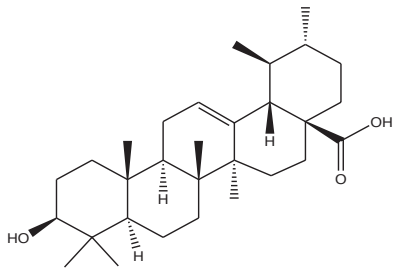


FIG. 9.21 Typical chromatogram of *Messua ferra* extract.

9.5.10 *Swertia chirata*

HPTLC fingerprint analysis of *S. chirata* leaf extract is shown in Table 9.12 and Figs. 9.22 and 9.23.



Ursolic acid (36)

TABLE 9.12 Chromatographic Conditions for HPTLC Fingerprint Analysis of *Swertia chirata* Leaf Extract

Sample preparation	Take 1 g of sample material and add 10 mL methanol and macerate it for 12 h and filter
Phytochemical references standard (PRS)	Dissolve a quantity of ursolic acid in methanol to produce a solution containing 1 mg per milliliter
Stationary phase	TLC aluminum plate precoated with silica gel 60F254
Application	Apply sample separately to the plate with bands of 2 mm, 10 µL of each test solution, phytochemical references standard, and test solution, leaving 6 mm between the bands
Mobile phase	Toluene:ethyl acetate:formic acid (7:3:0.2), 20 mL
Chamber for development	Twin-trough chamber, 10 cm × 20 cm
Conditions	Saturate the chamber with mobile phase for 30 min. The plate should be developed vertically for 8 cm
Detection	Examine in UV light at 254 nm
Amount of PRS	2.68% (w/w)

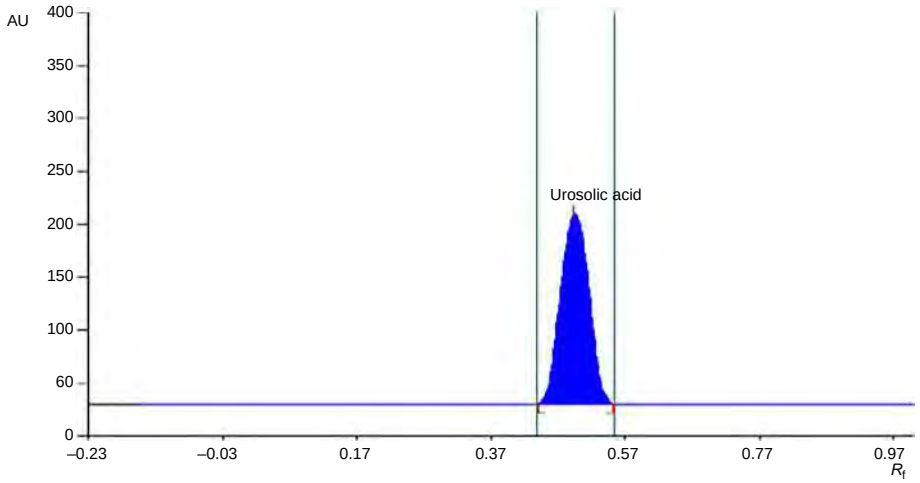


FIG. 9.22 Typical HPTLC chromatogram of ursolic acid.

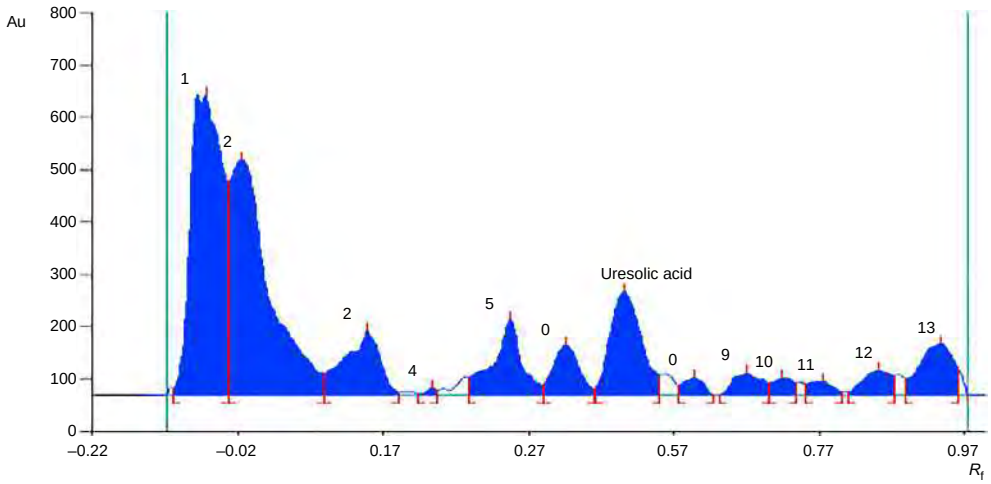


FIG. 9.23 Typical chromatogram of *Swertia chirata* extract.

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Chapter 10

High-Performance Liquid Chromatography for Analysis of Herbal Drugs

Chapter Outline

10.1 HPLC ANALYSIS FOR EVALUATION OF HERBAL DRUGS

High-performance liquid chromatography (HPLC) is a form of liquid chromatography in which the liquid mobile phase is mechanically pumped at high pressure through a column that contains the stationary phase to give fast and improved separation. Chromatography is a technique in which a mixture of components is separated on a stationary phase under the influence of a mobile phase. There have been various forms of the stationary phase over the course of time. Along with the development of different adsorbent materials, the focus has increased on analyzing particular classes of analytes by faster and more competent systems. HPLC is one of the most popular, modern, powerful, and versatile chromatographic separation techniques that have been routinely used to separate the components in a herbal extract or product. It helps to identify

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each component, to quantify separated components, and to obtain the chemical profile or fingerprint of a crude mixture (Weston and Brown, 1997). HPLC has also been used in many different ways to determine known or unknown substances in herbal products in order to assure their quality in the industry, as well as by the competent authorities. HPLC is extensively used for the analysis of food, beverages, perfumes, and other items that contain several naturally occurring secondary metabolites in the raw materials and the products derived therefrom. It is also used to detect and measure contaminants from spoilage or adulteration that may be harmful (Homans and Fuchs, 1970).

10.2 PREPARATION OF SAMPLE FOR HPLC

The basic process of preparation of samples for HPLC analysis constitutes the total dissolution of the sample in the solvent and filtration of the sample solution through microfilters (normally 0.45 µm). The nature of the result from any HPLC analysis frequently relies upon the sample preparation. The decision regarding a sample preparation method for HPLC analysis depends to a great extent on the type of sample to be evaluated and the HPLC mode utilized. For instance, if the sample substance is dissolvable in the mobile phase, a basic arrangement of sample solution is appropriate for HPLC examination. In cases in which the sample is not readily dissolvable in the mobile phase, an appropriate extraction condition should be used before HPLC examination. The selected extraction procedure ought to be quick and efficient, and it ought to incorporate the entire part of the low-molecular-weight compounds. This is generally accomplished by utilizing methanol (MeOH) or ethanol (EtOH). More fingerprints can be acquired by extraction with petroleum ether/n-hexane or chloroform (for lipophilic compounds) or water/water-acetone mixtures (for tannins, high polymeric procyanidins, and amino acids) as mobile phase. Polysaccharides and proteins can be identified using their sugar or amino acid fingerprints after enrichment and acidic or enzymatic hydrolysis. Relying on the nature of the main phytocomponent utilized in formulations or products, such as tablets and capsules, either a straightforward dissolution or an extraction step should be applied, followed by filtration, which must be utilized early in HPLC analysis. An appropriate sample preparation procedure is of special importance for the investigation of herbs and natural products, because it is important to remove the desired compound from the herbal material for further purification and characterization. Present day and traditional sample preparation procedures may incorporate at least one of the following processes:

- Solvent immersion.
- Solvent partitioning.
- Refluxing.
- Ultrasonic extraction.
- Solid-phase microextraction.
- Supercritical-fluid extraction.
- Pressurized-liquid extraction.
- Microwave-assisted extraction.
- Solid-phase extraction.
- Surfactant-mediated extraction.

Generally, a perfect sample preparation procedure ought to be safe, low cost, reproducible, fast, optimized to extract the maximum amount of the targeted analyte without compromising the structure, and able to exclude other compounds to limit any undesirable impedances (Sarker and Nahar, 2015).

10.3 NORMAL-PHASE CHROMATOGRAPHY

In normal-phase high-performance liquid chromatography (NP-HPLC), a polar stationary phase (usually silica) is employed and a nonpolar mobile phase (e.g., n-hexane and ethyl acetate) is used for separation of the analytes. In this type of chromatography, the analyte is retained based on its polarity. The rule is that the more polar the solute, the greater its retentivity. Due to the nonpolar nature of the mobile phase, an increase in the polarity of the mobile phase gives rise to decreased solute retention. The mechanism of separation is by both partition and adsorption mechanisms, but the dominant retention mechanism is adsorption. Because of this, normal-phase chromatography is also known as adsorption chromatography or liquid–solid chromatography. The polar stationary phase is characterized by the presence of hydroxyl groups (–OH); thus, if an analyte carries a permanent dipole or if it is dipole induced, then it will be attracted to the stationary phase by a dipole–dipole interaction. In normal phase chromatography, sample retention is directly proportional to the surface area of the stationary phase. It is, however, advisable to keep the surface area below 400 m^2/g, because higher surface areas can be achieved only when the pore size is small (Snyder and Kirkland, 1979).

For the isolation of hydrophobic compounds and long-chain alkanes, NP-HPLC is an appropriate method of chromatographic separation. Because of the fact that the interaction of different compounds with the stationary phase not only relies upon the functional groups present in the structure of the analyte atom, but also on its stereochemical characteristics, the impact of steric obstruction on interaction quality permits NP-HPLC strategies to determine geometric and positional isomers. Since the beginning of reversed-phase HPLC (RP-HPLC) in 1970, NP-HPLC has been superseded by RP-HPLC. The NP-HPLC mobile phase comprises a nonpolar solvent, for example, n-hexane or n-heptane, blended with a somewhat more polar solvent, for example, isopropanol, chloroform, or ethyl acetate There are four main factors, namely, solvent strength, localization, basicity, and UV cutoff, involved in the choice of solvents for NP-HPLC (Sarker and Nahar, 2015).

10.3.1 Mechanism of Retention

The major mechanism of retention in normal-phase chromatography is adsorption, as discussed previously. Two models have been proposed to describe the adsorption process. The first model is known as the "competition model," which assumes that the entire surface of the stationary phase is covered by mobile-phase molecules and that adsorption occurs due to the competition for the adsorption sites between the solute molecule and the mobile phase molecules (Snyder and Kirkland, 1979). The other model is the "solvent interaction model," which states that a bilayer of solvent molecules is formed around the stationary phase particles depending on the concentration of polar solvent in the mobile phase. In this model, retention results from the interaction of the solute molecule with this secondary layer of adsorbed mobile-phase molecules (Robards et al., 1994).

10.3.2 Stationary Phases for Normal-Phase Chromatography

The common stationary phases used in normal-phase or adsorption chromatography are porous adsorbents, such as silica and alumina, having polar hydroxyl groups on the surface. Silica is the preferred stationary phase because of its ready availability, low cost, and familiarity. However, for basic compounds, such as amines, which are very strongly retained on silica, it is advisable to use alumina. Apart from these porous adsorbents, a variety of polar-bonded phases are available in which functional groups, such as cyano [$-(CH_2)_3C{\equiv}N$], diol [$-(CH_2)_3OCH_2CH(OH)CH_2OH$], and amino groups [$-(CH_2)n NH_2$, where n is 3 or 4], are chemically bonded to the silica stationary phase (Salotto et al., 1990). These functional groups are relatively less polar than the hydroxyl group (–Si–OH) and therefore result in less retention than silica and alumina columns. Though various types of columns are available, they lack in selectivity. Thus, all compounds are eluted in the same order irrespective of the column selected, thus posing a great problem in identification (Weston and Brown, 1997). To overcome this difficulty, mobile phases need to be changed continuously.

10.3.3 Mobile Phases for Normal-Phase Chromatography

The mobile phases used in normal-phase chromatography are mainly nonpolar hydrocarbons, such as hexane, heptane, or octane, in which a small amount of a more polar solvent, such as 2-propanol, is sometimes added (Braithwaite and Smith, 1990). Solvent selectivity in normal-phase chromatography is controlled by the polarity of the solvent added. Additives having large dipole moments tend to interact mainly with solutes that have large dipole moments, such as nitro-compounds, nitriles, amines, and sulfoxides. Good proton donors, such as chloroform, m-cresol, and water, tend to interact mainly with basic solutes, such as amines and sulfoxides. On the other hand, good proton acceptors, such as alcohols, ethers, and amines, tend to interact best with hydroxylated molecules, such as acids and phenols (Weston and Brown, 1997).

10.4 REVERSED-PHASE CHROMATOGRAPHY

Reversed-phase chromatography is the most widely used HPLC in practice and used to separate analytes based on their hydrophobicity (Snyder and Kirkland, 1979). Reversed-phase chromatography (RP chromatography) is just the opposite of normal-phase chromatography. Here the stationary phase used is nonpolar, while the mobile phase is polar. Because of this, a reduction in mobile phase polarity results in a decrease in solute retention. Modern reversed-phase chromatography normally uses chemically bonded stationary phases with a functional group bonded to silica. As such, reversed-phase chromatography is also known as bonded-phase chromatography. However, in some special cases, polymeric stationary phases, such as polymethacrylate or polystyrene, or solid stationary phases, such as porous graphitic carbon, are also used.

A strong interaction occurs between the polar solvent and polar molecules in the mixture, while being passed through the column; however, there is less interaction between the hydrocarbon chains joined to the silica (the stationary phase) and

the polar moieties in the solution. As a result, polar moieties in the mixture invest a greater part of their energy moving with the solvent. Nonpolar moieties in the mixture are attracted with the hydrocarbon groups as a result of van der Waals forces. They exert less energy in solution in the solvent, and this reduces their speed when traveling through the column, thus giving rise to longer retention times. In RP-HPLC the polar molecules travel through the column more rapidly. RP-HPLC permits purification of most classes of compounds, incorporating compounds present in different herbal products, and is most commonly preferred when evaluating and trying to isolate and identify compounds in a complex mixture (Sarker and Nahar, 2015).

10.4.1 Mechanism of Retention

To describe the retention mechanism in reverse-phase chromatography, two models, namely, the "solvophobic model" and the "partitioning model," have been advocated. The solvophobic model states that the stationary phase acts like a solid on which retention of analytes occurs because of hydrophobic interactions between the analyte and the mobile phase. Due to this solvophobic effect, the analyte binds to the surface of the stationary phase, thereby reducing the surface area exposed to the mobile phase. This further increases the adsorption as the surface tension of the mobile phase increases (Miller, 2005). Thus, the retention of analytes is more due to the solvophobic interactions with the mobile phase than direct interactions with the stationary phase. On the contrary, in the partitioning model of retention, the role of the stationary phase is more important in the retention process (Weston and Brown, 1997). In this model, the analyte is thought to be fully anchored in the stationary-phase chains rather than adsorbed on the surface and therefore is considered to be partitioned between the mobile phase and a "liquid-like" stationary phase. Though the mechanism of retention on chemically bonded, nonpolar phases is still not fully clear, it is assumed that with the increase in chain length of the bonded material the retention mechanism follows a partitioning mechanism, while with shorter chain lengths the retention mechanism follows a pattern similar to the adsorption mechanism (Colin and Guiochon, 1977).

10.4.2 Stationary Phases for Reversed-Phase Chromatography

Stationary phases in reversed-phase chromatography are characterized by a functional group chemically attached to a silica support, also called bonded phases. Among the bonded phases the more popular one are the alkyl groups, such as $–CH_3$, $–C_4H_9$, $–C_8H_{17}$, and $–C_{18}H_{37}$, phenyl ($–C_6H_5$) groups, cyano [$(–CHz)_3CN$] groups, and amino [$(–CHz)_3NH_2$] groups, with retention increasing exponentially with chain length. The bonded-phase performance is affected by four factors: (1) the base silica and its pretreatment, (2) the functional group used, (3) quantity of material bonded to the silica (also called the carbon load), and (4) secondary bonding reactions (also called end capping).

The amount of carbon amalgamated into the stationary phase by the functional group is known as the carbon load and is measured as a weight percentage of the bulk silica packing. The carbon load can be varied by changing the functional group. The higher the carbon load, the greater is the retentivity of reversed-phase columns. Due to steric repulsion, all the silanol groups on the silica surface cannot react with the functional groups and usually only about 45% of the silanols take part in the reaction and are therefore bonded. Residual, unreacted, acidic silanol groups may cause retention of basic solutes, such as amines, giving rise to a mixed adsorption/partition retention mechanism. For this reason, the unreacted silanols are made inactive by treatment with a silating agent, such as trimethyl-chlorosilane ($Si(CH_3)_3C1$). This process is called end-capping. The functional group affects not only the carbon load, but also the selectivity and efficiency of the column. The functional group property controls selectivity, whereas the chain length controls column efficiency. Apart from this, few polymeric reversed-phase stationary phases are available. These have the advantage that they can be operated over a wider pH range than their silica counterparts. The disadvantage of this type of column is that they tend to be less efficient than silica-based ones and are often less retentive (Weston and Brown, 1997).

10.4.3 Mobile Phases for Reversed-Phase Chromatography

The choice of the mobile phases used in reversed-phase chromatography revolves around a polar solvent, such as water, to which a comparatively less polar solvent, such as acetonitrile or methanol, is added. Solvent selectivity can be controlled by the nature of the added solvent in the same way as discussed for normal-phase chromatography. Solvents with large dipole moments, such as methylene chloride and 1,2-dichloroethane, interact mainly with solutes that have large dipole moments, such as nitro-compounds, nitriles, amines, and sulfoxides. Solvents that are good proton donors, such as chloroform,

m-cresol, and water, interact preferentially with basic solutes, such as amines and sulfoxides, and solvents that are good proton acceptors, such as alcohols, ethers, and amines, tend to interact more with hydroxylated molecules, such as acids and phenols (Snyder and Kirkland, 1979).

10.5 FACTORS AFFECTING HPLC

HPLC is affected by four major factors. These are: (1) capacity, (2) efficiency, (3) selectivity, and (4) resolution. The capacity and selectivity of the column depend mainly on the column manufacturer, but the efficiency and resolution depend on the chromatographer to some extent. To obtain the best separation, band broadening should be minimized for optimizing the efficiency of the chromatographic system (Weston and Brown, 1997).

10.5.1 Capacity Factor

For better separation, a column should be able to retain samples and separate sample components efficiently. The capacity factor, k_R', of a column measures the strength of the interaction of the sample with the packing material according to the following equation:

$$k_R^{'} = t_R - t_o / t_o = V_R - V_o / V_o$$

where t_R corresponds to the time taken for a specific solute to reach the detector (retention time) and t_o is the time taken for nonretained species to reach the detector (holdup time). The value for k' remains the same if volumes are used in place of times: V_R is the volume of solution that travels through the detector before a specific peak is obtained (retention volume), and V_o is the volume of solvent traveling through the detector during the time of injection and the presence of the nonretained species (void volume). The void volume is the volume of the column except the packing material. The capacity factor of a column, though, depends mostly on the packing material, though it can be changed to a certain extent by varying the solvent strength. The capacity factor of the column is directly proportional to the ability to retain solutes. The resolution of a separation can be improved by using a column with higher capacity factor. But as a higher capacity factor results in longer analysis times, adjustments between resolution and analysis time must be made. Generally, a k' value between 2 and 5 is observed when there is good balance between analysis time and resolution. Still, k' values between 1 and 10 are usually acceptable.

10.5.2 Resolution

Resolution (R) signifies the degree of separation between neighboring solute bands or peaks. It depends on the selectivity (α), efficiency (N), and capacity (k') of the column. In general, an R value greater than 0.8 is adequate for accurate quantification of two peaks. A value of 1, for two equally sized peaks, occurs due to an overlap of about 2% of one band over the other. The most useful way to change resolution is to change the selectivity or the capacity factor of the column. The procedure to increase the efficiency of the column by increasing the column length or flow-rate velocity is less important, as the resolution is directly proportional to the square root of the number of theoretical plates. In this way, increasing the number of theoretical plates by incorporating a second column increases resolution by as little as a factor of 1.4, if it is desirable to have increased resolution, a column having a higher capacity factor is required. This increase in capacity factor will also increase the analysis time, so adjustments must be made between resolution and analysis time (Swartz, 2010; Weston and Brown, 1997).

10.5.3 Selectivity

The selectivity (α) of the chromatographic system depicts the difference in retention times (or volumes) between two given peaks and describes the effectiveness of a chromatographic system for the separation of two compounds.

Selectivity is usually defined in terms of α, where

$$\alpha = t_2 - t_0 / t_0 = v_2 - v_0 / v_1 - v_0 = k_2^{'} / k_1^{'}$$

The selectivity of a column mainly depends on the packing material, in spite of the fact that the chromatographer has some control using the mobile phase or temperature. The value for α can go from unity (1), in case the retention times of the two compounds are the same ($t_2 = t_1$), to infinity if the primary component of interest is eluted in the void volume.

In the event that α is approaching 1, then regardless of the number of theoretical plates or the period of time the components reside on the column, no separation will take place. The most useful way to increase the value of α is to change the ratio of the mobile phase. When changing the solvent ratio does not impart satisfactory change, changing the nature of the solvent will be sufficient (Weston and Brown, 1997).

10.5.4 Efficiency

When a sample mixture is introduced to the head of a column, the width of the column is extremely restricted. However, during component elution at the end of the column, the band widths tend to increase. This phenomenon occurs because, as the sample mixture passes through the column, different sample components interact with, and are held to different extents, by the stationary phase. This interaction, alongside the long distance traveled by the sample components through the stationary phase, causes the increase in band width, a process known as band broadening. The efficiency of a column is represented by a number that portrays peak broadening as a function of retention, and it is represented in terms of the number of theoretical plates *N*. Two main theories have been created to describe column efficiency, both of which are used as part of a modern chromatographic procedure. The plate theory, proposed by Martin and Synge, gives a straightforward and useful approach to quantify column efficiency, while the rate theory developed by van Deemter et al. provides a way to estimate the contributions to band broadening and, accordingly, to optimize the efficiency. The fundamental expressions derived in the plate theory are commonly applicable to all types of column chromatography. Despite the fact that the relationships are applicable only to Gaussian peaks, for convenience they are also used for nonsymmetrical peaks.

The main assumption in the plate theory is that a quick equilibrium is formed between the stationary phase and solvent system. The major drawback of the plate theory is that it does not include the impact of band broadening on separation, nor does it consider the impact of chromatographic factors, for example, particle size, stationary-phase loading, eluent viscosity, and flow rate, on column performance. According to the chromatographic model proposed in the plate theory, the chromatographic column is considered to include a number of thin sections or "plates," each of which enables a solute to equilibrate between the stationary and mobile phase. The greater the number of theoretical plates (*N*), the more effective the column is thought to be. The migration of a solute along the length of the column is seen as a stepwise exchange from one theoretical plate to the next. The thinner the theoretical plates, the greater the number that can be visualized inside a given length of column. These terms are connected by the relation: $H = L/N$, where *L* is the length of the column (millimeters). In this way, the smaller the height equivalent to a theoretical plate (HETP, or *H*), the greater is the efficiency of the column. As a rule of thumb, the *H* value decreases with the size of the stationary phase, low mobile-phase flow rates, less viscous mobile phases, higher separation temperatures, and smaller solute molecule sizes. The efficiency, *N*, is characterized in terms of the retention time (t_R) of the solute, estimated at the peak apex, and the standard deviation, σ, of the solute population in the peak measured as the peak width (Swartz, 2010; Weston and Brown, 1997).

10.6 INSTRUMENTAL FEATURES IN HPLC ANALYSIS

The basic components of a high-performance liquid chromatographic system consist of:

(a) Eluent containers for the mobile phase,
(b) A pump to move the eluent and sample through the system,
(c) An injection device to allow sample introduction,
(d) A column to provide solute separation,
(e) A detector to visualize the separated components,
(f) A waste container for the used solvent, and finally,
(g) A data collection device to assist in interpretation and storage of results.

The pump, injector, column, and detector are connected with tubing of narrow inner diameter. The technical features of an HPLC Instrument are explained in Fig. 10.1. The inner diameter of the tubing that is used between the injector and column and also between the column and the detector must be as narrow as possible (0.01 in. or less for analytical work) to minimize band broadening. The choice of detector is based on the intrinsic properties of the solute. Often, more than one detector can be used to maximize sample information and confirm peak identities. For example, an absorbance detector could be placed in series with a conductivity detector for the visualization of a charged, chromophoric solute.

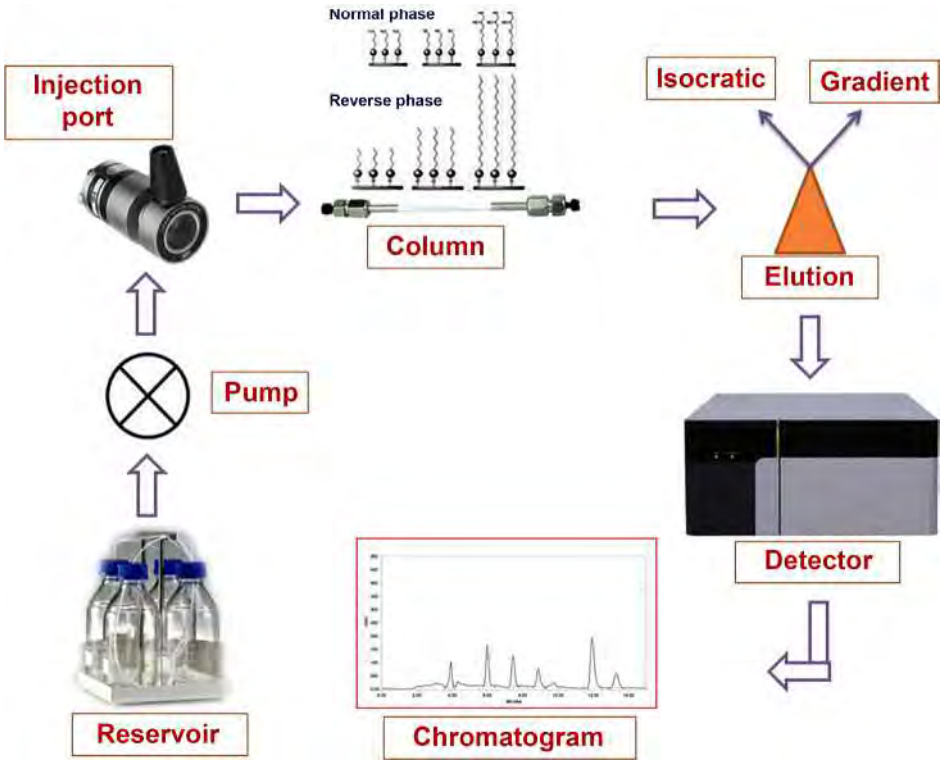


FIG. 10.1 Instrumentation of HPLC.

10.6.1 Solvent Delivery Systems

The function of the solvent delivery system is to deliver the mobile phase (eluent) through the chromatograph, accurately and reproducibly. The solvent delivery system comprises the pump, check valves, flow controllers, pulse dampeners, and pressure transducers, each one of which needs to be maintained to guarantee reproducible flow rates. The pump should be primed, reliable, and easy to maintain. It ought to be impervious to corrosion from the eluents used and be equipped to deliver the required flow rates with minimum holdup volume for fast solvent changes. Mobile phase delivery should be pulse free to guarantee minimal baseline noise from the pump.

10.6.2 Pumps

The pumping systems used in HPLC can be classified according to the mechanism by which the pump delivers the eluent. Each of these classifications is considered below.

10.6.2.1 Syringe Pumps

The syringe, or positive displacement, pump remains well known for applications requiring "pulseless" solvent delivery, for example, in capillary LC (in which usual flow rates are under 100 µL/min) or in microbore HPLC associated with an interface to mass spectrometry. The syringe pump is basically a substantial barrel syringe having volume of 10–50 mL, with the plunger associated with to a digital stepping motor or precision screw drive. With the forward movement of the plunger, the eluent is driven through the chromatograph with a pulseless stream. However, the runtime is limited by the volume of the syringe, and no flow occurs during the refill step.

10.6.2.2 Reciprocating-Piston Pumps

The reciprocating-piston pump is the most common design in modern HPLC. The pump head comprises two arrangements of moving parts: the check valves and the seal-piston assembly. The cam and connecting rod convert the rotational movement of the motor into linear development of the cylinder. With each stroke, the piston moves a small volume of liquid (40–400 mL) from a chamber fitted with check valves. Amid the fill stroke, the piston is pulled back into the chamber.

The inlet check valve rises from its seat because the approaching solvent is at a higher pressure than the pressure inside the liquid compartment of the pump head. Concurrently, the outlet check valve drops down onto its seat; the pressure on the column side of the pump is also higher than that inside the pump head. As a result of the two check valves, the solvent can enter the liquid chamber directly from the solvent reservoir. During the delivery stroke, the piston moves into the chamber and pressurizes the liquid present in it. As the pressure inside the chamber is currently more than atmospheric pressure, the inlet check valve is compelled to close. At the point when the pressure inside the pump head surpasses the pressure on the column side of the pump, the outlet check valve opens and the mobile phase flows toward the column. The mode of pumping of solvents can be classified into two types:

10.6.2.3 Isocratic Pumping Systems

Isocratic elution is normally used for the elution of analytes from the column. In isocratic elution, the mobile phase ratio is kept consistent all through the investigation. The mobile phase can be made of a single solvent or a combination of two or more miscible solvents. The main prerequisites of isocratic pumps are accuracy and smoothness of flow. Because the pump conveys only a single solvent system, basic, cheap pulse dampeners and rudimentary flow or pressure feedback control circuits can be utilized.

10.6.2.4 Gradient Pumping Systems

Gradient pumping systems are able to deliver more than one solvent during the analysis. The mixing of two solvents to form a gradient is referred to as a binary gradient, three solvents make a ternary gradient, and a quaternary gradient is formed by the use of four distinct solvents. The mixing of the solvents can occur in one of two different ways: high-pressure mixing or low-pressure mixing (Fig. 10.1).

10.6.3 Columns

The column is one of the major parts of the HPLC system, providing separation of a mixture into individual components. The selectivity, limit, and efficiency of the column are influenced by the type of the packing material or the construction materials.

10.6.3.1 Stationary Phases

HPLC packings are microparticles of various size, shape and porosity. The surface of the particles can be changed by either physical or chemical processes to provide access to any of the classic methods of chromatography. Silica gel is the major stationary phase for adsorption chromatography, in spite of the fact that the use of other metal oxides, for example, alumina and zirconia, and also carbon and hydroxyapatite, have been accounted for. Polymeric stationary phases, for example, polystyrene-divinyl benzene, have likewise been used, in spite of the fact that these resins are typically utilized as supports for bonded phases. Silica packings are prevalent because they can withstand the high pressures created when columns of 10–30 cm packed with 3–10 μm particles are utilized. Silica is plentiful, cheap, and accessible in an assortment of shapes, sizes, and degrees of porosity. Additionally, functional groups can be quickly attached to the silanols, and the chemistry of the bonding interactions is well understood. The major drawback of silica is its lack of stability at high and low pH (above pH 8 or below pH 2), limiting the eluents that can be used. Resin-based packings, for example, polystyrene divinyl benzene, alongside acrylic-based polymers, are being used progressively in HPLC columns. The packings are mostly used as a part of size-exclusion and ion-exchange chromatography; however, although they can be utilized over a wide pH range (pH 1–13), they are more restricted as far as pressure is concerned than silica-based columns. Resin-based columns also tend to be more restricted with organic modifier concentrations (Weston and Brown, 1997).

10.6.3.2 Pore Size

Porous microparticles are the most well-known stationary phase adsorbents used as a part of modern HPLC. The role played by pore size is important, as the pores supply the surface with which the sample interacts. Particles having small pores provide a high surface area and hence provide more retention. Proteins, which are large molecules, are unable to pass through the small pores. Therefore, for those molecules, a larger pore size packing is ideal. Porous particles are seldom used owing to low efficiencies. Despite the fact that pore sizes are normally depicted by a single number, packing materials do not have discrete pore sizes. Rather, the packing is depicted as a statistical distribution of pore sizes and shapes. The distribution can be wide or narrow, depending upon the manner in which the silica was made and the

subsequent treatments. For most methods of chromatography, a narrow distribution of pore size is ideal, although size-exclusion chromatography is as exception. Pellicular materials comprise a solid spherical bead of relatively large inner diameter with a thin external surface of stationary phase. In the event that the inner core is covered with a permeable silica layer before being covered with the stationary phase, the molecule is known as a superficially porous particle. Pellicular materials have come up as the most widely recognized materials for ion exchange chromatography. They provide higher efficiencies (bring down HETP) than porous particles of a similar size, yet are limited to small sample loadings due to the low active surface area. Other materials are more easily packed into columns, with the consequent advantages of ease of packing, long-term stability, and lower price. Porous microparticles are completely porous materials, which are either irregular or spherical in shape. Because of the numerous advantages of spherical microparticles, for example, better stability at high pressure, larger sample volume limit, and improved detector sensitivity, irregular microparticles are rarely utilized. Porous microparticles are generally chosen for obtaining the highest efficiency and speed, when peaks must be separated for identification, for trace analysis, or for complex mixtures necessitating a large peak capacity.

10.6.3.3 Particle Size

The particle size of the stationary phase decides the quantity of theoretical plates per unit length that can be produced. Small particle sizes give rise to high efficiencies; however, increased back pressure can occur because of the reduced column permeability. On the other hand, when small particles are packed into shorter columns, the speed of investigation will increase for a similar efficiency.

10.6.3.4 Internal Diameter of Column

The internal diameter of a column influences the sample load, the peak dilution, and the flow rate. The larger the inner diameter, the greater is the loading capacity and the higher the flow rate. Moreover, peak dilution increases with internal diameter and thus decreases mass sensitivity. Most analytical columns extend from 2 to 5 mm in diameter. Narrow-bore (or smaller) columns with 2 mm or less diameter are used for high-sensitivity applications in which the amount of sample is small, or with high solvent and disposal costs.

10.6.3.5 Column Length

Column length influences both the effectiveness and the speed of the separation. The analysis time is directly proportional to the column length. However, the column efficiency tends to increase with length. As a rule, short columns are used for simple separations. Analytical columns can vary from 30 to 300 mm in length.

10.6.4 Detector

The detector converts changes in the column discharge to electrical signals, which are recorded by the data system. Detectors are called selective or universal depending on the property measured. Selective (solute property) detectors, for example, fluorescence detectors, measure a physical or chemical characteristic that is specific for the solute(s) present in the mixture; only those components which have that characteristic will be distinguished. Universal (bulk property) detectors measure a physical property of the component present in the eluting solvent. Hence, with refractive-list (RI) detectors, for instance, all the solutes that have a unique refractive index in relation to that of the eluent will be distinguished. Selective detectors are more sensitive than universal detectors, and they are comparatively more widely used. Universal detectors are mainly used in preparative chromatography requiring a universal response due to the large sample size. Detectors may also be classified as those that measure UV absorbance and RI, which fall into the nondestructive classification. Nondestructive detectors are regularly utilized in series to get additional qualitative data. Destructive detectors comprise electrochemical and mass spectrometric detectors in which a small amount of the sample is modified by the detector itself (Weston and Brown, 1997).

10.6.4.1 UV–Visible Absorbance Detector

The UV–visible (UV–Vis) absorbance detector is the most common HPLC detector in use today because many compounds of interest absorb in the UV (or visible) region (from 190 to 600 nm). Sample concentration, output as absorbance, is determined by the fraction of light transmitted through the detector cell by Beer's Law:

$$A = \log\left(I_0 / I\right) = \varepsilon bc$$

where A corresponds to absorbance, I_0 denotes the incident light intensity, I stands for intensity of the transmitted light, ε is the sample extinction coefficient, b stands for the path length of the cell in cm, and c denotes the molar sample concentration. UV absorbance results because of the change of electrons from π–π*, n–π*, or n–σ* molecular; aromatic compounds absorb strongly at or below 260 nm, compounds containing one or more double bonds (e.g., carbonyls, olefins) at around 215 nm, and aliphatic compounds at around 205 nm. There are three distinct categories of UV detectors: fixed-wavelength detectors that depend on particular wavelengths, and variable and photodiode array detectors (PDAs) that depend on one or more wavelengths created from a broad spectrum lamp. Fixed-wavelength detectors, the mainstay of early HPLC frameworks, are low in price and simple to use, however, their use is limited nowadays. The most widely recognized fixed-wavelength detectors utilize the 254 nm wavelength created by a low-pressure mercury lamp. Variable wavelength detectors can be customized to work at the absorbance maximum of an analyte or at a more selective wavelength.

They can likewise be modified to change wavelengths amid a chromatographic analysis to make up the responses of various analytes. In a variable-wavelength detector, light created from a broad spectrum source (for UV deuterium is normal, tungsten is utilized for visible wavelengths) is guided through an opening to a diffraction grating that separates the light out into its constituent wavelengths. The grating is then turned to send a single wavelength of light through an opening, through the detector cell, to a photodiode. PDAs have an optical path similar to variable-wavelength detectors with the exception that the light passes through the flow cell before hitting the grating, enabling it to spread the spectrum over an array of photodiodes.

10.6.4.2 Fluorescence Detectors

Fluorescence detectors (FL) analyze the optical light emission by solute molecules after they have been energized at a higher energy wavelength. They can be exceptionally sensitive for compounds that have native fluorescence or that can be made to fluoresce by derivatization. The light source is normally a wide range deuterium or xenon flash lamp. The excitation wavelength (frequently near the UV λ_{max}) is chosen by a filter or monochromator between the lamp and the flow cell, at all times at a higher energy (lower wavelength) compared with the emission wavelength. FL detectors can be more than 100 times as sensitive as a UV detector, making them especially valuable for trace investigations, or when the sample material is limited or at a low concentration. Care ought to be taken to appropriately choose and watch over mobile phase constituents as some buffers or solvents can give rise to background fluorescence, and solvents that are not appropriately degassed can prompt quenching, both influencing sensitivity.

10.6.4.3 Electrochemical Detector

Electrochemical (EC) detectors are one of the most delicate and selective HPLC detectors available. EC detectors require the use of electrically conductive HPLC solvent system as mobile phase (including buffer) and, when appropriately used and maintained, are the leading standards for response levels for HPLC investigations of compounds, for example, catecholamines and neurotransmitters. EC detectors for HPLC comprise three different electrodes; a working, a counter (additional), and a reference electrode. Generally, the materials used in electrodes are carbon, gold, silver, or platinum. A constant potential difference is maintained between the working electrode and the reference electrode. Current generated from the electrochemical reaction as components are oxidized or reduced at the working electrode is adjusted by a current flowing in the inverse direction at the opposite electrode.

10.6.4.4 Conductivity Detector

The conductivity detector is a bulk-property detector measuring the conductivity of the mobile phase. Conductivity detectors are specific detectors for ion chromatography or ion exchange separations when the analyte does not possess a UV chromophore in its structure. In the case of a conductivity detector, the resistance between two electrodes in the flow cell is measured. For some applications, especially ion chromatography in which conductive buffers are essential in the mobile phase, a suppressor column is used after the analytical column for reducing the background conductance of the mobile phase (Weston and Brown, 1997).

10.6.4.5 Refractive Index Detector

The refractive index (RI) detector is also called a universal bulk property detector, and it is the first and oldest LC detector. RI detectors analyze the difference in optical refractive index between the mobile phase and the analyte, requiring no chromophore on the analyte to be present. Therefore, RI detection has been used effectively for the investigation of sugars,

triglycerides, and organic acids. The most widely recognized RI detector configuration is the deflection refractometer in which the light from a tungsten lamp as a source is directed through a couple of wedge-shaped flow cells (reference and sample). Static mobile phase is passed through the reference cell and the column effluent is passed through the sample cell. As the light goes through the two detector cells, it gets refracted in a different manner, measured by a couple of photodiodes that convert the signal into a quantifiable output voltage. Modern RI detectors use thermostatted flow cells because of the vulnerability of RI measurements to temperature variances.

10.6.4.6 Evaporative Light Scattering Detector

An evaporative light scattering detector (ELSD) works on the principle of evaporation (nebulization) of the mobile phase with subsequent measurement of the light scattered by the resulting particles. After passing through the column, the analyte, along with the mobile phase, is nebulized in nitrogen or an air carrier gas stream in a heated drift tube and any nonvolatile particles stay suspended in the gas stream. Scattering of the light of the column effluents is recognized by a photocell mounted at an angle to the incident light beam. Carrier gas flow rate and drift tube temperature must be balanced for the mobile phase that is used. The detector response is proportional to the amount of analyte present, and although the sensitivity decreases for volatile analytes, unlike the UV detector, chromophores are not required and it has a greater response than an RI detector. ELSD also has an advantage over RI detection in that the response does not depend on the solvent, so that gradients can be utilized, and the response is not sensitive to temperature or flow rate variations.

10.6.4.7 Corona Charged Aerosol Detection

Corona charged aerosol detection (CAD), also known as corona discharge detection (CDD) is an innovative technique, gradually gaining momentum, in which the HPLC column eluent is first nebulized with a nitrogen (or air) carrier gas to give rise to droplets that are then dried to eliminate the mobile phase, forming analyte particles. The essential stream of analyte particles is subjected to a secondary stream of nitrogen (or air), which is positively charged because of having passed through a high-voltage platinum corona wire. The charge moves through diffusion inversely to the stream of analyte particles and is also moved to a collector where it is measured by a highly sensitive electrometer, producing a signal directly proportional to the amount of analyte present. Because the whole procedure includes particles and direct measurement of charge, CAD is exceptionally sensitive, gives a consistent response, and has a wide dynamic range, which offers some genuine advantages, especially when analyzing compounds devoid of UV chromophores. When compared with other universal-type HPLC detectors, similar to RI and ELSD, CAD has been observed to be much less demanding to use, and unlike RI, has room for gradients.

10.6.4.8 Detectors Hyphenated with HPLC

Hyphenation or combination between HPLC and more advanced and sensitive detection technique, for example, a mass spectrometer (MS) or nuclear magnetic resonance (NMR) spectrometer, has expanded the ability for isolating and solving structural problems of complex natural products. At times, various identification procedures are used, for example, LC–MS, LC–MS–MS, and LC-NMR-MS. These days, an MS detector, mainly in combination with a UV–Vis or PDA detector, is likely the most sought after detection method used for HPLC, particularly when analyzing complex mixtures of compounds involving natural product extracts or herbal products. Whenever an MS detector is used, isolated compounds emerging from the column can be distinguished on the basis of their mass spectral information. The ionization techniques used as a part of HPLC-MS are mainly soft ionization techniques, for example, electrospray ionization mass spectrometry (ESI-MS), that show mainly the molecular ion species, with a few fragment ions mainly utilized in HPLC-MS. Once in a while, tandem mass spectrometry (MS–MS), which gives rise to fragments through collision-induced dissociation of the molecular ions, is also used. An HPLC-MS system does not really permit an entire and unambiguous online identification of a component, except if it is a well-known natural product and corresponding spectroscopic data is accessible in databases for comparison. Detailed description of LC–MS–MS analysis of herbal drugs has been described in Chapter 11. The nature of the MS response always depends upon various factors, for example, the nature of the compounds to be analyzed, the solvent and buffer utilized as the mobile phase, the flow rate, and the type of interface used. In this manner, it regularly causes problems in regard to the reproducibility of data. NMR, though probably the least sensitive of all detection techniques, is also used as a detector for HPLC as it offers the most helpful structural data toward the structure determination of natural products. A UV–Vis indicator is also utilized side by side as a primary detector along with an NMR detector. NMR has not exactly turned into a widely acknowledged detector for any HPLC task, essentially because of its lower level of sensitivity (Sarker and Nahar, 2015).

10.6.5 Properties of Detectors

A suitable on-line detector provides versatility, high sensitivity, the ability to continuously analyze column discharge, a low noise level, a broad linearity of response, a stable baseline, and it is independent of flow rate and temperature variations and responds to all types of compounds. It is tough, not excessively costly, and is capable of correctly measuring the peak volume without increasing its volume considerably. The terms noise, sensitivity, and linearity are mainly used as a part of portraying detector performance.

10.6.5.1 Noise

Noise is characterized as a variation of the output signal that cannot be ascribed to the solute passing through the cell. It can occur due to numerous causes, including instrumental effects, temperature variations, line voltage changes, and changes in flow rates. There are three types of detector noise: short-term, long-term, and drift. Short-term noise extends the trace and shows up as "fuzz" on the baseline, while, with long-term noise, the variation in the baseline shows up as "valleys" and "peaks." Consistent movement of the baseline either up or down is called drift.

10.6.5.2 Sensitivity

Sensitivity is the ratio of detector response (peak height) to the sample concentration. A larger peak is given for a given solute concentration by highly sensitive detectors. More important than the size of the signal, however, is the signal-to-noise (S/N) ratio, which measures the amount of signal noticeable over the baseline noise. If a specific detector creates a large signal, but noisy, the sensitivity will be endangered. The sensitivity of an optical detector, for example, an absorbance locator, may be increased by replacing the flow cell with another of longer path length. If this is done, the signal will be increased along with the noise. A detector with a higher S/N ratio is a more sensitive detector.

10.6.5.3 Accuracy

Accuracy is a measure of the closeness of a measurement to the actual value. Precision is a measure of the reproducibility of measurements. For some detectors, the accuracy of a measurement is performed by user calibration. However, in the case of some detectors, for example, PDAs, the accuracy depends on internal calibration. The "linear dynamic range" of the detector is the maximum linear response divided by the detector noise. The detector response is taken to be linear if the variation of response for two concentrations of a given compound is proportional to the difference in concentration between the two samples. Most detectors become nonlinear as the sample concentration is increased (Weston and Brown, 1997).

10.7 VALIDATION OF RP-HPLC METHODS

The validation of an analytical procedure takes place after its development and before its introduction into routine use. Validation is the confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled. The purpose of method validation is to demonstrate that an analytical method is suitable for its intended purpose and, for a quantitative method, provides a reasonable estimate of the true value of the sample tested. Appropriate performance characteristics, such as accuracy and precision, must be demonstrated before making decisions based on test data. Method validation involves assessing method performance against predefined criteria, established based on the sample specifications and the type of measurement to be performed, for example, assay, identification, or limit test. A rigorous assessment of method performance versus predefined criteria provides assurance that the method will consistently provide a fit-for-purpose performance. An analytical method should be validated prior to use. In addition, the method itself and associated validation criteria and data should be re-evaluated periodically to ensure that the method remains fit for the purpose (Barnett et al., 2013). The following characteristics are recommended for HPLC assay validation: accuracy, precision, specificity, linearity, range, quantitation limit, and robustness.

10.7.1 Accuracy

Accuracy is a measure of agreement of the measured value with the known or reference value. It is generally established by measuring samples of known composition across the range of expected concentrations and demonstrating the values, which are within the preestablished criteria defined by the user. In ISO documents, the accuracy of an analytical procedure expresses the closeness of agreement between a test result and the accepted reference value. The closeness of an agreement observed is the result of the sum of the systematic (δ) and random (2σ) errors. The accuracy is then the expression of the

sum of the trueness and precision. Accuracy can be estimated from reference materials (RMs), interlaboratory comparison (proficiency test), recovery of the spiked amount of analyte added to test samples, or comparison of results with those of an alternative method. Practically, it is through the list of samples selected for the tests that the analyst can show that the applicability of the method is correctly covered in terms of the types of matrices and ranges of concentrations (Waksmundzka-Hajnos and Sherma, 2010).

10.7.2 Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under stipulated conditions. Precision measures only the distribution of random errors linked to the analytical procedure and does not relate to the true value. The measure of precision is usually computed as a standard deviation or relative standard deviation (RSD) of the test results and is divided into three levels: repeatability, intermediate precision, and reproducibility (Waksmundzka-Hajnos and Sherma, 2010).

- Repeatability (also called intraassay precision) is a measure of the ability of an LC method to provide consistent measurements within a given run or sample sequence. It is assessed by measuring the precision of multiple test results and confirming adherence to predefined criteria.
- Intermediate precision is a measure of the ability of a method to provide consistent measurements within a given laboratory when testing is performed by different analysts using different equipment.
- Reproducibility is a measure of the ability of a method to provide consistent performance over time in different labs, with different analysts, using different equipment. This is very difficult to assess but most closely approximates the precision that the method will display over its lifetime (Barnett et al., 2013).

10.7.3 Specificity

The validation procedure should confirm that the analytical method is able to determine an analyte without interference from other components present in the sample (impurities, degradation products, matrix components). The terms selectivity or specificity are often used. The International Union of Pure and Applied Chemistry (IUPAC) states that specificity is the correct and preferred term if a method is free from interferences. From a chromatographic point of view, the term selectivity is often preferred. It refers to a method that may distinguish the responses of a number of chemical entities from each other in a complex mixture. The degree of selectivity depends on the affinities of analytes and other compounds with both stationary and mobile phases and the use of a detector adapted to the analyte properties. It is clear that, in a separation technique, a specific analytical method, based on the absence of interferences, must be selective. A relevant technique to highlight matrix effects is the standard additions method (Waksmundzka-Hajnos and Sherma, 2010).

10.7.4 Linearity

Linearity is established by demonstrating a mathematical relationship between the detector response and analyte concentration. While many LC methods used for routine testing rely on calibration curves exhibiting a straight line, other relationships are acceptable, provided they are appropriate.

10.7.5 Range

The validated method should state the range of concentrations over which the method has been shown to meet validation criteria.

10.7.6 Quantitation Limit

The method QL should be confirmed to meet preestablished criteria when the sample response is near the QL. The QL is established in a manner similar to the DL: (1) visual evaluation of the peak, (2) peak S/N, or (3) calculated from the response and the standard deviation of the slope of the calibration curve (Barnett et al., 2013).

The limit of detection (LOD) is the lowest concentration of analyte that can be reliably detected or distinguished from blank. The limit of quantification (LOQ) is the lowest concentration of analyte that can be quantitatively determined with an acceptable level of precision and accuracy. They characterize the measuring instrument more than the method. An

experimental design to characterize the calibration curve, as well as the LOD and LOQ, can be used. It is recommended to prepare at least five different standard solutions to measure the instrumental response. The levels of concentrations must be regularly distributed in the entire selected working range. For each level, five replicates are prepared, starting from a standard of definite purity. Each repetition is made on an independently prepared solution to respect the independence of measurements using statistical formulas of computation. Another approach to estimate the lower LOQ can be used. The calculated RSDs of measurements, which define precision, are plotted against concentration levels close to the LOQ, and a curve is fitted to this plot. When the curve crosses the previously defined required precision, the corresponding concentration level is equal to the LOQ (Waksmundzka-Hajnos and Sherma, 2010).

10.7.7 Robustness

Robustness is the ability of the method to provide suitable performance when small changes are made. For LC methods, it is traditionally demonstrated by measuring the effect of small, deliberate changes in method parameters (e.g., flow rate or column temperature) on performance and is usually assessed during method development. Although not described in ICH Q2 (R1), multifactor studies are often performed when assessing method robustness (Thompson et al., 2002; Borman et al., 2011). The data acquired during robustness testing and other validation studies are used to establish system suitability. System suitability parameters are method-specific criteria that an LC system must meet in routine use (Wiggins, 1991).

10.8 CHEMOMETRICS AND PRINCIPAL COMPONENT ANALYSIS

Chemometrics is a technique for extracting data from multivariate chemical information using the two methods of statistics and mathematics. With the progress of computational techniques, chemometrics has become an important technique for faster investigation of results/information and shorter product development time. It is, for the most part, applied for at least one of three basic roles to:

- Investigate the nature of association in information.
- Track properties of materials continuously.
- Prepare and use multivariate classification models.

This technique has the ability to analyze and model a diverse variety of data types for an even more diverse set of applications (Sarker and Nahar, 2015). Chemometrics can be performed by pattern recognition methods (unsupervised and supervised) when a qualitative evaluation is considered and also in a multivariate calibration for quantitative purposes. Principal component analysis is an unsupervised pattern recognition technique used for handling multivariate data without prior knowledge about the samples under investigation.

10.9 CASE STUDIES FOR HPLC IN THE ANALYSIS OF SOME PHYTOCHEMICAL GROUPS AND HERBAL PRODUCTS

10.9.1 Alkaloids

Alkaloids are plant metabolites that occur in several important families, such as Papaveraceae, Ranunculaceae, and Solanaceae, consisting of about several compounds. The basic properties of alkaloids lead to a solution of high polarity when dissolved in water. This in turn results in a decreased affinity for the lipophilic stationary phases applied. Alkaloids are present in aqueous solutions in both ionized and nonionized forms and therefore difficult to separate chromatographically. The mobile-phase pH plays a big role in the separation of such analytes with acid–base properties (Fonseca et al., 2004; Rosés, 2004). In most of the cases, a silica column, a strongly polar modifier, such as acetonitrile, and a medium-polar diluent (dichloromethane) are appropriate for this purpose. In one study, the addition of water to the mobile phase has been shown to improve the separation of ergot alkaloids (Wang et al., 2004).

For analyzing indole alkaloids from the *Haraldiophyllum* species on a silica column, a mixture of ethyl acetate and n-hexane was found useful (Guella et al., 2006), and for the analysis of alkaloids from *Psychotria suterella* a mixture of methanol and chloroform was found useful as eluent (Santos et al., 2001). Separation of strychnine and brucine from the extract has been carried out in an aminopropyl stationary phase. The mobile phase consisted of acetonitrile with an acetate buffer (Wong et al., 2002).

An analysis of Camptothecin alkaloid was carried out on an AQUASIL C18 stationary phase consisting of a C18 group and an additional polar group, thus giving new selectivity compared with a traditional C18 (Kitajima et al., 2002). A gradient

RP-HPLC method has been established by Ayyangar and Bhide (1988) for separation of five principal alkaloids (morphine, codeine, thebaine, noscapine, and papaverine), three minor alkaloids (laudanosine, cryptopine, and narceine), and meconic acid in gum opium samples.

10.9.2 Phenolics

Phenolic compounds consist of an aromatic ring containing one or more hydroxyl substituents and range from simple molecules to highly polymerized compounds. Phenolic compounds in many cases are polymerized into larger molecules, such as the proanthocyanidins (condensed tannins) and lignins. Phenolic acids may also occur in plants as esters or glycosides conjugated with other natural compounds, such as flavonoids, alcohols, hydroxyl fatty acids, sterols, and glucosides. Reversed-phase (RP) LC is the preferred mode of separation for phenolic compounds. The C18 phase is indispensable for the separation of phenolics. The column length ranges from 100 to 250 mm and the internal diameter from 2.1 to 4.6 mm. Particle sizes are in the usual range of 3–10 µm. Monolithic columns and columns packed with small particles have been utilized in the analysis of phenolics in plants. By decreasing the particle size and the internal diameter of the column, analysis time can be significantly reduced. Gradient elution is preferred due to the complexity of the phenolic profile, although isocratic elution is also used sidewise.

Binary systems comprising an aqueous component (acetic, formic, or phosphoric acid) and a less polar organic solvent, such as acetonitrile or methanol, are commonly used. In some cases, acetonitrile leads to better resolution in a shorter analysis time than methanol, and it generally gives sharper peak shapes, resulting in a higher plate number. However, methanol is often preferable to acetonitrile because of its nontoxic properties and the possibility of using higher percentages in the mobile phase. In some cases, tetrahydrofuran (THF) and 2-propanol, as less polar solvents with high elution strength, have also been used. In particular, separation of the more polar phenolic acids is highly dependent on the pH of the mobile phase because they are weak acids with pK_a values of about 4. Thus, a weakly acidic mobile phase will suppress ionization and enhance the separation on an RP column. In the separation of phenolic acids, the pH of the mobile phase should be about 2–2.5 with additives, such as acetic, formic, or phosphoric acid, or phosphate, citrate, or ammonium acetate buffer. The buffer concentrations typically vary from 5 to 50 mM. Most HPLC analyses of phenolic compounds are performed at ambient temperature, but moderately higher temperatures between 30 and 40°C have also been recommended. In RP-HPLC, the retention is based on hydrophobic interactions, and the more polar compounds (e.g., phenolic acids) elute first, followed by those of decreasing polarity. Hence, the elution order is typically phenolic acids < cinnamic acids < flavonoids, although overlap of the individual members of different classes is inevitable because of the diversity of the compounds. In cinnamic and phenolic acids, polarity is increased most by hydroxy groups at the 4-position, followed by those at the 3- and 2-positions. Methoxy and acrylic groups reduce polarity and hence increase retention times. A method developed for the determination of phenolic compounds in fennel (*Foeniculum vulgare*) allowed over 100 samples per day to be analyzed (Križman et al., 2007). Chromatographic parameters, such as column temperature and injection volume, were found to be crucial in obtaining adequate selectivity and resolution, consequently allowing short run times.

10.9.3 Coumarin

Coumarins are part of the group of benzopyrones called benzo-α-pyrones, which consist of a benzene ring joined to a six-member heterocyclic pyrone ring with an oxygen atom in the α-position. They can be classified into the group of naturally occurring cinnamic acid lactones, alternatively called phenylpropanoids. The use of normal stationary-phase columns for the resolution of natural compounds is limited in the studies. Linear furocoumarins, such as psoralen, bergapten, xanthotoxin, and isopimpinellin, isolated from three varieties of *Apium graveolens*, were examined by normal-phase HPLC equipped with a variable wavelength detector set at 250 nm; the mobile phase consisted of a mixture of ethyl acetate (0.1%) and formic acid (0.1%) in chloroform (Beier et al., 1983). Reversed-phase HPLC is suitable for most applications. Highly durable stationary phases with variable carbon chains and perfect reproducibility make it ideal for routine analyses, as well as complicated purifications. Generally, separation of neutral coumarins is possible using a C18 reversed-phase column eluted with water/acetonitrile or methanol/water mobile phases, their combinations, and various additives, such as phosphoric acid. Alternatively, it is possible to employ THF or acetic acid as additives; these are well suited for the separation of phenolic coumarins by stepwise elution with an increasing concentration of aqueous methanol (Thompson and Brown, 1984).

Furocoumarins isolated from acetone extracts of the dried fruits of *Tetradium daniellii* were analyzed using a LiChrospher column (250 × 4.0 mm I.D., 5-µm particle size) employing this gradient at a 1 mL/min flow rate (Stevenson et al., 2003). Prior to separating some simple coumarins, such as esculin, esculetin, fraxin, and fraxetin, from Cortex Fraxini extracted

into 50% ethanol, chloroform, ethyl acetate, or n-butanol, filtering is recommended. A C18 column (100×4.6 mm, 3 µm) eluted with a mixture of acetonitrile, methanol, and 0.01% phosphoric acid (2:1:12, v/v/v), with detection at 340 nm, should then be employed for successful separation (Wu et al., 2007). Efficient simultaneous chromatographic separations and quantitative analyses of 24 fragrance allergens, including coumarin, were achieved using conventional reversed-phase HPLC coupled with DAD. For this purpose, *p*-anisaldehyde was used as an internal standard, and a C18 column (250×4.6 mm I.D.) combined with a guard column was eluted isocratically with an acetonitrile:water mobile phase, with flow rates in the range 0.7–1.0 mL/min. The DAD was scanning in the wavelength range 190–500 nm. This method was used for representation of commercially available scented products. Quantitative assays were performed by means of the internal standard procedure. The calibration graphs for each standard were constructed from triplicate injections of five solutions with different concentrations and plotting the analyte against the internal standard peak areas (Villa et al., 2007). The specific fluorescence the unique natural trait of coumarins can be harnessed for our cause in HPLC applications as well. Use of a fluorometric detector instead of DAD was proved to work well for the determination of 7-hydroxycoumarin (umbelliferone), which is a product formed via coumarin 7-hydroxylation and 7-ethoxycoumarin O-deethylation by P450 enzymes. It was carried out using an HPLC apparatus equipped with a C18 5-µm analytical column (150×4.6 mm I.D.), supplemented with a C18, 5-µm guard column. The flow rate was 1.2 mL/min, and the excitation and emission wavelengths of the detector were 338 and 458 nm, respectively (Yamazaki et al., 1999).

10.9.4 Flavonoids

Flavonoids are a large group of phenolic plant constituents. These compounds consist of two benzene rings (A and C) that are connected by an oxygen-containing pyrene ring (B). Therefore, flavonoids can be regarded as chromane derivatives with a phenyl substituent in the C-2 or C-3 position. Flavonoids are often hydroxylated in positions 3, 5, 7, 3′, 4′, and/or 5′. Frequently, one or more of these hydroxyl groups is methylated, acetylated, prenylated, or sulfated. Flavonoids are mainly present in plants as O- or C-glycosides. Aglycones (the forms lacking sugar moieties) occur less frequently. At least eight different monosaccharides or combinations of these (di- or trisaccharides) can bind to the different hydroxyl groups of the flavonoid aglycone. The large number of flavonoids is a result of the many different combinations of flavonoid aglycones and these sugars. Although flavonoids are compounds of wide structural diversity and polarity, the systems used for their separation are usually similar. The most often used columns are filled with reversed-phase C18 sorbent. Packings of the C8 type have been employed for separation of more polar flavonoids, for example, aglycones and glycosides of isoflavones (Barnes et al., 1998). RP-HPLC with UV detection or DAD was used for the determination of bioactive flavonoids in the rhizome of *Alpinia officinarum* (Tao et al., 2006), the fruits of *Silybum marianum* (Minakhmetov et al., 2001), *Flos inulae* (Geng et al., 2007), and herb of *Artemisia annua* from different sources (Bilia et al., 2006). Camarda et al. (Wang and Li, 2007) compared the antiproliferative activity and flavonoid composition of Citrus juices. The use of HPLC for monitoring the flavonoid content in hairy-root cultures of *Scutellaria baicalensis* has been reported (Kovács et al., 2004). For determination of rutin in plant extracts, a method using a C18 column, isocratic elution with acetonitrile–aqueous acetic acid, and UV detection at 252 nm has been elaborated (Deineka et al., 2004). There are reports on the use of RP-HPLC in chemotaxonomic investigations, for example, in identification of *Epilobium* species (Onagraceae) (Tóth et al., 2006) and *Epimedium* species (Chen et al., 2007a,b) with flavonoids as chemotaxonomic markers.

10.9.5 Lignans

Lignans are a group of phenolic compounds found throughout the plant kingdom. Structurally, lignans consist of two phenylpropane units linked together with β,β-bonds. In some plants from the Magnoliale and Piperale family, some lignans have been identified that are linked together with other carbon–carbon bonds; these are called neolignans. In trees, trimeric (sesquilignans) and tetrameric lignans (dilignans) occur, representing a group called oligolignans. For analytical purposes, normal-phase columns have been used mainly for lipophilic lignans, such as lignans present in *Podophyllum* species (Fay and Ziegler, 1985) or in sesame seed oils (Kamal-Eldin et al., 1994). For most lignans, which are more hydrophilic, normal-phase columns are generally used only for isolation or purification at a preparative or semipreparative scale.

Three lignans in Podophyllum resin, specifically, podophyllotoxin and α- and β-peltatin, were separated using 1.8% ethanol in chloroform as the mobile phase (Treppendahl and Jakobsen, 1980). Using n-heptane–dichloromethane–methanol (90:10:4) as the mobile phase, seven diastereoisomers of podophyllotoxin were successfully separated (Lim and Ayres, 1983). Furthermore, a method was developed for quantification of podophyllotoxin in different varieties of Podophyllum resins using a mobile phase consisting of n-hexane–methanol–tetrahydrofuran–acetic acid (85:10:4:1) (Fay and Ziegler, 1985). With RP columns, gradient elution is usually applied, with a slightly acidic mobile phase because of the acidity of

the phenolic groups. Methanol or acetonitrile have been extensively used as organic solvents in the mobile phase. For the separation of mixtures containing both diastereomers and functional-group derivatives, for example, mixtures of methanol and acetonitrile or dimethyl sulfoxide are required (Lim and Ayres, 1983). The most widely used RP column is RP-18 (octadecylsilica); however, RP-8 columns are more suitable for separation of more hydrophilic lignans, such as HMR isomers or HMR acid and Con acids (Smeds et al., 2005; Eklund et al., 2004a,b).

10.9.6 Terpenes (Mono, Sesquiterpenes, Diterpenes, and Triterpenes)

Terpenes are derived biosynthetically from units of isoprene, which has the molecular formula C_5H_8. The in vivo precursors are isopentenyl pyrophosphate and its isomeric dimethylallyl pyrophosphate. In biosynthesis, the isoprene units are linked together to build up the skeletons of the terpenes as a multiple of $(C_5H_8)_n$. Monoterpenes consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. The addition of a further C5 (isoprene) unit forms the C15 sesquiterpenes. Adding more of these C5 units yields the diterpenes (C20), sesterpenes (C25), triterpenes (C30), tetraterpenes (C40), and polyterpenes ($C > 40$). With regard to the monoterpenes, these substances can be further divided into three groups depending on whether they are acyclic, monocyclic, or bicyclic. Within each group, the monoterpenes may be unsaturated or have functional groups and be alcohols, aldehydes, or ketones. The majority of natural terpenoids have cyclic structures with or without functional groups, sometimes with an aromatic structure (thymol). Also included among the monoterpenes for biosynthetic reasons are the monoterpene lactones, known as iridoids. Like the monoterpenes, the sesquiterpenes are grouped according to their basic carbon skeleton. The common ones are acyclic, monocyclic, and bicyclic. Although HPLC is not recommended for terpene analysis, attempts to monitor volatile terpenes are not lacking. The separation of enantiomeric volatile terpenes is difficult because they lack functional groups that provide the hydrogen-bonding, dipole, π–π, or charge-transfer interactions required for most chiral recognition mechanisms. Cyclodextrins have been very successful in the separation of these compounds because of their ability to provide enantiomeric selectivity through an inclusion mechanism.

HPLC is now a very popular and reliable technique for the qualitative and quantitative analysis of diterpenes. Qualitative HPLC analysis of diterpenes in natural samples is most often applied for identification or fingerprinting of plant materials (used, e.g., in the production of natural drugs). Another important application of fingerprint analysis is chemotaxonomic analysis, which allows the discovery of chemotaxonomical markers describing differences between related taxonomic units (for example, fingerprint-based chemotaxonomy of *Taxus* species) (Ge et al., 2008). The qualitative HPLC separation of tanshinones in *Salvia miltiorrhiza* followed by pharmacological activity profiling (MAO A and iNOS inhibitory effects) of effluent microfractions was elaborated, and in this way compounds of high pharmacological activity could be found (Dittmann et al., 2004). Diterpenes are rather medium-polar compounds, so reversed-phase systems are most often used: RP-18 and RP-8 stationary phases and mixtures of polar organic solvents with water as eluents; ultra-high-performance liquid chromatography (UPLC) columns are sometimes used (Ge et al., 2008). Dolfinger and Locke showed the results of optimization of chromatographic systems in the analysis of taxanes. They drew particular attention to the usefulness of various fluorinated stationary phases (linear perfluorohexyl, branched propyl perfluorophenyl, perfluorophenyl) and a C-8 RP stationary phase, eluted with a gradient of acetonitrile in water (Dolfinger and Locke, 2003). Other examples present normal-phase separation on a cyano-bonded column eluted with hexane: methanol (Citoglu et al., 2005) or a silica gel column eluted with chloroform: methanol (Dimitrijević et al., 1996) or 0.8% of 2-propanol in hexane (Kamm et al., 2002).

10.9.7 Triterpenes

Triterpenes are a large group of naturally occurring substances with relatively complex cyclic structures consisting of a carbon skeleton based on six isoprene units. They are formed by "head-to head" condensation of two farnesyl pyrophosphate units. The product of this reaction is an acyclic C30 hydrocarbon, squalene. HPLC is the most powerful and the most frequently used technique for triterpene and steroidal determination because it can deal effectively with nonvolatile, highly polar compounds. It has been used extensively for determination of both aglycones and intact saponins. The separations are usually performed on normal- (silica gel) and reversed-phase (C8, C18) columns, of which C18 is definitely preferred, but other modified silica gel supports, including NH2 and DIOL, are occasionally used. Carbohydrate and NH2-modified columns have been shown to be very effective in the separation of glycoalkaloids (Saito et al., 1990), but some steroidal saponins were also successfully analyzed (Xu and Lin, 1985). β-Chaconine, α-chaconine, and α-solanine were nicely separated with a μ-Bondapack NH2 column in the reversed-phase mode in less than 7 min (detection limit 5–15 ppm) (Bushway et al., 1979). Borate anion-exchange chromatography depends on the formation of borate complexes with *cis*-diols in the saccharide moiety. The formation of these complexes in some cases significantly improves the resolution and separation of isomeric glycosides that are not separated on a reversed-phase support (Yamaguchi et al., 1988). After separation, genuine

saponins can be recovered by removing borate as volatile methyl borate by repeated codistillation of the eluate with methanol. Resolution of closely related compounds can also be improved by the application of a hydroxyapatite support. The hydroxyapatite is more hydrophilic than a silica gel support and allows separation of two glycosides differing only in a terminal pentose (Kasai et al., 1987).

10.9.8 Carotenoids

Carotenoids are an important class of naturally occurring compounds that are widely distributed in nature. They are a class of hydrocarbons (carotenes, e.g., β-carotene, lycopene) and their oxygenated derivatives (xanthophylls, e.g., lutein, β-cryptoxanthin) consisting of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining nonterminal methyl groups are in a 1,5-positional relationship (IUPAC, 1972). Reversed-phase HPLC (RP-HPLC) has been a method of choice for carotenoid analysis, with the use of both C18 and C30 stationary phases. There are also reports of the use of C8 columns for the resolution of different carotenoid classes (Frassanito et al., 2005). Selectivity in carotenoid separation is influenced mainly by stationary-phase bonding density, its chemistry (monomeric vs polymeric surface modification), and alkyl chain length. In addition, free silanols influence the separation of polar carotenoids, while nonpolar carotenoids are relatively insensitive to this stationary-phase property. Polymeric and more densely bonded stationary phases have been proved to provide better separations of carotenoids (Sander et al., 2000). In reversed-phase liquid chromatography (RPLC), carotenoids are eluted according to their increasing hydrophobicity and decreasing polarity (Dugo et al., 2008). The elution order also depends on the type of cyclization present in the compound. On reversed-phase columns, polar carotenoids with two hydroxyl groups, such as lutein, elute earlier, followed by monohydroxy carotenoids (e.g., zeinoxanthin and β-cryptoxanrhin) and finally by the carotenes. Carotenoids with 10 conjugated double bonds (c.d.b.), such as zeinoxanthin and α-carotene, are eluted earlier than those with corresponding polarity and 11 c.d.b, for example, β-carotene and β-cryptoxanthin. According to several researchers, the elution order of the β-carotene *cis* isomers can change depending on the characteristics of the C18 column and mobile phase employed (Hamano and Mercadante, 2001). Photodiode array detection is recommended in the case of carotenoids, as the identification of the pigments is easier due to rapid and advanced spectral analysis (Oliver et al., 1998). For example, β-carotene and zeaxanthin, which have the same chromophore but different hydroxyl groups, show an equivalent absorption spectrum. However, the maxima in the spectra of the compounds are defined by the presence of particular chromophores, and the chromatographic behavior may be determined by differences in the carbon number or the presence of different functional groups between them (Mendez-Robles et al., 2004). UV detection at 450 nm is recommended by the majority of authors to determine the presence of carotenoids in the analyzed samples (Marinova and Ribarova, 2007). Baseline resolution of *cis* and *trans* isomers of lutein in saponified marigold extracts using a C30 column was reported by Delgado-Vargas and Paredes-Lopez (1997). High levels of lutein were found in the analyzed dried petals. Rivas identified four major esters of lutein in marigold extracts: dipalmitate, myristate–palmitate, palmitate–stearate, and dimyristate (Rivas, 1991). Bakó et al. reported on the HPLC investigation of the carotenoid composition of the stems, leaves, petals, and pollen of *Calendula officinalis* L. (Bakó et al., 2002). In the petals and pollen, the main carotenoids were flavoxanthin and auroxanthin, while the stems and leaves mostly contained lutein and β-carotene.

Rosa canina is an herb commonly applied in traditional medicine. Its lipophilic fraction was proved to have antioxidative and antiinflammatory properties (Chrubasik et al., 2008). HPLC analysis of saponified *R. canina* fruit extracts, on a C18 column, revealed that the major carotenoids were β-carotene, lycopene, β-cryptoxanthin, rubixanthin, zeaxanthin, and lutein (Hodisan et al., 1997). The pair lutein and zeaxanthin remained unresolved under isocratic conditions, but their separation was somewhat better after the application of gradient elution. Marinova and Ribarova reported that blackberries had the highest levels of carotenoids in comparison with other investigated berries (Marinova and Ribarova, 2007). The HPLC analysis of these fruits revealed they were also characterized by the highest content of β-cryptoxanthin and β-carotene. The carotenoid content of *Capsicum annum* fruits has been investigated by means of HPLC by Deli et al. (1996). In the chromatograms, 56 peaks were detected, and 34 carotenoids were identified. In the ripe fruits, capsanthin, capsorubin, zeaxanthin, cucurbitaxanthin A, and β-carotene were found to be the main carotenoids, the remainder being capsanthin 5,6-epoxide, capsanthin 3,6-epoxide, karpoxanthin, cucurbitaxanthin B, violaxanthin, cycloviolaxanthin, antheraxanthin, capsanthone, nigroxanthin, β-cryptoxanthin, and several *cis* isomers and furanoid oxides. In the unripe fruits the main carotenoids are lutein, zeaxanthin, and β-carotene. An extract of dandelion (*Taraxacum officinale*) was analyzed by HPLC using a C30 column, as a result of which six geometric carotenoid isomers were separated (Meléndez-Martínez et al., 2006).

10.9.9 Some Case Studies With HPLC Analysis of Few Herbs and Their Components

This section highlights several methods or techniques developed by researchers for HPLC evaluation of few herbs and their components being used in different systems of medicine.

10.9.9.1 Estimation of Aristolochic Acid in Medicinal Plants

An RP-HPLC procedure with a silica gel RP-18 reversed-phase column for the determination of aristolochic acids I, II in medicinal plants has been developed. The mobile system of 0.3% ammonium carbonate solution–acetonitrile (75:25, v/v) with pH 7.5 was the optimal buffer to clearly separate aristolochic acids I, II within 20 min (Lee et al. 2001).

10.9.9.2 Estimation of Ginsenosides in Panax ginseng

The separation of ginsenosides in methanolic extracts was achieved with a water: acetonitrile gradient system using a C-18 reversed phase column. The column used for separation was the Hypersil Elite (Runcorn, Cheshire, England) C18 (250 × 4.6 mm I.D., 5 µm). The initial condition was set at 10% of B, gradient up to 40% B in 15 min, and up to 100% B in the next 10 min, before returning to the initial condition for 10 min. Detection was at 203 nm. The flow rate was set at 1.0 mL/min. The system precision by HPLC was found to be less than 2% (RSD, $n = 6$) for all the ginsenosides used in this work. A linearity between 40 and 500 mg/L was achieved for all ginsenosides analyzed in this work. The quantitation of the ginsenosides was performed above the lowest concentration of standard used for calibration at 40 mg/L (Lee et al., 2002).

10.9.9.3 RP-HPLC for Fingerprint Quality Evaluation of Hypericum japonicum

A novel, simple, and accurate fingerprint method was developed using high-performance liquid chromatography–photodiode array detection (HPLC–DAD) for the quality control of *H. japonicum* thunb (Tianjihuang). It is a Chinese herbal medicine used for the treatment of several bacterial diseases, infectious hepatitis, gastrointestinal disorder, internal hemorrhage, and tumors. The column was a reversed-phase column (LiChrospher 100 RP18e, 5 µm, 250 mm × 4.0 mm I.D. Merck, German). Separation was performed by linear gradient elution using acetonitrile (4%–27% in 100 min) and buffer solution (water–KH_2PO_4–H_3PO_4, pH 3.0, 96%–73% in 100 min). The flow rate was 1.0 mL/min. The detection wavelength and column temperature were set at 300 nm and 28°C, respectively. The reference fingerprint was developed by comparing fingerprints of the same plant from different locations. Using the reference fingerprint, the raw herbs of *H. japonicum* can be identified (Yang et al., 2005).

10.9.9.4 Estimation of Mangiferin in Mangifera indica

Mangiferin, a polyphenolic xanthone glycoside from *M. indica*, is used as a traditional medicine for the treatment of numerous diseases. One study developed and validated a reversed-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of mangiferin from the bark extract of *M. indica*. RP-HPLC analysis was performed by isocratic elution with a low pressure gradient using 0.1% formic acid: acetonitrile (87:13) as a mobile phase with a flow rate of 1.5 mL/min. The separation was performed at 26°C using a Kinetex XBC18 column as stationary phase and the detection wavelength was 256 nm. The proposed method was validated for linearity, precision, accuracy, LOD, LOQ, and robustness by the International Conference on Harmonization guidelines. In linearity, the excellent correlation coefficient of more than 0.999 indicated a good fit to the curve and also good linearity. The intra- and interday precision of <1% of the RSD of the peak area indicated the high reliability and reproducibility of the method (Naveen et al., 2017).

10.9.9.5 Simultaneous Estimation of Ten Compounds in Tibetan Herb, Pterocephalus hookeri *by UPLC*

A UPLC-photodiode array (PDA) method was developed to compare the chemical composition of two different medicinal components of *Pterocephalus hookeri*. Samples were chromatographically separated in succession using a Waters Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm) and gradient elution (0.2% phosphoric acid aqueous-acetonitrile). Using a partial least-squares discriminant analysis and one-way analysis of variance, attempts were made to distinguish different medicinal parts of *P. hookeri*. Regression equations for 10 compounds showed good linear regression ($R^2 > 0.9994$). The RSDs of precision, stability, repeatability, and recovery were under 5%. Compared with the aerial plant part, the root had significantly higher levels of sylvestroside I ($P < 0.01$), cantleyoside ($P < 0.001$), dipsanosides B ($P < 0.01$), and dipsanosides A ($P < 0.01$), but significantly lower levels of loganic acid ($P < 0.001$), chlorogenic acid ($P < 0.01$), and isochlorogenic acid ($P < 0.01$) (Tang et al., 2017).

10.9.9.6 Determination of Baicalin and Puerarin by RP-HPLC

HPLC methods for the determination of baicalin in *Scutellariae Radix* and puerarin in Puerariae Radix were established for the quality control of Chinese medicinal preparations containing these drugs. The samples were separated on a Cosmosil X column with 0.03% phosphoric acid–acetonitrile (79:21), 0.03% phosphoric acid–acetonitrile (87:13), and 2% acetic acid–methanol (79:21) as mobile phases at flow rates of 1 mL/min. Very satisfactory and reproducible results were obtained within 25 min for baicalin and 50 min for puerarin (Wen et al., 1993).

10.9.9.7 Simultaneous Estimation of Hydrastine and Berberine

Hydrastis canadensis L. is an ingredient of several dietary supplements intended for enhancing general immunity. Many *H. canadensis* products are currently available in the United States, either alone or in combination with Echinacea as dietary supplements. In most products, the content of the main active alkaloids of *H. canadensis*, hydrastine and berberine, is not indicated on the label. A high-performance liquid chromatography (HPLC) method has been developed for the detection and quantification of hydrastine and berberine in a number of products obtained from the United States market. The method uses a Phenomenex Luna C18 column, a mobile phase consisting of solvent A (100 mM sodium acetate/acetic acid, pH 4.0) and solvent B (acetonitrile/methanol; 90/10, v/v). Elution was run at a flow rate of 1.0 mL/min, with a linear gradient of 80%–40% A in B over 20 min and ultraviolet detection at 290 nm. A wide range of content variation was observed for both alkaloids in the tested samples (Abourashed and Khan, 2001).

10.9.9.8 Estimation of Boswellic Acid From Market Formulations Containing Boswellia serrata *Extract*

A simple, rapid, and reproducible RP-HPLC method has been reported for the estimation of boswellic acids, the active constituents in *B. serrata* oleogum resin. The chromatographic separation is performed using a mobile phase consisting of acetonitrile–water (90:10, % v/v) adjusted to pH 4 with glacial acetic acid on a Kromasil 100 C18 analytical column with a flow rate of 2.0 mL/min and detection at 260 nm. The elution times are 4.30 and 7.11 min for 11-keto β-boswellic acid (11-KBA) and 3-acetyl 11-keto β-boswellic acid (A-11-KBA), respectively. The calibration curve is linear in the 11.66–58.30 and 6.50–32.50 µg/mL ranges for 11-KBA and A-11-KBA, respectively. The limits of detection are 2.33 and 1.30 µg/mL for 11-KBA and A-11-KBA, respectively. The mean recoveries are 98.24%–104.17% and 94.12%–105.92% for 11-KBA and A-11-KBA, respectively. The inter- and intraday variation coefficients are less than 5%. Thus, the method can be successfully applied for the estimation of boswellic acids from the market formulations containing *B. serrata* extract (Shah et al., 2008).

10.9.9.9 RP-HPLC Analysis of the Phenolic Compounds in Menta pulegium

Extracts of aromatic plants of Greek origin *M. pulegium* were examined as potential sources of phenolic compounds. RP-HPLC with UV detection was employed for the identification and quantification of the phenolic antioxidants, present in methanolic extracts. The separation was achieved on a Waters Spherisorb 5 µm ODS2 4.6×250 mm column at ambient temperature. The mobile phase consisted of water with 1% glacial acetic acid (solvent A), water with 6% glacial acetic acid (solvent B), and water/acetonitrile (65:30, v/v) with 5% glacial acetic acid (solvent C). The most abundant phenolic acids were ferulic acid (1.1–280 mg/100 g of dry sample) and caffeic acid (1.2–60 mg/100 g of dry sample) (Proestos et al., 2005).

10.9.9.10 Validated RP-HPLC Method for the Standardization of Phyllanthus niruri *(Herb and Commercial Extracts) Using Corilagin*

P. niruri L., commonly known in Brazil as "quebra-pedra," has long been used in the treatment of diverse diseases in several system of medicine. The therapeutic effects of *P. niruri* are attributed to various compounds present in the plant, including the hydrolysable tannin corilagin. In the present study, high-performance liquid chromatography (HPLC-/PAD) profiles of leaves and commercial extracts of *P. niruri* were examined and three compounds were found to be present in all of the samples studied. These compounds were corilagin, rutin, and ethyl 3,4,5-trihydroxybenzoate. Corilagin, which has been proposed as a phytochemical marker for *P. niruri*, was employed as an external standard in the development and validation of a rapid and efficient qualitative and quantitative HPLC assay for the analyte. The HPLC profiles of leaves and of various commercial extracts of *P. niruri* were obtained using a Phenomenex Luna C18 column (250×4.6 mm I.D.; 5 µm particle size; 100 Å pore size) protected by a C18 guard column (4.0×3.0 mm I.D.; 5 µm particle size; 100 Å pore size). The mobile phase comprised 1% aqueous acetic acid (solvent A) and acetonitrile (solvent B), and a linear gradient elution from 5% to 100% B in 40 min at a flow rate of 1.0 mL/min was employed. During further investigations, the

chromatographic parameters (column, mobile phase, flow rate, and elution program) were optimized in order to provide better separation of the components in a shorter run time. Validation of the optimized method was performed using a Waters (Milford, MA, USA) Alliance model 2795 liquid chromatographic system equipped with a model 996 PAD and a Phenomenex Luna phenyl-hexyl column (250 × 4.6 mm I.D.; 5 µm particle size; 100 Å pore size) protected by a phenyl–hexyl guard column (4.0 × 3.0 mm I.D.; 5 µm particle size; 100 Å pore size). The mobile phase comprised a mixture of solvents A and B, as above, and was delivered at a flow rate of 1.0 mL/min, the sample injection volume was 20 µL, and the column was held at room temperature during the analysis. The final gradient employed in the developed analytical method was: 0–12 min, 13%–37% B; 12–13 min, 37%–100% B; 13–15 min, 100% B; 15–16 min, 100% to 13% B; and 16–30 min, isocratic at 13% B. The chromatogram was monitored at 267 nm, and UV spectra of individual peaks were recorded in the range of 200–400 nm (Colombo et al., 2009). Standardization and quality evaluation of several herbs by HPLC has been described in Table 10.1 with respective chromatographic conditions, marker, and reference to the methods developed for further use.

10.10 HPLC STANDARDIZATION OF SOME MEDICINAL PLANT WITH THEIR CHROMATOGRAMS

This section deals with several case studies of HPLC analysis with specific medicinal plant extracts studied at my laboratory. I would like to thank all my research team members for their contribution in developing the HPLC profiles of those plants mentioned in this section.

10.10.1 *Tinospora cordifolia* HPLC Quantification of Tinosporaside

Bahadur et al. (2016) developed and validated an RP-HPLC method for determination of tinosporaside from a hydroalcoholic extract of *T. cordifolia.* The HPLC system was composed of a rheodyne-7725 injection valve with a sample loop (20 µL), vacuum degasser, quaternary pump, and photodiode array detector, with data acquisition by Empower 2 software (Waters 600, Milford, MA, USA). Chromatography was performed on a Spherisorb C18 column (250 mm × 4.6 mm, 5 mm; Waters, Ireland) fitted with a C18 guard column (10 mm × 3.0 mm). The sample elution was performed at 25°C and detected at the ultraviolet wavelength of 254 nm. *T. cordifolia* extract was standardized by RP-HPLC under isocratic conditions using the external standard calibration technique. Bioactive compound was identified by comparing with the respective retention time (R_t) of the tinosporaside as standard. A calibration curve was plotted by plotting peak areas against concentrations, and five standard marker ranges from 100 to 500 µg/mL. A standard compound showed a good linearity between concentrations and peak area, with a correlation coefficient (r^2) of 0.998. An optimum separation was achieved using the mobile system at the volume ratio of acetonitrile and Milli-Q water in the ratio of 75:25 (v/v) with a flow rate of 1 mL/min. The R_t of tinosporaside was found to be 9.13 min. The percentage amounts of standard constituent (tinosporaside) present in the crude extract were found to be 1.64% (w/w). RP-HPLC chromatograms of tinosporaside and methanolic extract of *T. cordifolia* are shown in Fig. 10.2.

The developed HPLC method can be used for routine analysis and standardization of *T. cordifolia* crude drugs, extracts, and/or finished products using ursolic acid and oleanolic acid as appropriate markers (Bahadur et al., 2016).

10.10.2 *Mucuna pruriens*

M. pruriens extract was standardized by Chaudhary et al. (2013) by RP-HPLC analysis using an isocratic elution technique by the external standard method. The mobile phase composition was optimized to methanol and 1% (v/v) acetic acid in water (5:95, v/v), which was degassed and filtered through a membrane (0.45 mm) prior to being run in the column. The temperature of the column was kept at 25°C, and each injection volume was 20 mL. The flow rate was set at 1 mL/min. The quantitative estimation of levodopa present in the plant extract/fractions was determined using a calibration curve of standard levodopa. Detection of the compound was performed at 280 nm. Peak identification was achieved by comparison of the retention time of the standard peak with that of the extract. The linearity was evaluated by regression analysis using six different concentrations of the standards (100–1000 mg/mL). RP-HPLC chromatograms of levodopa and extract of *M. pruriens* are shown in Fig. 10.3.

The coefficient of determinants (r^2) was greater than 0.99, which represents that the data are very close to the line of best fit. The chromatograms were found to be directly proportional to the concentrations of the calibration solutions in the range of 100–1000 mg/mL. The retention times of the standard and the reaction mixture were found to be 6.96 and 6.980 min, respectively (Chaudhary et al., 2015).

TABLE 10.1 HPLC Standardization of Herbs for Quality Evaluation

Plant Name With Family	Column and Mobile Phase	Markers (BRS and PRS)	Detection Wavelength and Detector Used	References
Tinospora crispa (Menispermaceae)	C18 column The mobile phase consisted of acetonitrile (ACN) and 0.1% formic acid in water in gradient mode	Berberine	254 nm Photodiode array detector	Syarifah et al. (2017)
Polygonum multiflorum (Polygonaceae)	Agilent Zorbax XDB-C18 column (5 µm, 4.6 mm × 250 mm) The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (B) in a gradient mode	Gallic acid, procyanidins B1	275 nm Diode array detector	Li et al. (2017)
Nelumbo Nucifera (Nelumbonaceae)	Dionex C18 column (5 µm, 120 Å, 4.6 mm × 150 mm) The mobile phase consisted of 0.1% TFA–water and methanol in gradient mode	Neferine, ethanone, isoquinolinol, isoquinolinediol	205 nm Diode array detector (DAD)	Ryu et al. (2017)
Chimonanthus nitens (Calycanthaceae)	Elite Hypersil ODS2 column (250 × 4.6 mm, 5 µm) The mobile phase consisted of water (A) and acetonitrile (B), in gradient mode	Scopoletin, isofraxidin, rutin, scoparone	344 nm Diode array detector (DAD)	Zhou et al. (2016)
Magnolia officinalis (Magnoliaceae)	YMC-Pack-ODS-AQ (250 × 4.6 mm I.D. S-5 µm, 12 nm) The mobile phase consisted of acetonitrile (A) and 0.2% phosphoric acid (v/v, B) in gradient mode	Rutin, afzelin, hyperoside, isoquercitrin, quercetin-3-O-α-L-rhamnoside, honokiol and magnolol	280 nm Diode array detector (DAD)	Yi et al. (2016)
Murraya koenigii (Rutaceae)	Waters Spherisorb 5 mm ODS2, 250 × 4.6 mm (Ireland) The mobile phase consisted of methanol and 0.5% acetic acid in water (90:10, v/v) in isocratic mode	Mahanine, mahanimbine	254 nm Diode array detector (DAD)	Pandit et al. (2011)
Terminalia bellerica (Combretaceae)	Waters Spherisorb C18 column, 250 × 4.6 mm, 5 µm The mobile phase consisted of methanol:water:acetic acid (85:14:1, v/v) in isocratic mode	Gallic acid	270 nm UV–visible detector	Chaudhary et al. (2012)
Bacopa monnieri (Plantaginaceae)	Stainless steel column (250 × 4.6 mm) packed with octadecylsilane bonded to porous silica The mobile phase consisted of water:acetonitrile in gradient mode	Bacoside A	205 nm Diode array detector (DAD)	Agarwal and Murali (2010)
Commiphora wightii (Combretaceae)	Stainless steel column (250 × 4.6 mm) packed with octadecylsilane bonded to porous silica The mobile phase consisted of water:acetonitrile (55:45) in isoacratic mode	Guggulusterone	242 nm Diode array detector DAD	
Glycyrrhiza glabra (Combretaceae)	Stainless steel column (250 × 4.6 mm) packed with octadecylsilane bonded to porous silica The mobile phase consisted of glacial acetic acid:acetonitrile:water (6:30:64) in isocratic mode	Glycyrrhizin	254 nm Diode array detector (DAD)	

Ocimum tenuiflorum (Combretaceae)	Stainless steel column (250×4.6 mm) packed with octadecylsilane bonded to porous silica The mobile phase consisted of glacial acetic acid:acetonitrile:water (6:30:64) in isocratic mode	Ursolic acid and oleanolic acid	205 nm Diode array detector (DAD)	
Phyllanthus amarus (Combretaceae)	Stainless steel column (250×4.6 mm) packed with octadecylsilane bonded to porous silica The mobile phase consisted of methanol:water (65:35)	Phyllanthin and hypophyllanthin	230 nm Diode array detector (DAD)	
Phyllanthus emblica (Combretaceae)	Stainless steel column (250×4.6 mm) packed with octadecylsilane bonded to porous silica The mobile phase consisted of water:acetonitrile in gradient mode	Gallic acid	270 nm Diode array detector (DAD)	
Piper longum (Combretaceae)	Stainless steel column (250×4.6 mm) packed with octadecylsilane bonded to porous silica The mobile phase consisted of water:acetonitrile (40:60) in isocratic mode	Piperine	270 nm Diode array detector (DAD)	
Plectranthus barbatus (Combretaceae)	Stainless steel column (250×4.6 mm) packed with octadecylsilane bonded to porous silica The mobile phase consisted of water:acetonitrile (55:45) in isocratic mode	Forskolin	220 nm Diode array detector DAD	
Withania somnifera (Combretaceae)	Stainless steel column (250×4.6 mm) packed with octadecylsilane bonded to porous silica The mobile phase consisted of water:acetonitrile in gradient mode	Withanolides A and B	220 nm Diode array detector (DAD)	
Gardeniae fructus (Rubiaceae)	SinoChrom ODS-BP C18 column The mobile phase consisted of aqueous solution with 0.1% formic acid and acetonitrile in a gradient elute mode	Geniposidic acid	240 nm Diode array detector (DAD)	Yin et al. (2015)
Bergenia crassifolia (Saxifragaceae)	Reversed phase Luna C18(2)-HST HPLC column The mobile phase consisted of mobile phase A (0.1, v/v % TFA in water) and mobile phase B (0.1, v/v % TFA in methanol) in gradient mode	Gallic acid, arbutin and bergenin	280 nm Diode array detector (DAD)	Boros et al. (2014)
Aegle marmelos (Rutaceae)	250×4.6 mm I.D., 5-μm particle, Purospher STAR RP-18 end capped column The mobile phase consisted of 55:45 (%, v/v) methanol–water containing 0.1% acetic acid in isocratic mode	Marmelosin, umbelliferone and scopoletin	300 nm UV–visible detector	Shinde et al. (2014)
Coffea arabica (Rubiaceae)	Purospher reverse-phase C18 endcapped column (250×4.6 mm, 5 μm) The mobile phase consisted of potassium dihydrogen orthophosphate A with acetonitrile B	Chlorogenic acid	274 nm Diode array detector (DAD)	Tripathi et al. (2014)
Dipsaci Radix (Dipsacaceae)	ODS C18 column (250×4.6 mm, 5 μm) The mobile phase consisted of acetonitrile and water in gradient mode	Loganin	212 nm UV–visible detector	Zhao et al. (2013)

Continued

TABLE 10.1 HPLC Standardization of Herbs for Quality Evaluation—Cont'd

Plant Name With Family	Column and Mobile Phase	Markers (BRS and PRS)	Detection Wavelength and Detector Used	References
Scutellariae Radix (Lamiaceae)	YMC Pack Pro C8 column (150×4.6 mm^2, 3 mm) The mobile phase consisted of 0.1% formic acid:acetonitrile (70:30, v/v) in isocratic mode	Baicalein, baicalin, wogonin and wogonoside	280 nm UV–visible detector	Islam et al. (2012)
Curcuma longa (Zingiberaceae)	YMC ODS-A C18 column The mobile phase consisted of acetonitrile and 0.1% formic acid in water in gradient mode	Curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC)	270 nm UV–visible detector	Li et al. (2011)
Morus alba (Moraceae)	ODS column The mobile phase consisted of methanol–water–acetic acid (18:82:0.1, v/v/v) in isocratic mode	Mulberroside A	320 nm UV–visible detector	Piao et al. (2011)
Hypericum empetrifolium (Hypericaceae)	Hypersil ODS (125 mm×4 mm; 5 µm) The mobile phase consisted of 20 mM ammonium acetate (solution A) and acetonitrile (solution B) in gradient mode	Hypericin and hyperforin	287 (hyperforin) and 590 nm (hypericin)	Tawaha et al. (2010)
Tripterygium wilfordii (Celastraceae)	LiChrospher RP-18 column (250×4.6 mm I.D.; particle size 5 µm) The mobile phase consisted of acetonitrile:H_2O in the ratio of 39:61 (v/v) in isocratic mode	Triptolide	210 nm UV–visible detector	Li and Wang (2005)
Fructus Psoraleae (Fabaceae)	RP-C8 column The mobile phase consisted of acetonitrile and 0.1% acetic acid solution in water in gradient mode	Psoralen	310 nm Diode array detector (DAD)	Qiao et al. (2007)
Pericarpium Citri Reticulatae (Rutaceae)	Hypersil BDS C18 (5 µm, 250 mm×4.6 mm) column The mobile phase consisted of acetonitrile and water in gradient elution mode	Hesperidin	280 nm Diode array detector (DAD)	Yi et al. (2007)
Salvia plebeian (Lamiaceae)	Zorbax Eclipse XDB-C18 column (250 mm×4.6 mm, 5 µm) The mobile phase consisted of A (0.5%, v/v aqueous glacial acetic acid) and B (methanol) in gradient mode	Caffeic acid	342 nm Diode array detector (DAD)	Jin et al. (2008)
Oldenlandia diffusa (Rubiaceae)	Zorbax Eclipse XDB-C18 column (250 mm×4.6 mm, 5 µm) The mobile phase consisted of methanol–water (83:17 containing 0.2% NH_4OAc, pH 6.74) in isocratic mode	Oleanolic acid, ursolic acid	210 nm Diode array detector (DAD)	Liang et al. (2008)
Polygoni multiflori (Polygonaceae)	C18 reversed-phase packing column (3.0×150 mm, 5 µm) The mobile phase consisted of methanol:water:phosphoric acid = 600:400:1 in isocratic mode	Emodine	254 nm Diode array detector (DAD)	Jiao and Zuo (2009)
Isatis indigotica (Brassicaceae)	ODS-3 Inertsil 5 µm column (25 cm×4.6 mm I.D.). The mobile phase consisted of water A–acetonitrile B in gradient mode	Indigotin and indirubin	210 nm Diode array detector (DAD)	Zou et al. (2005)

Platycladus orientalis (Cupressaceae)	Agilent Eclipse XDB-C 18 column (3.5 µm, 150 mm × 4.6 mm, I.D.) The mobile phase consisted of methanol (E), acetonitrile (F) and 18 mM sodium acetate buffer adjusted to pH 3.5 with glacial acetic acid (G) in gradient mode	Rutin	356 nm Diode array detector (DAD)	Lu et al. (2006)
Vitis vinifera (Vitaceae)	Eclipse XDB C-18 reversed-phase column (250 mm × 4.6 mm length, 5 µm particle size) The mobile phase consisted of A: 2.0% acetic acid in distilled water and B: methanol in gradient mode	Gallic acid	278 nm Diode array detector (DAD)	Sagdic et al. (2011)
Cephaelis acuminate (Rubiaceae)	Shimadzu ODS-VP column The mobile phase consisted of methanol–acetonitrile–0.1% phosphate acid in water (9:3:88) in isoacratic mode	Emetine	205 nm Diode array detector (DAD)	Gui-ru et al. (2013)
Hippophae rhamnoides (Elaeagnaceae)	HIQ SIL C18 column The mobile phase consisted of methanol–acetonitrile–water (40:15:45, v/v/v) containing 1.0% acetic acid in isocratic mode	Catechin	279 nm Diode array detector (DAD)	Zu et al. (2006)
Chrysosplenium alternifolium (Saxifragaceae)	C18 Hypersil ODS column (5 µm, 125 mm × 4 mm, I.D.) The mobile phase consisted of water with an acetonitrile 10%–100% (v/v) in gradient mode	Chrysosplenosides, chrysosplenols	345 nm Diode array detector (DAD)	Olszewska and Gudej (2009)
Gentiana ottonis (Gentianaceae)	Column: Nova Pak C18 (150 × 3.9 mm), and Nova Pak C18 The mobile phase consisted of acetonitrile:water = 5: 95% to 65: 35% in 50 min	Flavone C-glycoside; Swertisin	Detection at 254 nm	Wolfender et al. (1997)
Radix puerariae (Fabaceae)	Column: HP ECLIPSE XDB-C18 column (150 × 4.6 mm) The mobile phase consisted of methanol:water = 22:78	Puerarin	Detection: UV 250 nm	Guo et al. (2001)
Adhatoda vasica (Acanthaceae)	Column: Merck Hibar C18 (250 × 4 mm, 10 µm) The mobile phase consisted of acetonitrile:0.1 M phosphate buffer:glacial acetic acid (15:85: 1)	Vasicine and Vasicinone	Detection: 300 nm	Srivastava et al. (2001)
Papaver somniferum L. (Papaveraceae)	Column: Merck Durasil C18 (250 × 64.6 mm, 10 µm) The mobile phase consisted of acetonitrile:0.1 M phosphate buffer:glacial acetic acid, 20:80:0.4, pH 3.8	Morphine, codeine, oripavine, codeinone, reticuline, thebaine, papaverine, narcotine	Detection 240 nm	Singh et al. (2000)

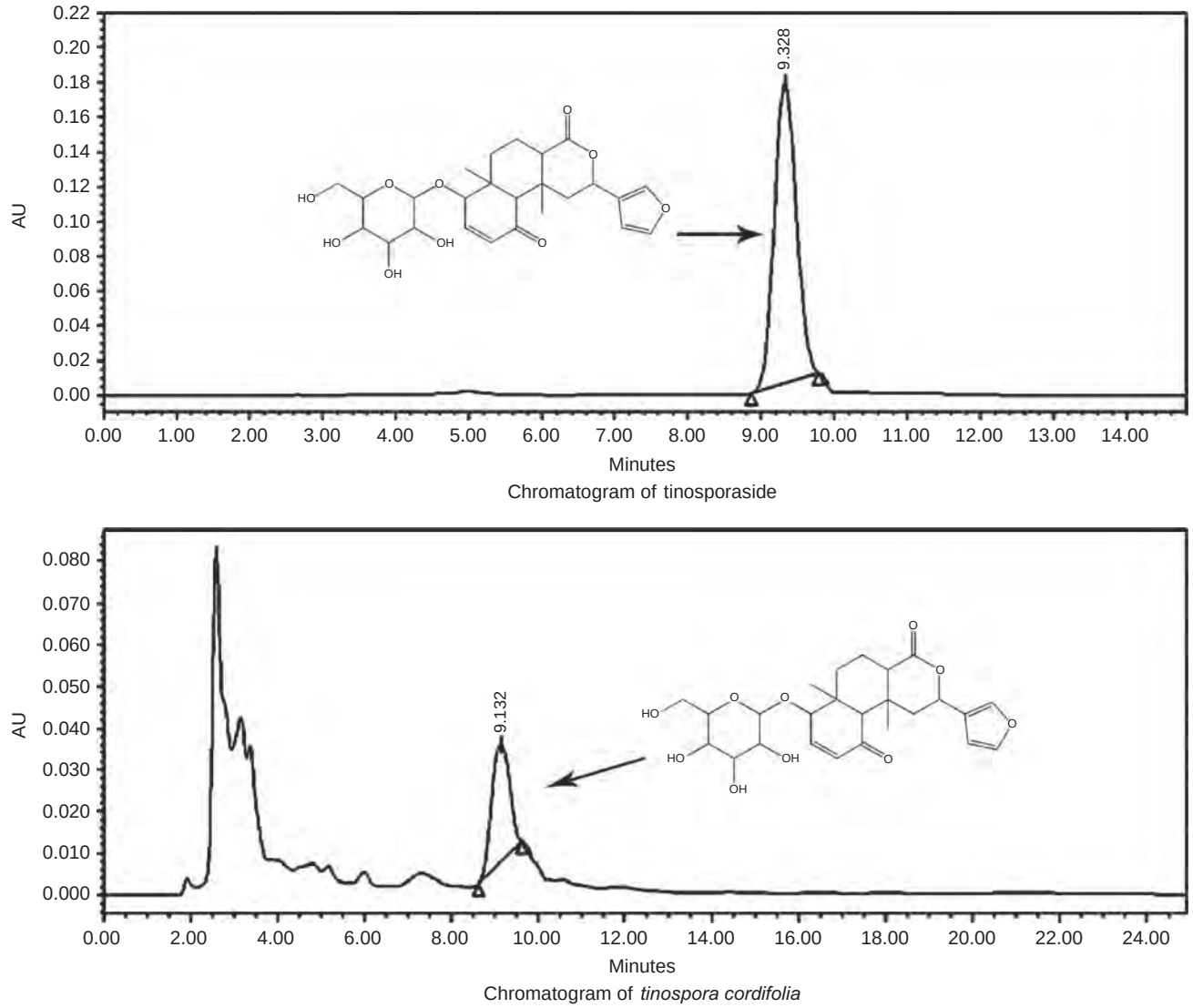


FIG. 10.2 RP-HPLC chromatograms of tinosporaside and methanolic extract of *Tinospora cordifolia*. *(Reproduced with permission from Bahadur, S., Mukherjee, P., Milan Ahmmed, S., Kar, A., Harwansh, R., Pandit, S., 2016. Metabolism-mediated interaction potential of standardized extract of Tinospora cordifolia through rat and human liver microsomes. Indian J. Pharmacol. 48, 576–581).*

10.10.3 *Swertia chirata*

The lyophilized extract of *S. chirata* was standardized by using RP-HPLC. Ursolic acid was used as the standard. A Waters RP-HPLC system (Milford, MA, USA) equipped with 600 quaternary pump, Rheodyne-7725i injector with a loop size of 20 µL, a PDA, and a C18 column (Waters Spherisorb, Ireland) with a length of 250 mm and width of 4.6 mm, a 5-µm particle size was used as the stationary phase. The peak area was calculated with Empower-2 software. A standard stock solution of ursolic acid (1 mg/mL) and *S. chirata* extract (1 mg/mL) were prepared in methanol (HPLC grade). Both the standard and extract solutions were filtered through a NYL (0.45 µm) syringe filter. Aliquots of 20 µL of both solutions were injected into the rheodyne injector port separately. Elution was carried out with an optimized mobile phase containing methanol:water 1% acetic acid (90:10, v/v) at a flow rate of 1 mL/min and at wavelength of 210 nm. The RP-HPLC method of ursolic acid was validated in terms of linearity, repeatability, intermediate precision, sensitivity, and recovery, as per the recommended guidelines of the International Conference on Harmonization (ICH). A quantitative measurement was performed by comparing the retention time (R_t) and peak area of ursolic acid with the extract. Standard ursolic acid showed good linearity in the range from 20 to 100 µg/mL on the calibration curve.

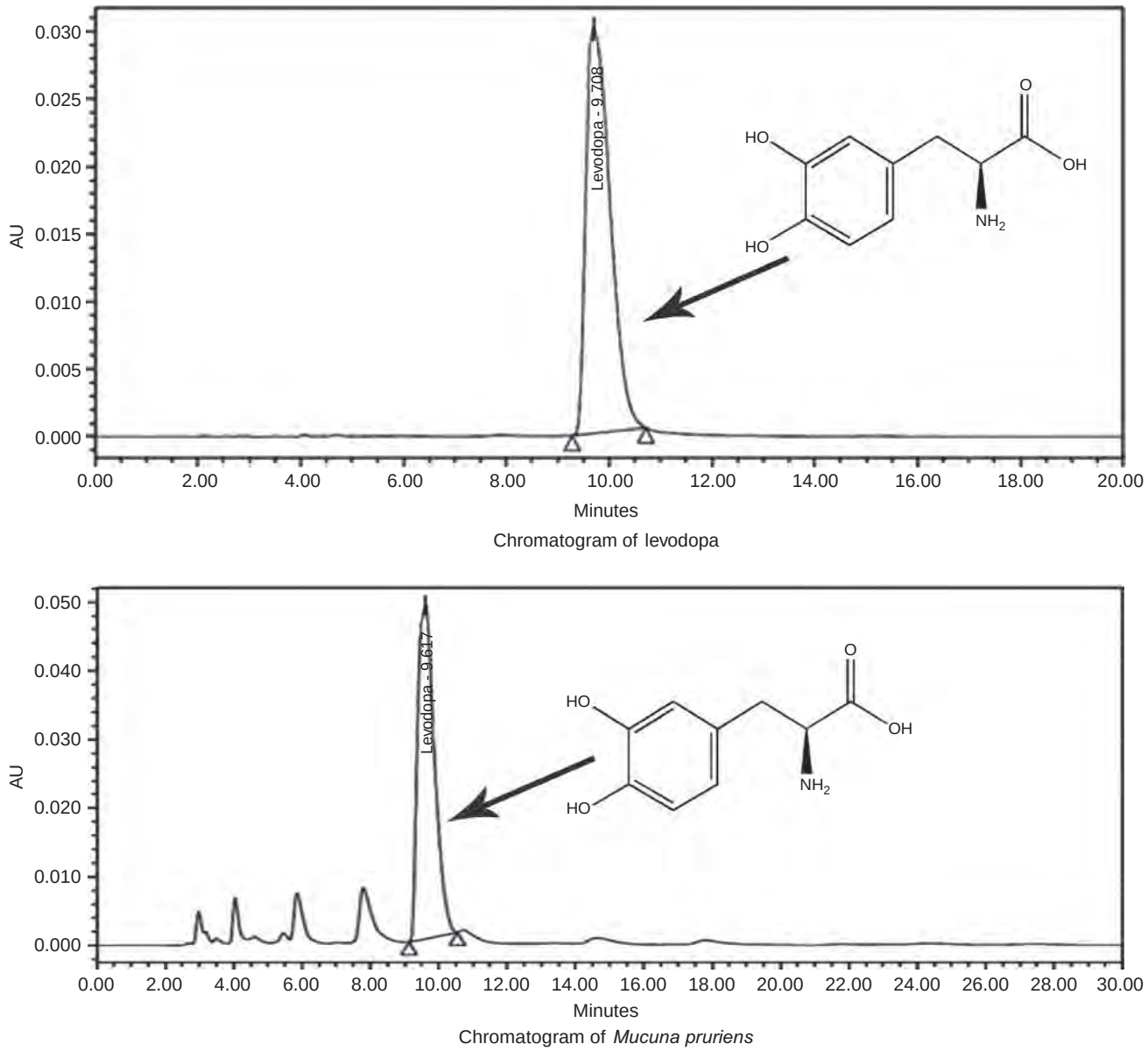
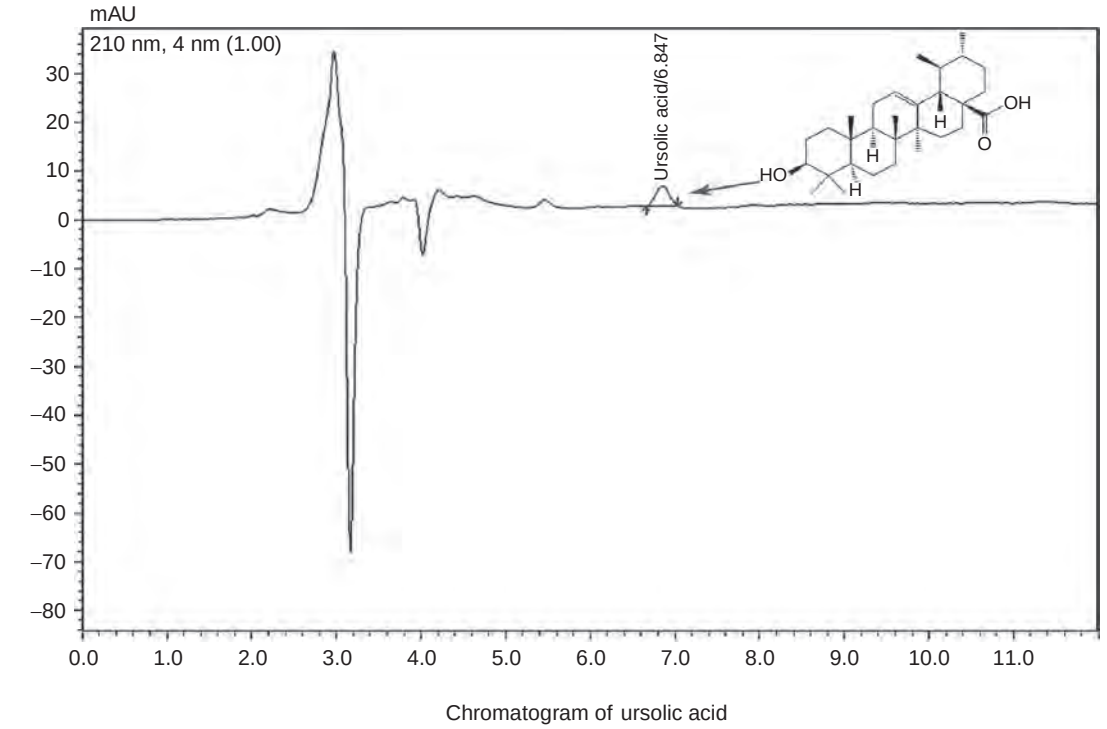


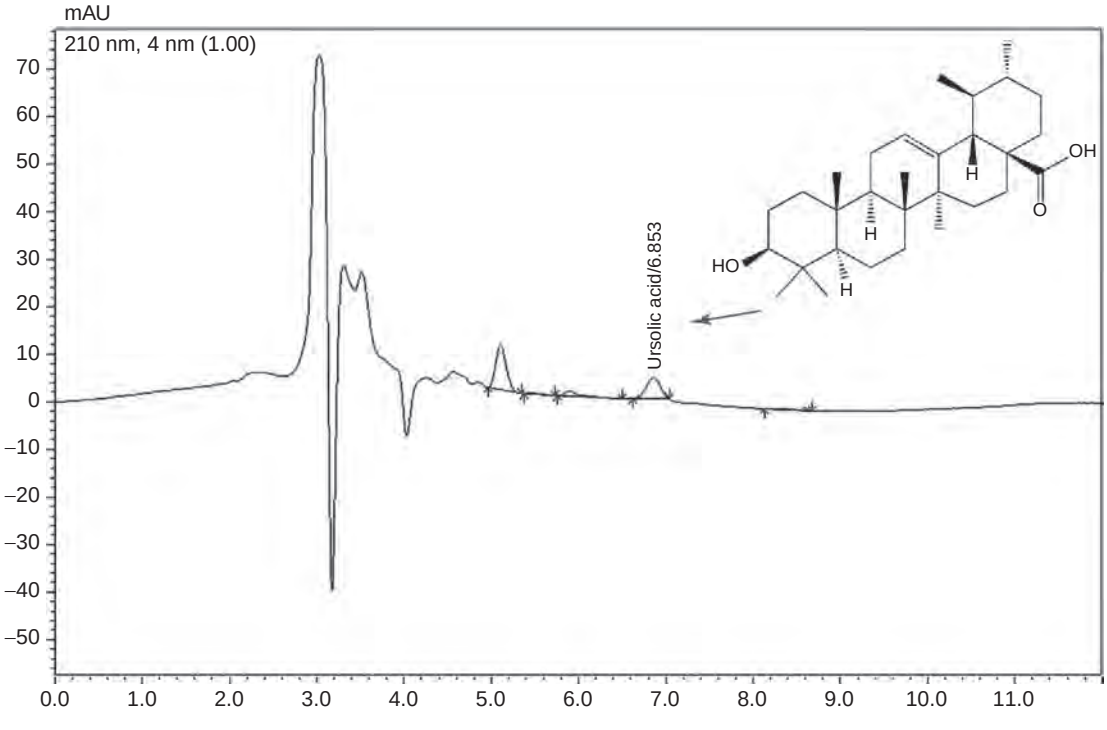
FIG. 10.3 RP-HPLC chromatograms of the standard levodopa and extract of *Mucuna pruriens. (Reproduced with permission from Chaudhary, S.K., De, A., Bhadra, S., Mukherjee, P.K., 2015. Angiotensin-converting enzyme (ACE) inhibitory potential of standardized* Mucuna pruriens *seed extract. Pharm. Biol. 53, 1614–1620).*

RP-HPLC chromatograms of ursolic acid and extract of *S. chirata* are shown in Fig. 10.4. The correlation between concentration and peak area was found to be good, with a correlation coefficient (r^2) value of 0.998. The % RSD was estimated to assess instrumental precision. The RSD values of instrumental precision and intra-assay precision of ursolic acid was estimated to be 2.0%, indicating the RP-HPLC method for ursolic acid was precise. The LOD and LOQ of ursolic acid were found to be 0.15 and 0.315 mg/mL. This result shows that the method was sensitive enough to detect the ursolic acid in the extract. The recovery measures the closeness between the theoretically added amount and the practically obtained amount and was performed by spiking with a known amount of ursolic acid in the sample.

The presence of ursolic acid in the SC extract was identified and quantified by comparing with the retention time (R_t 7.71 min) of standard ursolic acid. These results were considered satisfactory and acceptable for subsequent quantitative analysis. The ursolic acid present in *S. chirata* was found to be 2.66% (w/w) (Ahmmed et al., 2016).



Chromatogram of ursolic acid



Chromatogram of *Swertia chirata*

FIG. 10.4 RP-HPLC chromatograms of the standard ursolic acid and methanolic extract of *S. chirata*. *Reproduced with permission from Ahmmed, S.M., Mukherjee, P.K., Bahadur, S., Harwansh, R.K., Kar, A., Bandyopadhyay, A., Al-Dhabi, N.A., Duraipandiyan, V., 2016. CYP450 mediated inhibition potential of Swertia chirata: an herb from Indian traditional medicine. J. Ethnopharmacol. 178, 34–39.*

10.10.4 *Trigonella foenum-graecum*

Ahmmed et al. (2015) standardized an extract of *T. foenum-graecum* with respective biomarker trigonelline by using RP-HPLC. An RP-HPLC system (Shimadzu Prominence, Kyoto, Japan) equipped with two Shimadzu LC-20AD UFLC reciprocating pumps, a variable Shimadzu SPD-M20A Prominence PDA detector, and a Rheodyne manual injector with a loop size of 20 µL was used. The peak area was calculated with LC solution software. The analysis was carried out in isocratic conditions using a C18 reverse-phase column having dimension of 250 mm (length) × 4.6 mm (width) with a particle size of 5 µm (Phenomenex Luna C18, Torrance, CA, USA). Samples were filtered through a Whatman NYL 0.45 µm syringe filter and an aliquot of 20 µL of each sample was injected into the injector port. Elution was carried out with methanol: water containing 0.5% acetic acid (60:40) at a flow rate of 1 mL/min and the eluate was monitored at 254 nm. Trigonelline (1 mg/mL) solution was prepared in methanol as a stock solution. A calibration curve was plotted by diluting the stock solution in the concentration range of 200–1000 µg/mL. RP-HPLC chromatograms of trigonelline and extract of *T. foenum-graecum* are shown in Fig. 10.5.

The trigonelline present in *T. foenum-graecum* extract was identified by comparing the retention time (R_t) in chromatographic peaks of the standard with that of the extract. The percentage of trigonelline present in *T. foenum-graecum* was determined by constructing a calibration curve. Standard trigonelline exhibited good linearity in the range from 200 to 1000 µg/mL along the calibration curve. A good correlation between concentration and peak area was obtained, with a correlation coefficient (r^2) value of 0.997. The peak of trigonelline in the extract was identified by comparing with the R_t of the standard of trigonelline ($R_t = 4$ min). The content of trigonelline was found to be 3.38% (w/w) in the *T. foenum-graecum* extract (Ahmmed et al., 2015).

10.10.5 *Syzygium aromaticum*

Reverse-phase HPLC (RP-HPLC) standardization of clove oil (*S. aromaticum)* with standard eugenol was performed on an RP-HPLC Shimadzu Prominence, Kyoto, Japan. Clove oil (10 mg/mL) and standard eugenol (1 mg/mL) were dissolved in methanol for the chromatography analysis. An isocratic mobile phase (methanol:water, 75:25) condition was maintained throughout the process. The samples were eluted from the column at room temperature with a flow rate of 1 mL/min, which was detected at 254 nm. RP-HPLC chromatograms of eugenol and extract of *S. aromaticum* are shown in Fig. 10.6.

LC solution software was used for the study of the chromatogram. Identification and quantification of eugenol was verified by peak areas obtained in HPLC analysis. The retention time of eugenol in the extract was found to be 13.633 min, which was similar to the retention time of standard eugenol. The calibration curve of eugenol shows a linear relationship between the peak area and standard eugenol concentration with a correlation coefficient of $r^2 = 0.9958$. The amount of eugenol in clove oil was found to be 0.5 µg/mL (Dalai et al., 2014).

10.10.6 *Centella asiatica*

C. asiatica extract has been standardized by RP-HPLC analysis with respect to asiaticoside as a standard. A chromatographic separation of the extract and fractions was performed with a Waters liquid chromatographic system. A Waters Spherisorb (Ireland) C18 column (250 × 4.6 mm, 5 µm particle size) was used as a stationary phase for the separation. The mobile phase of a mixture of methanol:water:acetic acid (70:29:1, v/v) pH (7.2) was delivered at a flow rate of 1 mL/min with an injection volume of 20 mL for detection at 210 nm. The prepared solutions were filtered through a Whatman NYL 0.45 mm syringe filter prior to injection. Standard stock solutions of isolated asiaticoside as standard and samples were prepared by weighing 10 mg accurately and dissolving them with methanol in a 10 mL volumetric flask by ultrasonication. A calibration curve was plotted with standard asiaticoside by suitable dilution of the stock solution with the mobile phase, where five sets of asiaticoside standard dilutions were made to get the desired concentrations in a linearity range of 10–500 mg/mL. The retention time of asiaticoside was found to be 4.27 min. The calibration curve for asiaticoside was constructed by plotting the mean peak area against the concentration.

RP-HPLC chromatograms of asiaticoside and extract of *C. asiatica* are shown in Fig. 10.7. The linearity of the calibration curves was tested by regression analysis and found to be linear in the concentration range of 10–500 mg/mL, with good correlation between the concentration and mean peak area, with a correlation coefficient (R^2) of 0.929. The HPLC chromatogram of the asiaticoside standard compound, *C. asiatica* methanolic extract, and *C. asiatica* n-butanol fraction showed specific peaks at retention times of 4.277, 4.234, and 4.358 min, respectively, at a wavelength of 210 nm. The content of asiaticoside in the *C. asiatica* methanolic extract and *C. asiatica* n-butanol fraction were found to be 3.3% (w/w) and 4.27% (w/w), respectively (Nema et al., 2013).

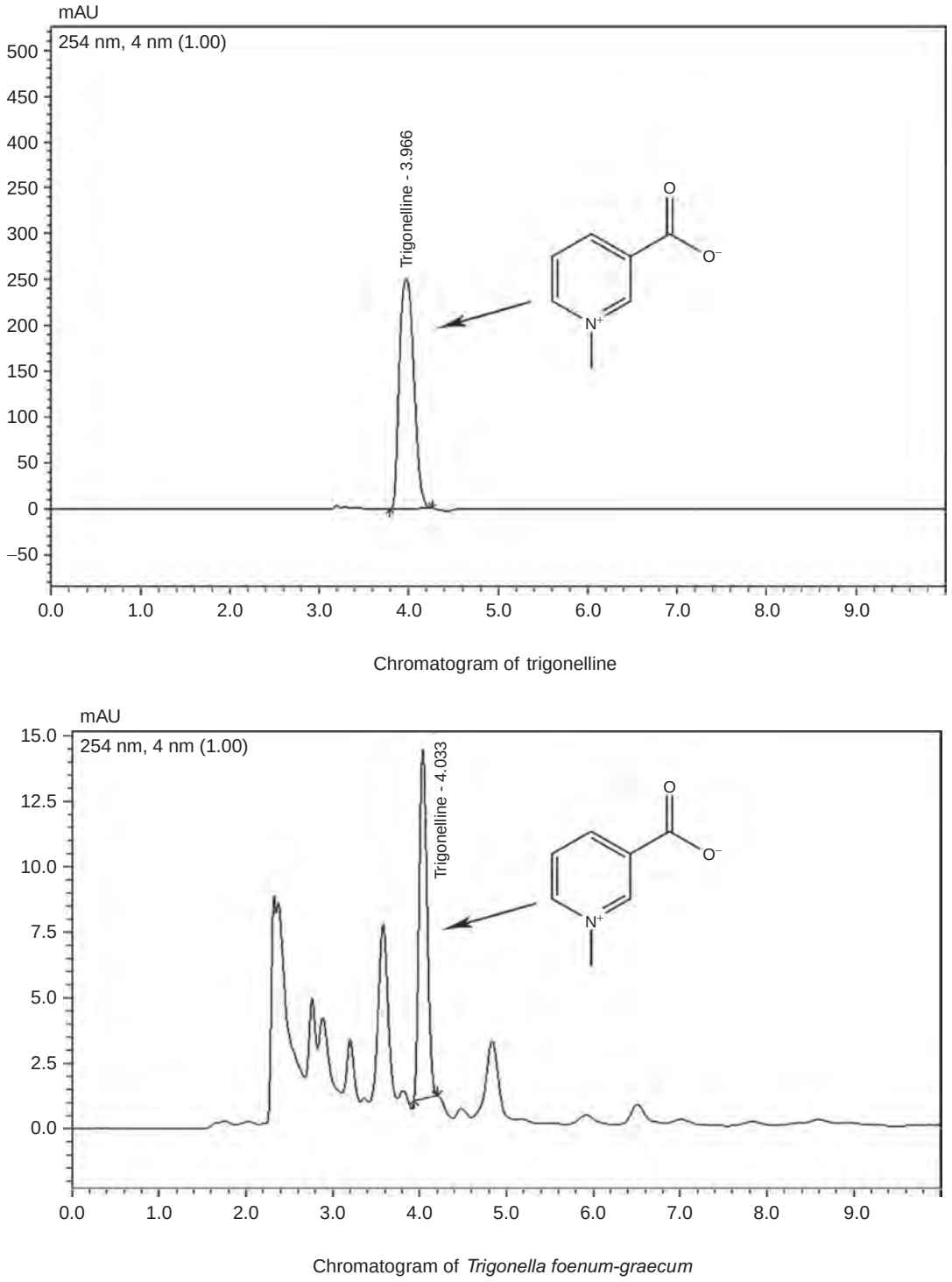


FIG. 10.5 HPLC chromatograms of the standard trigonelline and methanolic extract of *Trigonella foenum-graecum*. *Reproduced with permission from Ahmmed, S., Mukherjee, P., Bahadur, S., Kar, A., Mukherjee, K., Karmakar, S., Bandyopadhyay, A., 2015. Interaction potential of* Trigonella foenum *graceum through cytochrome P450 mediated inhibition. Indian J. Pharmacol. 47, 530–534.*

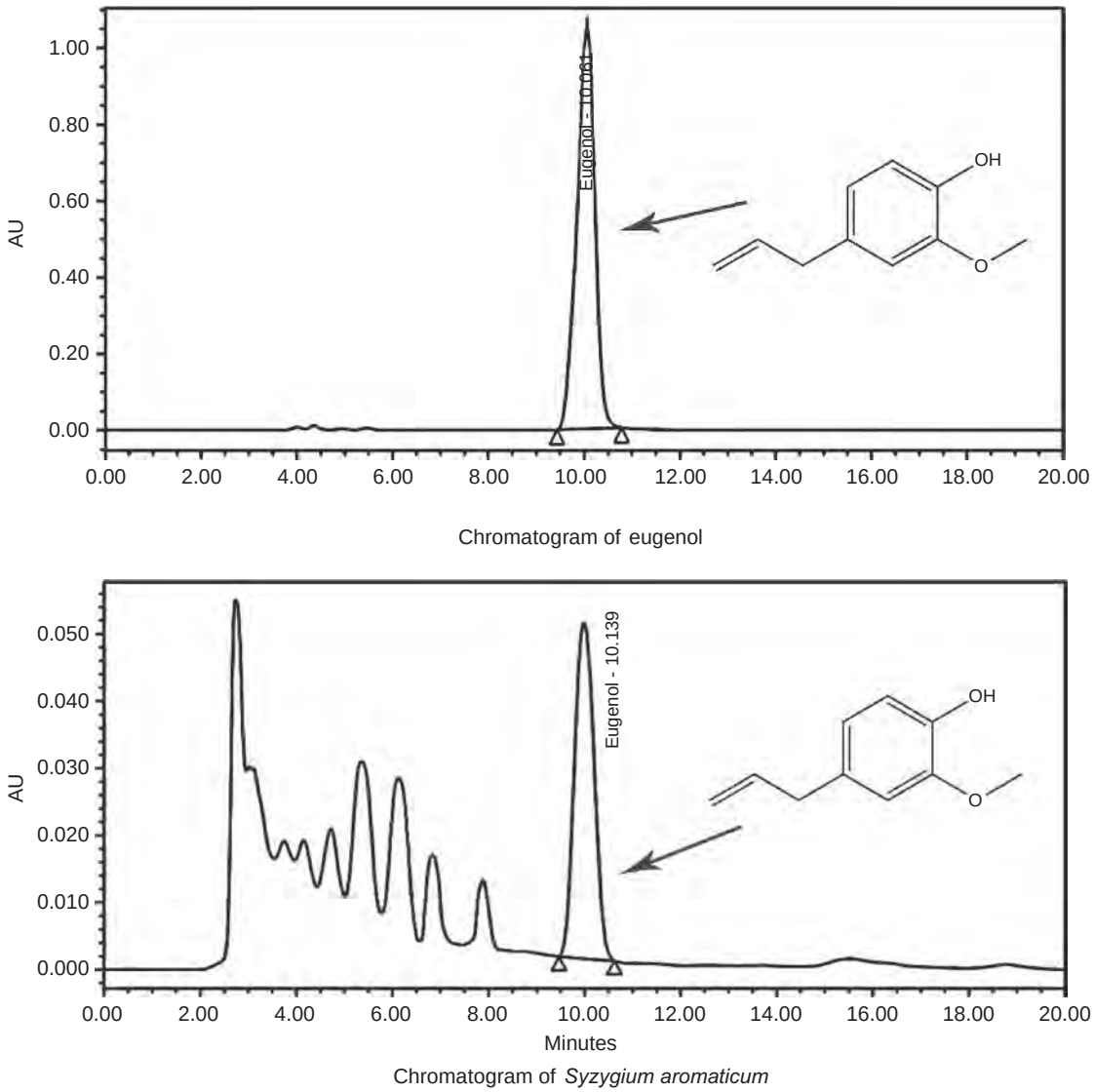


FIG. 10.6 RP-HPLC chromatograms of the standard eugenol and clove (*Syzygium aromaticum*) oil. *Reproduced with permission from Dalai, M.K., Bhadra, S., Chaudhary, S.K., Bandyopadhyay, A., Mukherjee, P.K., 2014. Anti-cholinesterase activity of the standardized extract of Syzygium aromaticum L. Pharmacogn. Mag. 10 (Suppl. 2), S276–S282.*

10.10.7 *Piper longum*

Chaudhary et al. (2013) standardized the extracts of *P. longum* with the phytomarker piperine. The extract and fractions were precisely weighed, extracted with methanol in a sonicator and filtered through a 0.45-μm filter. An aliquot of 20 μL was injected into the HPLC column (Waters Spherisorb C18 column, 250 × 4.6 mm, 5 μm) and elution was carried out using methanol:water:acetic acid (85:14:1, v/v/v) as mobile phase at a flow rate of 1 mL/min; the elute was monitored at 343 nm. RP-HPLC Chromatograms of piperine and extract of *P. longum* are shown in Fig. 10.8. The quantification of piperine in the crude extract and fractions was made using HPLC. The concentration of piperine in the extract and fractions was calculated from the experimental peak areas by interpolation to the standard calibration curve. The content of piperine was on the order of PLE (2.34% ± 0.20%, w/w) (Chaudhary et al., 2013).

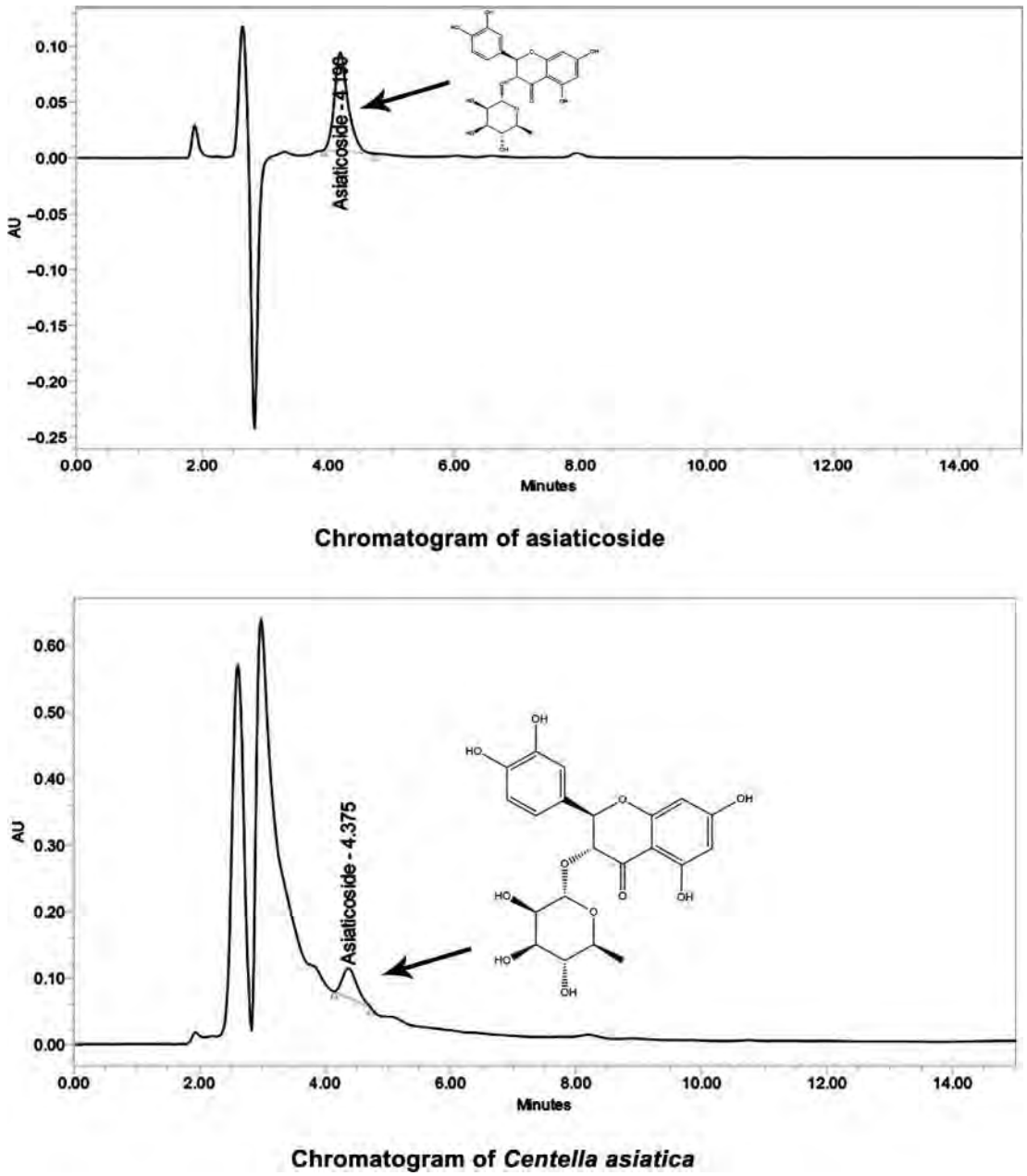


FIG. 10.7 RP-HPLC chromatograms of the standard asiaticoside and *Centella asiatica* methanolic extract. *Reproduced with permission from Nema, N.K., Maity, N., Sarkar, B.K., Mukherjee, P.K., 2013. Matrix metalloproteinase, hyaluronidase and elastase inhibitory potential of standardized extract of* Centella asiatica*. Pharm. Biol. 51, 1182–1187.*

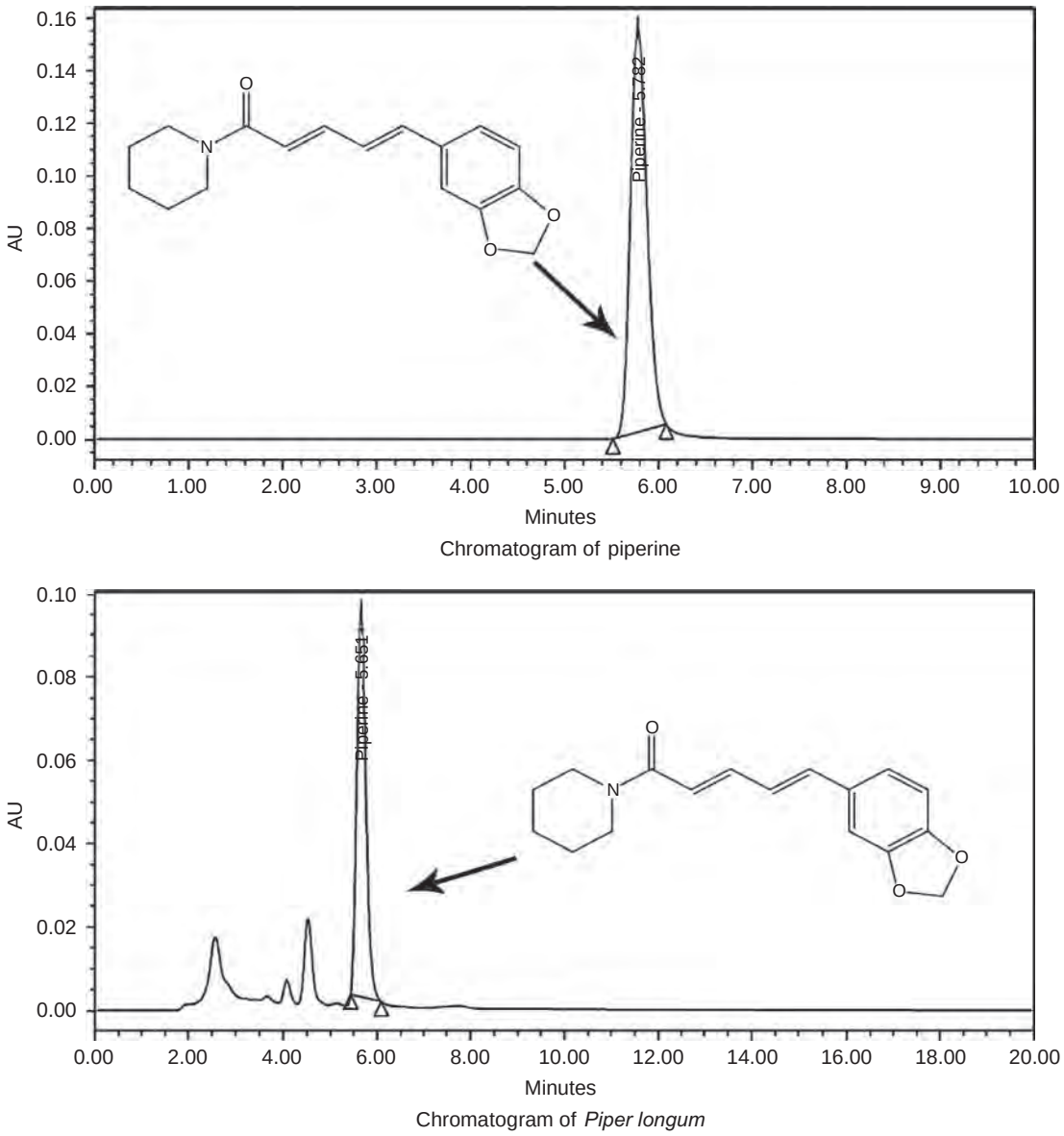


FIG. 10.8 RP-HPLC chromatograms of standard piperine and *Piper longum* methanolic extract. *Reproduced with permission from Chaudhary, S.K., Mukherjee, P.K., Maiti, N., De Kumar, A., Bhadra, S., Saha, B.P., 2013. Evaluation of angiotensin converting enzyme inhibition and anti-oxidant activity of Piper longum L. Indian J. Tradit. Knowl. 12, 478–482.*

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Chapter 11

LC–MS: A Rapid Technique for Understanding the Plant Metabolite Analysis

Chapter Outline

11.1 BASIC THEORY AND PRINCIPLE

The concept of mass spectrometry (MS) was first proposed by J.J. Thomson of Manchester, England during cathode-ray-tube experimentation in 1897. Later, in 1953, Wolfgang Paul invented the quadrupole and quadrupole ion trap for which he was awarded the Nobel Prize in Physics. This was followed by development of electrospray ionization (ESI) in 1968. After a few years, another scientist, Horning, developed atmospheric pressure chemical ionization (APCI) in 1974. In 1983, Vestal and Blakely proposed a technique involving a heated liquid stream for the introduction of a sample in a mass analyzer, called thermospray. There has been tremendous development in the mass spectrometric field during the last decade. Major advancements in the mass spectrometric analyzer led to some newer hybrid technology with robust applications (Griffiths, 2008).

MS is a powerful analytical technique used for the identification and quantification of both unknown and known compounds and also to elucidate the structure and chemical properties of different molecules. The process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass-to-charge (m/z) ratios and relative abundances. MS is also widely applicable for structure elucidation of some biomolecules (peptides and proteins), natural products, and some organic compounds. The first step in the mass spectrometric analysis of the compounds is to produce gas-phase ions of the compound, basically by electron ionization in order to undergo fragmentation. Thus, each molecular ion produces primary product ions that are subsequently separated in the mass spectrometer according to their mass-to-charge ratio, and detected in proportion to their abundance. Thus, a mass spectrum

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of the molecule is produced, which can be represented in a graphical form (a plot of ion abundance vs mass-to-charge ratio). The structure and nature of the precursor molecule can be interpreted from the mass spectrum. In the case of any pure compound, a molecular ion corresponds to a higher *m*/*z* value, which indicates the molecular mass of the compound.

A mass spectrum is a plot of the mass-to-charge ratio of positively charged ions against their relative abundance. The most intense peak in the mass spectrum is called the base peak and is assigned a relative intensity of 100%. A molecular ion is formed by the removal of one electron of lowest ionization potential form the analyte molecule. The molecular ion is detected as a mass-to-charge ratio that corresponds to the molecular weight of molecule. The molecular ion peak gives the molecular weight of compound. The ions produced from the molecular ion by cleavage of bonds are called fragment ions. They have lower masses and used as building blocks to reconstruct the molecular structure. Most of the fragmentation occurs due to heterolytic and homolytic cleavage of molecular bonds. In many analyses, the compound(s) of interest are found as part of a complex mixture and the role of the chromatographic technique is to provide separation of the components of that mixture to allow their identification or quantitative determination. From a qualitative perspective, the main limitation of chromatography in isolation is its inability to provide an unequivocal identification of the components of a mixture even if they can be completely separated from each other. Identification is based on the comparison of the retention characteristics, simplistically the retention time, of an unknown with those of reference materials determined under identical experimental conditions. The combination of the separation capability of chromatography to allow "pure" compounds to be introduced into the mass spectrometer with the identification capability of the mass spectrometer is advantageous, particularly as many compounds with similar or identical retention characteristics have quite different mass spectra and can therefore be differentiated. The power of MS lies in the fact that the mass spectra of individual compounds are unique to allow their identification with a high degree of confidence. The combination of HPLC with MS therefore allows more definitive identification and the quantitative determination of compounds that are not fully resolved chromatographically.

The LC/MS system consists of an HPLC pumping system, injector, and column coupled to a mass spectrometer through some type of evaporative ionizing interface. A computer system coordinates the components of the system together by providing control of the HPLC for flow, solvent gradient, and remote starting of injection and the gradient run. It also provides control of the mass spectrometer scan range and lens, and accesses and processes data from the ion detector's amplifier. All of this is done through either a remote control interface or through A/D (analog-to-digital; data input) and D/A (digital-to-analog; control) microprocessor cards in the computer system module. The digital data from the A/D card is then processed by the computer's software to provide a total ion chromatogram (TIC) and the molecular weights of the compounds in the peaks detected using the mass spectrometer's spectral data (Cai et al., 2018; McMaster, 2005).

However, although LC–MS profiling has become more advanced, it may not always be sensitive enough to detect and characterize metabolites at trace levels. Hence, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed for analyzing small molecules and offers higher sensitivity and selectivity in the trace analysis of multicomponent or multiconstituent substances (de Hoffmann and Stroobant, 2003).

11.2 IONIZATION SOURCES

An LC–MS system consists of several elements, which include:

- A sample inlet to introduce the compound that is analyzed (e.g., LC).
- An ionization source to produce ions from the sample; one or several mass analyzers to separate the various ions.
- Detectors that detect the ions based on the *m*/*z* ratio.
- A data processing system that aids in analog-to-digital conversion for processing of mass spectral data.

However, some mass spectrometers combine the sample inlet and the ionization source and others combine the mass analyzer and the detector. In an MS system, the gaseous phase ions are generated by ionization of the analyte molecule in a vacuum. In the case of ESI, the samples get volatilized and then ionize in a discrete way. It is difficult to ionize most of the biological molecules due to their high molecular weights and high polarities, as it limits their volatility. Although a number of ionization techniques have been developed over the years for the analysis of nonvolatile and thermally labile compounds, there are four major techniques involved (Yamashita and Fenn, 1984):

(i) Electrospray ionization (ESI)
(ii) Matrix-assisted laser desorption/ionization (MALDI)
(iii) Fast atom bombardment (FAB) and
(iv) Atmospheric pressure chemical ionization (APCI).

Among several ionization processes, proton transfer is considered to be the most important phenomenon in LC–MS analysis. For example, the analyte molecules (M) are converted into protonated molecular ions (MH+) by accepting a

TABLE 11.1 Different Ionization Sources in Mass Spectrometry

Ionization Source	Event
Electrospray ionization (ESI)	Evaporation of charged droplets
Nanoelectrospray ionization (nanoESI)	Evaporation of charged droplets
Atmospheric pressure chemical ionization (APCI)	Corona discharge and proton transfer
Matrix-assisted laser desorption ionization (MALDI)	Photon absorption/proton transfer
Desorption/ionization on silicon (DIOS)	Photon absorption/proton transfer
Electron ionization (EI)	Electron beam/electron transfer
Chemical ionization (CI)	Proton transfer
Fast atom/ion bombardment (FAB)	Ion desorption/proton transfer

proton transferred from molecule M, based on the acid–base reaction principle. In this case, the analyte molecule serving as a Bronsted acid (proton donor) releases a proton to the Bronsted base (proton acceptor), and thus ionizes it. The ionization capacity of the reagent ion depends on its proton affinity values (Kang, 2012). Table 11.1 describes the different types of ionization techniques involved in an LC–MS/MS study.

11.2.1 Electrospray Ionization (ESI)

In ESI, the analyte solution is converted into charged droplets (in aerosol form) by electrostatic spraying of the sample. Sometimes N_2 gas is used to nebulize the sample. On nebulization, the solvent and analyte molecules produce small droplets with a net positive or negative charge, which depends on the polarity of the applied voltage. Thereafter, the solvents get vaporized and eventually the ions become free to enter in the mass analyzer. The major principle behind the ionization process is protonation/deprotonation. In fact, it was observed that some proteins, peptides, oligonucleotides, etc., offer protonation or deprotonation in the presence of acid/base functionality in their structure. Multiple charging also enables mass spectrometers with limited *m*/*z* ranges to analyze higher-molecular-weight molecules. ESI has some very impressive attributes that allow it to be used for a wide variety of biological problems. Perhaps the most practically useful attribute of ESI is its ability to couple MS and liquid separation techniques (Glish and Vachet, 2003). ESI has two notable disadvantages:

- The flowing nature of ESI demands a constant flow of sample in which there is a possibility of wasting sample material in each run. This can be addressed by using mass analyzers that have an inherently higher duty cycle and by developing pulsed ESI sources.
- Susceptibility of ESI to ion suppression effects: At higher salt concentration (>~1 mM), the formation of an analyte ion is usually hindered, which creates an urgent need for desolation of biological samples before analysis. In addition, when complex mixtures of compounds are present, the higher-concentration analytes can suppress ion formation by lower-concentration analytes (Kebarle, 2000).

11.2.2 Atmospheric Pressure Chemical Ionization (APCI)

CI consists of producing ions through a collision of the molecule to be analyzed with primary ions present in the source. Molecules can be ionized by transfer of an electron, a proton, or other charged species, and either positive or negative ions can result. CI relies on gas-phase chemical reactions that take place between the analyte of interest and ions generated from a reagent gas. When coupled with LC, the composition of the reagent gas is derived from the mobile-phase solvents. The initial electrons are produced by a discharge from a corona needle. APCI is a method that is typically done using a similar source as ESI, but instead of putting a voltage on the spray itself, the voltage is placed on a needle that creates a corona discharge at atmospheric pressure. This discharge creates ions, in theory mostly H_3O^+ or water clusters. The sample is injected into the discharge by a spray created by a flow of liquid combined with a heated gas that volatilizes the sample. The ions are formed by proton transfer from the H_3O^+ or the water clusters to the sample. These ions are then extracted into the same opening vacuum that is used for electrospray. Another variation of this technique is atmospheric pressure

photoionization (APPI), in which the initial ionization is performed by photoionization, usually of a dopant that absorbs the light and is added to the sample flow.

In the case of APCI, some analyte compounds suppress the formation of ions. For example, sometimes amines are added to improve the chromatographic behavior of some samples, which in turn protonates the analyte molecule. As a result, the selection of additives is of utmost importance for analyzing complex mixtures. APCI can be combined with liquid chromatographic systems in a manner similar to ESI. APCI offers less susceptibility to matrix interference from salts and it can also ionize the weakly polar analytes in solution. Hence, high-sensitivity APCI is quite applicable in pharmacokinetic studies for monitoring drug and metabolite concentrations in in vivo systems. However, a major drawback of APCI is the decomposition of thermally labile compounds during the nebulization process (Byrdwell, 2001).

The most stable products after ionization (for example, the analytes with the highest basicity) will usually be observed to the exclusion of less stable products (analytes with the lowest basicity) (Rosenberg, 2003).

11.2.3 Atmospheric Pressure Photoionization (APPI)

The APPI source represents an advancement in atmospheric pressure sources. The basic principle is to use photons to ionize gas-phase molecules. In APPI, the sample in solution is evaporated by applying heated nebulizer, similar to APCI. As the analyte solution gets vaporized, it readily interacts with the photons emitted from a discharge lamp. These photons, in a gas-phase reaction, lead to ionization of the sample molecules. Three main steps are responsible for detection by photoionization in the vapor phase: vaporization of the eluent, production of the photo-ions by interaction between a photon emitted by a UV source and analytes, and detection through a mass spectrometer (Raffaelli and Saba, 2003). The major benefit of photoionization involves the ionization of those molecules which are not ionizable by APCI and ESI. It has been successfully demonstrated to provide high sensitivity to LC–MS analysis. A vacuum-ultraviolet lamp designed for photoionization detection in gas chromatography is used as a source of 10-eV photons. The mixture of samples and solvent eluting from an HPLC is fully evaporated prior to introduction into the photoionization region. There are some APPI sources, which have been developed to couple with ion-mobility spectrometry, used for the detection and quantitation of those classes of chemical compounds that are ionizable by a suitable vacuum-UV radiation (Raffaelli and Saba, 2003; Robb et al., 2000).

11.2.4 Thermospray

In a thermospray interface, a jet of vapor and small droplets is formed by heating the column effluent of an LC column or any other continuous liquid stream in a heated vaporizer tube. Nebulization takes place as a result of the disruption of the liquid by the expanding vapor that is formed upon evaporation of part of the liquid in the tube. A considerable amount of heat is transferred to the solvent prior to the onset of the partial inside-tube evaporation. The ionization of the analytes takes place by mixed mechanisms based on gas-phase ion-molecule reactions and ion-evaporation processes. The eluent from the column is vaporized and thus a portion of vapor is transferred to the mass spectrometer and rest of the vapor is pumped to waste. As a result, a supersonic jet of vapor, containing a mist of particles and solvent droplets, is created. There vaporization takes place in the presence of an electrolyte with the LC buffer and the droplets are charged. And finally, they enter into the ionization chamber. The reagent gas for ionization can be made either in a conventional way using energetic electrons from a filament or discharge electrode, or in a process called thermospray buffer ionization, in which the volatile buffer dissolved in the eluent is involved (Niessen, 2017).

11.2.5 Matrix-Assisted Laser Desorption/Ionization (MALDI)

Ions are produced by pulsed-laser irradiation of a sample, which are mixed on a probe that is inserted into the vacuum system, and after irradiation, the gas-phase ions that are formed are directed toward the mass analyzer. The sample is cocrystallized with a solid matrix that can absorb the wavelength of light emitted by the laser. The ions generated from a MALDI source are transferred into a vacuum system for mass analysis, similar to the ESI technique. A typical ionization of the analyte is observed in MALDI, but the mechanism is still to be explored. MALDI can rapidly provide information on the molecular weight for one or more analytes while coupled with certain mass analyzers (for example, time-of-flight—TOF). High-throughput behavior can also be observed by using sample plates with higher load. In fact, MALDI can ionize proteins directly from cell lysates and whole cells. Even though the pulsed nature of the technique is one source of MALDI's inherent sensitivity, it is also a source of difficulty when coupling to some mass analyzers (Nesvizhskii et al., 2007; Iwamoto and Shimada, 2017).

11.3 MASS ANALYZER

The mass analyzer is one of the major components in a mass spectrometer, which separates ions by the mass divided by their charge, that is, their *m*/*z* ratio. These can be molecular ions with positive charges, adducts made from a combination of molecular ions and solvent or mobile-phase components, fragmentation ions from a collision chamber, or negatively charged ions produced when the polarity is switched in the ionization chamber and in the draw-out and focusing lens. The purpose of the analyzer is to hold ions, select specific mass ions as the radio frequency is scanned, and move the selected ions into the ion detector for counting (McMaster, 2005). Analyzers can be divided into two basic types. Scanning analyzers consist of a flight tube occupied with the magnetic field, which transmits the ions of different masses successively along a timescale based on their mass-to-charge ratio. The other type of analyzers, namely, dispersive magnetic analyzers, TOF analyzers, ion trap mass analyzers, and orbitrap, allow the simultaneous transmission of all ions.

A mass spectrum may be considered to be a plot of the number of ions of each *m*/*z* ratio produced by an analyte upon ionization. After the production of ions using an appropriate ionization method, it is therefore necessary to separate the ions of different *m*/*z* ratios, determine these *m*/*z* values, and then measure the relative intensities of each group of ions. MS can be performed in two general data-acquisition modes:

(i) Full spectrum analysis or SCAN mode, in which a series of mass spectra are acquired. This mode is typically applied in qualitative analysis. In the scan mode, the instrument detects signals over a wide mass range (e.g., from 50 to 2000 *m*/*z*) during a short period of time. During this scan period, the MS electronics sequentially read the signals detected within narrower mass intervals until the full mass range is covered. The spectra represent the detected signal for the full mass range. Because full mass spectra are recorded, this mode of operation is typically selected for qualitative analysis of unknown analytes.

(ii) Selected-ion monitoring (SIM) mode, in which the ion abundances of preselected ions are acquired. SIM is applied in routine quantitative analysis. In quadrupole and magnetic sector instruments, acquisition in SIM mode provides a substantial gain in signal-to-noise (S/N) ratio (Niessen, 2006). MS can also be operated in the SIM mode. Other than scanning at a wide range of mass, they can be set to only monitor a few *m*/*z* ratios. As a result, the quadrupole is able to spend significantly more time sampling each of the *m*/*z* values, with a concomitant and large increase in sensitivity. Moreover, because the cycle time between data points is often shorter than it is in scan mode, quantitative precision and accuracy are improved through optimal peak-shape profiling. Because the *m*/*z* values to be sampled must be set in advance, SIM is favored for target compound analysis.

For analysis of multiple target compounds, SIM ion sampling choices can be time programmed to match compound elution time windows. The basis of selection of the mass analyzer is the desired resolution, which can be defined as the ability to differentiate between closely related signals (Price, 1991). In MS, these "signals" are the *m*/*z* ratios of the ions, with the resolution being defined mathematically as follows:

$$R = m / \Delta m,$$

where R is the resolution, m is the "*m*/*z*" to be measured and Δm is the difference (in Dalton) between this and the ion from which it is to be separated. It is to be noted that, due to its inherent characteristics of accurate mass measurements and multiple stages analysis, the integrated strategy of LC coupled with TOF–MS and ion trap mass spectrometry (IT-MS) has emerged as a very powerful tool in both the qualitative and quantitative study of herbal medicine (Brenton and Godfrey, 2010).

11.3.1 Quadrupole Analyzer

The quadrupole is composed of two pairs of metallic rods. Each opposing rod pair is connected together electrically, and the direct current (dc) charged surfaces of the analyzer are then swept with a changing radio-frequency (RF) signal that selects for different mass ions for each frequency, allowing them to follow a stable path to the detector. A direct current voltage is then superimposed on the RF voltage. Ions travel down the quadrupole between the rods. Only ions of a certain mass-to-charge ratio (*m*/*z*) reach the detector for a given ratio of voltages. Other ions have unstable trajectories and collide with the rods. This permits selection of an ion with a particular *m*/*z* and allows the operator to scan for a range of *m*/*z* values by continuously varying the applied voltage. This cascade of electrons amplifies the signal of a single contact for transfer to the data system. In the data system, the data received are converted to a chromatogram of signal strength vs elapsed time as a TIC. There are several advantage of triple quadrupole (MS/MS) systems in comparison with single quadrupole (MS) systems, including higher selectivity, resulting in less interference of coeluting compounds and matrix, better S/N allowing lower limits of quantitation, more reliable identification of detected analytes using multiple reaction monitoring (MRM) in comparison with SIM, a wider linear range of quantitation, and better accuracy and reproducibility, especially at low concentrations (Johnson et al., 1990).

11.3.2 Time-of-Flight (TOF)

The initial idea of measuring the mass of an ion by its flight time was put forward by Stephens in 1946. This very simple concept makes TOF an attractive method, but high demands on electronics and limited resolution constrain its applications. The principle of TOF a mass analyzer is based on the different velocities of two ions, created by a uniform electromagnetic force applied to all of the ions at same time, which causes them to accelerate down a flight tube. The ions are expelled from the source in the presence of plasma or laser desorption, after which they are accelerated toward the flight tube by a difference of potential applied between an electrode and the extraction grid. The ions are characterized based on their distribution in kinetic energy and velocity. Thus, the lighter ions travel more quickly and strike the detector first so that the *m*/*z* ratio of the ions is detected.

An LC–MS instrument with TOF detectors is found to be very suitable for detection in herbal components analysis as it is very well suited to perform structure elucidation or confirmation, especially for nontarget compounds. TOF provides information regarding the elemental composition of parent and fragment ions by its accurate mass measurement (<5 ppm) and full-scan spectral sensitivity. The TOF instrument is able to identify nontarget compounds because it can record the full spectrum at all times with accurate mass measurements, which cannot be done through the SIM or MRM techniques. The high resolution, accurate mass measurement capability, and full-scan spectral sensitivity of TOF instruments are found very applicable for the identification of unknown secondary metabolites present in plant extracts. The hyphenation of TOF with quadrupole systems also offers excellent mass resolution and sensitivity. In Qq-TOF, the last quadrupole section is replaced by a TOF analyzer. One of the main advantages of Qq-TOF instruments over triple quadrupole ones is the high mass resolution of TOF, typically around 10,000 (Williamson and Bartlett, 2007; Bristow, 2006).

11.3.3 Ion Trap

IT-MSs are more sensitive than a quadrupole. They can easily be switched between APCI, CI, and EI modes, and they have the potential to be used for MS/MS studies. In an ion trap detector, the ionized molecules enter the trap from a pinhole orifice on the side of the ring electrode and are held in three hyperbolic electrodes to trap ions in a three-dimensional space using static and radio-frequency voltages from the DC/RF source. Ion trap analyzers use three hyperbolic electrodes to trap ions in a three-dimensional space using static and radio-frequency voltages. Ions are then sequentially ejected from the trap on the basis of their *m*/*z* values to create a mass spectrum. Alternatively, a specific ion can be isolated in the trap by the application of an exciting voltage while other ions are ejected. An inert gas can also be introduced into the trap to induce fragmentation (Tozuka et al., 2003). An interesting feature of these ion trap analyzers is the ability to fragment and isolate ions several times in succession before the final mass spectrum is obtained, resulting in so-called MS^n capabilities (Payne and Glish, 2005).

11.3.4 FT-ICR

FT–ICR–MS instruments provide extremely high (mass-dependent) resolution, accurate mass determination, and a dynamic range of five orders of magnitude. In FT-ICR, the entering ions are trapped in circular well-defined orbits for extended periods by electrical and magnetic fields. They are excited by RF and generate current and convert by Fourier transform into orbital frequencies. The angular frequency of motion is called the cyclotron frequency. A charged particle in a magnetic field with a velocity vector perpendicular to the magnetic field experiences a force perpendicular to the plane defined by the velocity and the magnetic field. This force is always perpendicular to the direction of the velocity vector. Following the vector of velocity, it describes a circle and as a result, the charged particle (an ion) is trapped by the magnetic field on a circular trajectory. The frequency of this rotation is characteristic for each mass and magnetic field, and in a uniform magnetic field it is not dependent on initial coordinates and velocities of ions. The resolution increases with measurement time, and longer measurement times are only possible if extreme high vacuum (~10–9 mbar) is achieved in the ICR cell (Nikolaev and Kostyukevich, 2016).

11.4 DETECTORS

The detector generates a signal from incident ions by either generating secondary electrons, which are further amplified, or by inducing a current generated by a moving charge (similar to FT-ICR). In MS, the separation of bunches or streams of ions occurs according to their individual *m*/*z* ratios. The choice of detector depends on the design of the instrument and the type of experiment it was designed to perform. The earliest ion detectors consisted of photographic plates located at the end of the mass analyzer. All ions of a given *m*/*z* would impact at the same place on the photographic plate, making a spot. The darkness of the spot was indicative of the intensity of that particular *m*/*z*. The most common types of ion detector used are described in subsequent sections (de Hoffmann and Stroobant, 2003).

11.4.1 Photographic Plates

Photographic plates were a first-generation detector located behind the analyzer in earlier LC–MS instruments. In principle, ions having the same *m*/*z* ratio generally reach the plate at one particular position, by which the *m*/*z* values are determined. The relative abundances are measured based on the darkness of the spot observed. Simultaneous detection over a large *m*/*z* range is allowed.

11.4.2 Faraday Cup

The basic principle of a Faraday cup is that the incident ion strikes the dynode surface, which emits electrons and induces a current which is amplified and recorded. The dynode electrode is made of a secondary emitting material, such as CsSb, GaP, or BeO. The Faraday cup is a relatively insensitive detector but is very robust.

11.4.3 Electron Multiplier

Electron multipliers are probably the most common means of detecting ions, especially when positive and negative ions need to be detected on the same instrument. It consists of a series of dynodes maintained at increasing potentials, resulting in a series of amplifications. Another type of electron multiplier consists of curved ("horn" shaped) continuous dynodes in which amplifications occur through repeated collisions with the dynode surface. In both cases, ions pass the conversion dynode (depending on their charge) and strike the initial amplification dynode surface. Thus, a large number of secondary electrons are emitted, which are then attracted either to the second dynode or into the continuous dynode in which more secondary electrons are generated in a repeating process that ultimately results in a cascade of electrons.

11.4.4 Scintillation Detectors

In scintillation detectors, the ions initially strike a dynode, which results in electron emission. These electrons then strike a phosphorous screen, which in turn releases a burst of photons. The photons then pass into the multiplier where amplification occurs in a cascade fashion, much like with the electron multiplier. The main advantage of using photons is that the multiplier can be kept sealed in a vacuum, preventing contamination, and extending the lifetime of the detector.

11.5 DATA HANDLING, LIBRARIES, AND DATABASE

In earlier days, biochemical approaches typically focused on a very limited number of metabolites, keeping the results manually interpretable by researchers. However, being a very active field of research, metabolomics has made rapid progress, nowadays allowing modern instrumentation to measure thousands of metabolites simultaneously. This growing complexity of high-throughput small-molecule measurements constitutes a substantial challenge to researchers. The challenges arise in a derivation of biologically meaningful results given thousands of chemically distinct metabolites measured in a specific experiment. In order to answer this question, robust statistical methods are suitable for analysis and functional interpretation of the complex interactions between the analytes. The workflow of LC–MS-based metabolomics data processing is discussed in Chapter 17. Methodologies used to interpret high-throughput metabolomics data can be categorized as univariate and multivariate analysis.

- The univariate techniques include *t*-test, fold-change analysis, and Wilcoxon rank-sum test; analysis of variance (ANOVA) and other techniques are used to assess the statistical significance of each peak separately. They are used to find the significantly altered metabolites in either unpaired or paired studies. *P*-values are usually assessed in univariate methods, either through parametric approaches or permutation tests. However, univariate methods fail to discriminate between groups if there are only minor differences on single-molecule level, even if multimolecule combinations would delineate them on a systems level.
- Multivariate analysis methods seek to capture not only changes of single metabolites between different groups, but also to utilize the dependency structures between the individual molecules. Probably the most prominent multivariate analysis techniques applied in the field of metabolomics are principal component analysis (PCA), cluster analysis, and partial least-squares regression (PLS). A multivariate analysis considers the combinatorial effect of multiple variables. It can be further categorized into unsupervised and supervised techniques. One of the most popular unsupervised techniques in LC–MS-based metabolomic studies is PCA, which finds a series of orthogonal projection

directions that maximize the variance of the projected data. PCA has been extensively used in multiple studies to elucidate the metabolomics consequences in explorations of phenotypic and genotypic relationships in disease pathophysiology.

The workflow of LC–MS-based metabolomics data processing has been described in Chapter 17 (Fig. 17.3). Several unsupervised techniques, such as self-organizing map or two-mode clustering, have also been found to be suitable for analysis of metabolomics data. A recent extension to the PLS repository is the orthogonal-PLS (OPLS) method. OPLS–DA is a more powerful data analysis method, which not only can reflect the difference between species, but can also find substances that cause such differences between species. OPLS has drawn attention in metabolomics research recently with

TABLE 11.2 List of Libraries and Databases Used in Mass Spectrometry

Name	Link
PubChem	pubchem.ncbi.nlm.nih.gov
ChEMBL	www.ebi.ac.uk/chembl
Chemspider	www.chemspider.com
Universal Natural Product Database (UNPD)	http://pkuxxj.pku.edu.cn/UNPD/introduction.php
Metlin	http://metlin.scripps.edu
Isometlin	https://isometlin.scripps.edu/
Massbank	http://www.massbank.jp/
Global Natural Product Database (GNPS)	gnps.ucsd.edu
MzCloud	www.mzcloud.org
ChemBank	chembank.broadinstitute.org
ChemIDplus	chem.nlm.nih.gov/chemidplus
NCI	cactus.nci.nih.gov/download/nci
Antibase	https://application.wiley-vch.de/stmdata/antibase.php
Dictionary of Natural Products	http://dnp.chemnetbase.com
CAS	www.cas.org
ReSpect	http://spectra.psc.riken.jp/
Drugs DB	www.drugsdb.com
Beilstein Database/Reaxys	https://www.reaxys.com/reaxys
FooDB	www.foodb.ca
Supernatural Database	http://bioinf-applied.charite.de/supernatural_new/index.php
NIST	http://webbook.nist.gov/chemistry/
BMRB	http://www.bmrb.wisc.edu/metabolomics/
GolmMetabolome Database (GMD)	http://gmd.mpimp-golm.mpg.de
Phenol-Explorer	phenol-explorer.eu
HMDB (Human Metabolome Database)	www.hmdb.ca
MMCD	http://mmcd.nmrfam.wisc.edu/
NAPROC-13	http://c13.usal.es/c13
SDBS	http://sdbs.db.aist.go.jp
Spektaris	http://langelabtools.wsu.edu/spektraris/

a broad variety of applications, including molecular epidemiology, alternative medicine, and the monitoring of kidney transplant patients (Bartel et al., 2013).

Many libraries and databases have been found to be useful for the interpretation of the LC–MS data in order to shorten the timeframe for analysis of natural products. A few have been listed in Table 11.2.

There are several commercial software packages available for the processing of MS metabolomics data, such as Markerlynx, MarkerView, MassHunter, Metabolic Profiler, Metabolyzer, metAlign, and Phenomenome Profiler (Want and Masson, 2011). Application of the PLS–DA model was able to analyze the metabolic variations of the mixtures of Kansui and licorice by a UHPLC–QTOF/MS-based metabolomics study. The role of chemometrics and multivariate data analysis was found useful for authentication and batch control of herbal medicine. Data analysis tools, namely, PCA and HCA/SIMCA-P, were applied for NMR-based identification and quantitation of chemical constituents of some Vietnamese herbal medicine against rheumatic diseases. Some exploratory data analysis through CDA/AMIX, random forest/R computing systems were also reported in an NMR-based metabolomics study for authentication of medicinal plants (Kazi et al., 2013; McKenzie et al., 2011).

11.6 STATISTICAL METHODS FOR ANALYSIS OF LC–MS DATA

11.6.1 Principal Component Analysis (PCA)

Principal components (PCs) are variables in a new reference system, calculated hierarchically, and are a useful pattern recognition tool. The systemic variation in PCs generally arises from the variance observed in the original dataset, as well as the residual variance. The systematic variations are described in the first PCs, whereas the experimental noise and random variations are observed in the last ones. The PCs are linear combinations of the original variables. The PCs are represented graphically by their score in order to identify the similarity or differences in their behavior. The identification of the variables can be detected from the score plot. The results can be visualized in terms of separations achieved for the different classes in the space given by the relevant PCs (Kumar, 2017).

11.6.2 Cluster Analysis

Cluster analysis allows us to identify the samples (or descriptors) in a dataset in which the clustering methods follow the hierarchical model and the samples are categorized on the basis of the measure of their similarity (Massart et al., 1983). The cluster analysis of a group of the samples is presented as a dendogram, in which the *X*-axis (horizontal axis) represents the distance or dissimilarity between clusters and the *Y*-axis (vertical axis) is represented by the objects and clusters. The dendogram represents the similarity between the two clusters on the graph by joining them through horizontal lines. These horizontal lines further split into two lines, which is represented as a short vertical bar, which gives the distance (dissimilarity) between the two clusters. The results of hierarchical clustering are reflected in the specific measure of similarity. The clustering techniques function on the original variables or to the scores of the relevant PCs, which serves as a useful source of variation (Marengo et al., 2007). The two-way hierarchical clustering helps in identifying the cluster of samples to provide the information on the behavior of the variables in the different clusters. It is a widely used statistical tool that is used in proteomics and genomics studies. In combination with PCA, the hierarchical clustering model is able to identify the similarity among groups of variables and is also predict the behavior of the variables in the identified groups (Kamimura et al., 2000).

11.6.3 Soft-Independent Model of Class Analogy (SIMCA)

SIMCA is a widely known supervised pattern recognition method used in the field of chemometrics. This method is also useful when more variables than objects are available because it performs a substantial dimensionality reduction. It gives further information about the class memberships: each class is described by its relevant PCs. The samples belonging to each class are represented as SIMCA boxes. SIMCA classifies each sample by its relevant PCs and it is not affected by experimental uncertainty and random variations (Bylesjö et al., 2006).

11.6.4 Partial Least-Squares Discriminant Analysis (PLS–DA)

PLS–DA is a chemometrics technique used to optimize separation between different groups of samples, which is accomplished by linking two data matrices *X* (raw data) and *Y* (groups, class membership, etc.). The method is in fact an extension of PLS1, which handles single dependent continuous variables, whereas PLS2 (called PLS–DA) can handle multiple dependent categorical variables. The main advantage of this PLS–DA approach is the availability and handling of

highly collinear and noisy data, which are very common outputs from metabolomics experiments. In addition, it provides several statistics, such as loading weight, variable importance on projection, and regression coefficient. Some other statistical methods, such as principal component–discriminant function analysis (PC-DFA), support vector machines, and random forests (RF) have also been found suitable for LC–MS data analysis (Gromski et al., 2015).

11.6.5 Heat Map

Heat maps are an efficient method for visualizing complex datasets organized as matrices. In a biological context, a typical matrix is created by arranging the data such that each column contains the data from a single sample and each row corresponds to a single feature (e.g., a spectrum, metabolite). It is a combination of two independent procedures applied to a data matrix. A heat map performs two actions on a matrix. First, it reorders the rows and columns so that rows (and columns) with similar profiles are closer to one another, causing these profiles to be more visible to the eye. Second, each entry in the data matrix is displayed as a color, making it possible to view the patterns graphically (Melissa Key, 2012). Heat maps are a useful tool for presenting quantitative metabolomics data for identification of clusters across datasets and detailed analysis of metabolite features for optimizing metabolomic data visualization and deconvolution. Several biological relationships can be studied by analyzing the heat maps (Benton et al., 2015).

11.6.6 Quantitative Trait Loci (QTL)

Biologists have long strived to understand what causes phenotypic differences between two individuals. These include differences in morphology, disease susceptibility, and physiology, as well as potential metabolic differences underlying these higher-order phenotypes. The diversity between individuals is partitioned into both environmental and genetic variations. Most genetic variations studied to date tend to be qualitative such that there are one or more distinct and nonoverlapping phenotypic states. QTL mapping is currently the most commonly used approach to identify genomic regions associated with a specific complex phenotype by statistical analysis of the associations between genetic markers and phenotypic variation. A QTL analysis of metabolite levels resulted in the identification of genomic regions associated with metabolic variation in order to confirm their association with related biochemical pathways. A QTL study is also a very helpful tool to identify putative genetic predictors involved in several metabolic pathways in plant tissues. In sum, this model can be applied in high-quality breeding of various plant species by exploring genetic pathways (Carreno-Quintero et al., 2012).

11.7 LC–MS IN METABOLITE PROFILING AND DEREPLICATION OF HERBAL DRUGS

In natural product research, the term "metabolite" is defined as any molecule (MW of <1000 Da) present in plants. They are mainly of two types: primary and secondary metabolites. Among them, secondary metabolites include polyphenols, alkaloids, terpenes, polyketides, and hormones that offer several drug-like compounds due to their remarkable pharmacological activities. Metabolite profiling emphasizes a large number of targeted metabolites related to any specific class of compounds. The profiling of metabolites deals with the output of analytical techniques for qualitative and quantitative estimations of several secondary plant metabolites. These metabolites are sequentially assessed through different statistical processes and measurements of various spectral and chromatographic peaks. These modern techniques can be utilized for comprehensive analysis of the constituents present in plant samples.

In general, metabolite profiling allows us to quantitatively and semiqualitatively measure some marker compounds present in plants, often leading to the dereplication process (Wolfender et al., 2015). Dereplication is the process of testing sample mixtures that are active in screening in order to differentiate the novel compounds from active substances that have already been studied. The significant development of natural product-based research relies on the dereplication process, based on both targeted and untargeted metabolomics studies. As plant extracts contain a large number of secondary metabolites, sometimes isolation and purification to obtain novel bioactive compounds are very difficult due to high cost and the labor involved. The major advantages of the dereplication process are to avoid the isolation or purification processes with prior knowledge of the chemical compounds present in the plant and thus to offer a robust and time-saving approach in the natural product-based drug discovery process. In this background, the LC–MS-guided dereplication methodology offers a rapid tool for identification of metabolites and targeted isolation of compounds having significant biological activity (Jiang et al., 2017). There are several dereplication processes that have been reported in different plants, namely, *Melicope vitiflora*, *Ruta graveolens*, and *Ficus coronate*, based on their antibacterial activity guided LC–MS-based platform (Smyth et al., 2012). Another report on an LC–MS-based dereplication strategy concerned the metabolite profiling of Brazilian Lippia species. The dereplication process was carried out via some critical steps, as reported by Funari et al. (2012). These steps included elimination of noncoherent putative molecular formulae by heuristic filtering, verification of the occurrence of

remaining molecular formulae in databases, cross search with reported compounds in the Lippia genus, match with reported UV spectra, and finally, estimation of the chromatographic retention behavior based on the log *P* parameter of reference compounds (Funari et al., 2012).

Dereplication is also considered to be a very promising tool for metabolite profiling using ultra-high-pressure liquid chromatography coupled to high-resolution mass spectrometry (UHPLC–HR–MS) for early stage drug discovery. The integration of quantitative structure retention relationship models along with PLS regressions improves the dereplication process to a higher confidence level (Eugster et al., 2014). Several hepatoprotective triterpenoid saponins, namely, celosin H, celosin I, celosin J, and pseudoginsenoside, were obtained through dereplication-guided isolation from the seeds of *Celosia argentea* through HPLC coupled with ESI–tandem quadrupole–TOF–MS (Jiang et al., 2017). Boukhris and his coworkers reported a dereplication strategy for the identification of some phenolic compounds related to chlorogenic acid and dicaffeoylquinic acid derivatives and 18 flavonoids from *Anvillea radiate* (Boukhris et al., 2016). An LC–MS-based dereplication strategy offered some potential bioactives, namely, hyperfirin, adhyperfirin, hypericin, and protohypericin from the genus Hypericum based on their retention times and mass spectral data (Alali and Tawah, 2009). A novel dereplication strategy using UHPLC/Q–TOF–MS/MS has been successfully developed for characterization and identification of two parishins-type compounds (parishin J and parishin K) in an extract of *Gastrodia elata* (Li et al., 2015).

Thus, LC–MS-based metabolite analysis provides a comprehensive understanding of the spectrum of phytochemical constituents of plants. This approach can be very useful for characterization and identification of metabolites and can be helpful for evaluation of herbal medicine (Mukherjee et al., 2016). Metabolite profiling not only identifies the metabolites relative to the distribution of compounds with each other but also compares the nature of compounds. The application of targeted metabolomics is widely applied to identify specific metabolites from the complex mixture of biomolecules present in plant extracts. Targeted metabolomics measure some specific groups of chemically characterized annotated metabolites, whereas untargeted metabolomics are intended to analyze all the measurable analytes. In the targeted metabolite approach, quantification of the metabolites is performed through the use of internal standards and authentic chemical standards of each metabolite (Dudley et al., 2010). Some LC–MS-based secondary metabolite profiling (both targeted and untargeted metabolomics) of medicinal plants are reported in Chapter 17.

11.8 LC–MS IN QUALITY CONTROL AND THE IDENTIFICATION AND QUANTIFICATION OF ADULTERANTS

LC–MS-based metabolomics study is an important tool for identifying chemical variability in different species, which mainly varies due to their geographical location. Applying PCA and OPLS–DA, researchers found that the presence or absence of sutherlandioside B (SU1) and its derivatives is a key biomarker, responsible for their quality control purposes (Albrecht et al., 2012). The chemical composition of herbal drugs depends on different factors, such as growth of plants, environment, period of collection, drying process, and methods for extraction. These factors need to be included in the quality evaluation of herbal medicine. Modern analytical techniques can be used in quality evaluation and scientific validation of medicinal plants and their formulations to fulfill the demands of the market. Hence, urgent attention should be given to purity, quality, and several other standardization parameters, based on the major biological reference compounds. Natural products, including medicinal plants, have been the focus area for research in multidisciplinary fields for development of templates of new chemical entities. Several new lead molecules are being developed through hyphenated technology. The standardization, quality control, and biological evaluation of herbal drugs will be very useful for validating the ancient but effective claims for healthcare. Consecutively, to improve the accuracy and consistency of herbal/medicinal plant-based preparations worldwide, regulatory authorities are mandating research into new analytical methods for stricter standardization of herbal drugs. Such approaches have to be both objective and robust, and should address the reproducibility of the content of the chemical profiles. In order to rationalize the use of herbal products in different forms, more particularly the extracts/marketed product in therapy, as is being used nowadays, a need-based and novel concept of chemoprofiling is gaining momentum. Therefore, utmost attention is necessary for the promotion and development of HM through international coordination and harmonization. Metabolite profiling not only identifies the metabolites relative to the distribution of compounds with each other but also compares the nature of compounds (Mukherjee et al., 2016).

Adulteration in herbal medicine increases the impurity by adding some extraneous, improper, or inferior ingredients. Herbal medicines adulterated with conventional drugs, and plant materials have repeatedly been documented. Adulterations include the addition of orthodox drugs, the substitution of fake or inferior plant materials, and the addition of foreign materials. In order to maintain the quality, safety, and efficacy of herbal medicine, standardization is required to identify the potential contaminants present. Several chromatographic platforms, such as HPTLC and HPLC, have been widely used for the qualitative and quantitative analysis of adulterants. The LC–MS method is found to be more suitable for detection

of adulterants due to its robustness and higher sensitivity. There are several LC–MS-based methods that have been reported for carrying out quality evaluation and identification of adulterants in herbal drugs. One LC–MS method was developed to detect the adulteration of saffron samples with gardenia. In this study, geniposide was identified as an adulteration marker, detected in negative ESI mode (Guijarro-Díez et al., 2017). An LC–Q-TOF/MS method has been developed to detect homotadalafil, a synthetic analog of tadalafil present in various herbal products, used as an aphrodisiac (Lee et al., 2015a, b). Letsyo et al. reported the occurrence of pyrrolizidine alkaloids as adulterants found in several herbal medicines used in Ghana, identified by HPLC–ESI–MS/MS (Letsyo et al., 2017). Adulteration of grape seed extract was detected by chromatographic, coupled with mass spectrometric, techniques for quality control of marketed grape seed extract (Villani et al., 2015). Some synthetic hair-growth compounds were identified through ultra-high-pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) as adulterated products in different marketed natural products (Park et al., 2018). The high-pressure LC–ESI–MS–MS method was applied for the detection of the most common synthetic adulterants in herbal remedies, including analgesic drugs, antibiotics, antidiabetic drugs, antiepileptic drugs, aphrodisiacs, hormones and anabolic drugs, psychotropic drugs, and weight-reducing compounds (Bogusz et al., 2006). Rapid determination of sibutramine and its analog present in dietary supplements was performed by LC–triple quadrupole MS–TOF–MS (Zou et al., 2007; Date et al., 2008).

In combination with chemometrics, metabolite studies promise an effective quality control tool for the authenticity, efficacy, and consistency of different batches of herbal medicine (Bansal et al., 2014). Untargeted metabolomic profiling of different coffee species was carried out in an LC–HRMS-based platform, along with PCA and PLS–DA, to determine the interspecies differences in metabolomes, related to their botanic and genetic origins (Souard et al., 2018). A UPLC–MS-based comprehensive metabolomics strategy was developed for content estimation and various distribution patterns of lycopodium alkaloids present in different tissues of *Huperzia serrata*. The study confirmed the presence of *N*-methylhuperzine A (lycopodium alkaloid) as a distinct quality control marker present in Huperzia species (Wu et al., 2018). A comparative metabolomics study of different species rhizomes of *Zingiber officinale* were carried out in which six potential chemical markers were identified as effective quality control parameters (Mais et al., 2018).

11.9 CHEMOTAXONOMIC AND PHYLOGENETIC CHARACTERIZATION OF MEDICINAL PLANTS

MS-based metabolomics analysis assesses plant chemotaxonomic characteristics at the system level by analyzing the metabolic profile. Chemotaxonomic characterizations exhibit a wide variation in chemical diversity and distribution of various plant secondary metabolites, such as phenolics, alkaloids, terpenoids, and nonprotein amino acids. Chemotaxonomic metabolite profiling is useful to discover the correlation between different metabolites and bioactivities depending on their phylogenetic family. In 2015, Lee and his coworkers performed chemotaxonomic metabolite profiling of 62 indigenous Korean plant species, in which they analyzed the correlation between metabolites and bioactivities based on their phylogenetic family. In particular, Aceraceae, Betulaceae, and Fagaceae were distinguished from Rosaceae, Fabaceae, and Asteraceae based on the presence of several plant metabolites (Lee et al., 2015a, b). Another chemotaxonomic based phylogenetic classification of *Striga asiatica* was performed through HR–LC–TOF–MS, which confirmed that the Striga species belongs to the family Scrophulariaceae instead of Orobanchaceae (Huang et al., 2013). The chemotaxonomic classification of different Penicillium species in correlation with their antioxidant activity was studied to analyze the influence of species-specific variation affecting biological activity (Kim et al., 2012). MS-based profiling and characterization of some flavonoids were carried out in the leaves and roots of different Mediterranean and Mexican lupine species for analyzing their chemotaxonomic comparison (Wojakowska et al., 2013). The presence of tetracyclic kauran-type diterpenoids as chemotaxonomic markers for the classification and subdivision of genus Helichrysum has been reported. In this study, the structural characterization of the diterpenoids was confirmed through LC–MS analysis (Zanetsie Kakam et al., 2011). In addition, the application of hyphenated MS analysis was able to elucidate the phenotype–genotype relationship in medicinal plants and its contribution in the alteration of secondary metabolites involved in biochemical pathways. In this context, a phylogenetic hypothesis on the evolution of the secondary metabolites in Lychnophorinae species based on metabolomics and chemotaxonomical studies has been reported (Martucci et al., 2018).

11.10 LC–MS IN TARGET COMPOUND ANALYSIS

Most of the herbal drugs consist of a large number of secondary metabolites, including alkaloids, flavonoids, terpenoids, saponins, and phenolic acids. All of these metabolites are responsible for major pharmacological activity with greater therapeutic efficacy. The identification, as well as the characterization, of the metabolites is of great importance for

natural product-based drug screening processes. As the chemical compounds obtained from herbal drugs possess complex structure, so it is sometimes difficult to identify the chemical structures due to lower sensitivity (Zhang et al., 2017). An LC–MS-based study is very useful for identification and quantification of several targeted metabolites present in herbal medicine. The MS-based techniques also provide useful structural information on chemical constituents along with their qualitative and quantitative profiles. As plant extracts are very complex in nature, it is very important to separate those compounds in a chromatographic platform based on their physiochemical nature. The introduction of HPLC offers a versatile separation technique for separation of the natural product because of its sensitivity, choice of stationary and mobile phases, and usefulness of various sample matrices and different detection methods. UHPLC, with higher chromatographic resolution, represents and advance in separation science and can identify a large number of metabolites present in plants (Wolfender et al., 2015). The combination of UHPLC with an MS detector appears to be a suitable approach that fulfills the key requirements in terms of sensitivity, selectivity, and peak assignment for the rapid determination of analytes at low concentrations in complex matrices. The UHPLC–TOF–MS and UHPLC–QqTOF–MS platforms offer high-resolution and increased sensitivity for the analysis of targeted metabolites. It should also be noted that triple quadrupole (in SRM mode) coupled with UHPLC is most suitable for target compound analysis,

TABLE 11.3 Selected LC–MS Studies of Different Classes of Phytoconstituents

Chemical Compounds	Plant Name	LC Mobile Phase	Mass Source	Reference
Acids				
Aristolochic acid	*Asarum* spp.	10 mM ammonium acetate, methanol, acetonitrile, water (+0.1% formic acid)	ESI	Zhao et al. (2008)
Aristolochic acids (AA I, AA II, AA C, AAD)	*Aristolochia* and *Asarum* spp.	0.2% acetic acid in water and methanol	ESI	Yuan et al. (2007)
Chlorogenic acids and geniposidic acid	*Eucommia ulmoides*	0.5% acetic acid in methanol	APCI	Tong et al. (2008)
Caffeic, chlorogenic, protocatechuic acid	*Salvia miltiorrhiza*	0.2% formic acid, methanol, and acetonitrile	ESI	Guo et al. (2008)
Caffeic acids	Echinacea product	Water and methanol	GD EI	Castro et al. (2010)
Amino acids (derivatized)	*Gentiana dahurica*	30 mM formic acid, water, and acetonitrile	ESI	Sun et al. (2009)
Ginkgolic acids	*Ginkgo biloba*	Water containing 0.1% phosphoric acid (A), acetonitrile (B), and 70% acetonitrile containing 0.1% phosphoric acid	ESI	Lee et al. (2013)
Ganodermic acids	*Ganoderma atrum*	0.1% acetic acid in water and B (acetonitrile)	Ion trap instrument with an ESI	Li et al. (2012)
Alkaloids				
Pyrrolizidine alkaloids, lycopsamine, echimidine	*Symphytum officinale*	Water and acetonitrile both with 0.1% formic acid	ESI	Liu et al. (2009)
Aconitine, hypaconitine, mesaconitine	*Aconitum carmichaelii*	0.1% formic acid in water and acetonitrile	ESI	Wang et al. (2009a, b)
Steroidal alkaloids	*Fritillaria* spp.	Water and ACN both with 0.03% diethylamine	ESI	Zhou et al. (2008)
Oxoprotoberberine alkaloids	*Coscinium fenestratum*	0.1% formic acid in water and methanol	ESI	Deevanhxay et al. (2009)
Senkirkine and senecionine	*Tussilago farfara*	0.1% formic acid in 20 mM ammonium acetate and 0.1% formic acid in acetonitrile	ESI	Jiang et al. (2009)

Continued

TABLE 11.3 Selected LC–MS Studies of Different Classes of Phytoconstituents—cont'd

Chemical Compounds	Plant Name	LC Mobile Phase	Mass Source	Reference
Phenolics and flavonoids				
Curcumin and derivatives	*Curcuma domestica*	0.1% acetic acid in water and ACN	ESI	Herebian et al. (2009)
Liquiritin, liquiritigenin, isoononin	*Glycyrrhiza uralensis*	0.1% formic acid in water and in acetonitrile	ESI	Jayaprakasam et al. (2009)
Flavonoid aglyca and glycosides	*Chrysanthemum morifolium*, *Artemisia annua*	0.1% formic acid in water and ACN	ESI + APCI	Lai et al. (2007)
28 flavonoid glycosides	*Trigonella foenum-graecum*	2% AA, B: ACN with 2% AA	ESI–IT	Benayad et al. (2014)
Flavonoids phenolic acids A: 0.1% FA B: ACN with 0.1% FA	*Chenopodium hybridum*		ESI–QqQ–IT	Podolak et al. (2016)
Terpenes				
Diterpenes (ginkgolides) and bilobalide	*G. biloba*	0.01% FA, MeOH with 0.01% FA	ESI–QTOF	Luo et al. (2013)
Triterpene saponins	*Bacopa monnieri*	A: 0.1% FA B: ACN with 0.1% FA	ESI–QTOF	Nuengchamnong et al. (2016)
Triterpene saponins (glycyrrhizic acid and derivatives)	*Glycyrrhiza glabra*	A: 0.05% TFA B: CAN with 0.05% TFA	ESI–IT ESI–QqQ	Montoro et al. (2011)
Ginsenosides	*Panax ginseng*	A: 0.1% FA B: ACN with 0.1% FA	ESI–QTOF	Chu et al. (2013)
Asiatic acid, asiaticoside, madecassoside	*Centella asiatica*	0.1% acetic acid in water and acetonitrile	ESI	Shen et al. (2009)
Miscellaneous				
Phenols, alkylphthalides, phthalide dimers	*Ligusticum chuanxiong*, *Angelica sinensis*, *A. acutiloba*, *Cnidium officinale*	Acetonitrile and water	ESI	Yi et al. (2007)
Hyperforin and polyprenylated derivatives	*Hypericum perforatum*	0.01% phosphoric acid in water and acetonitrile	ESI	Charchoglyan et al. (2007)
Glucosinolates	*Isatis tinctoria*, *I. indigotica*	10 mM aqueous ammonium formate (pH 4.6) and acetonitrile	ESI–MS	Mohn and Hamburger (2008)
Alkamides	*Echinacea purpurea*	0.2% formic acid in water and ACN	ESI	Woelkart et al. (2008)

whereas TOF–MS analyzers are suitable for nontargeted analysis. Table 11.3 summarizes some selected studies on target compound analysis of medicinal plants/herbal drugs.

11.11 LC–MS IN PHARMACOKINETICS AND BIOEQUIVALENCE STUDY OF HERBAL DRUGS

The identification and measurement of the phytoconstituents present in herbal drugs is necessary to explore the therapeutic basis and action mechanisms. After administration of an herbal medicine, it gets absorbed into the blood and generates several therapeutic activities. The bioavailability is a crucial factor to assess the efficacy of herbal medicine as it is mandatory that the active components reach the target site. Therefore, the absorption and efficacy of HM is required to understand the

mechanism of ADME of herbal medicine (Stylos et al., 2017). Unfortunately, the concentrations of the compounds present in the blood are very low, and thus it is difficult to detect in vivo. In this context, the integration of MS-based technologies offers higher sensitivity and specificity for the qualitative and quantitative measurement of the metabolites. The fact that it provides extra sensitivity, specificity, and good separation in complex samples makes LC–MS/MS the ultimate tool in the determination of many types of chemical compounds, such as phytochemicals. The serum pharmacochemistry approach has been developed for the screening, identification, and quantification of bioactive components from herbal drugs to reveal their therapeutic effects after oral administration. This study mainly reflects the interaction between the body and drugs in conjunction with metabonomics technologies by using metabolic biomarkers to evaluate the therapeutic effect of HD, thus validating the therapeutic claim (Yan et al., 2015). In 2016, Zhang and his coworkers performed chromatographic fingerprinting and serum pharmacochemistry of Sanziguben Granule (TCM) to identify the bioactive components for a quality control study (Zhang et al., 2016).

Visnagin is a furanocoumarins derivative obtained from *Ammi visnaga*. This plant has several pharmacological activities against kidney stones, cardiovascular diseases, and different fungal, bacterial, and viral diseases. A sensitive and highly selective LC–MS method was developed to determine visnagin in rat plasma (Vanachayangkul et al., 2009). An LC–MS/MS method with orthogonal *Z*-spray electrospray interface system was developed to determine the pharmacokinetics and oral bioavailability of curcumin and demethoxy curcumin from *Curcuma longa*. In this study, several pharmacokinetic parameters, namely, AUC, $t_{1/2}$, C_{max}, and T_{max}, of curcumin and demethoxy curcumin were determined (Ireson et al., 2002). Another comparative pharmacokinetics study of quercetin, kaempferol, and isorhamnetin present in *Ginkgo biloba* extract was reported by Chen et al. (2010). The pharmacokinetic parameters of mangiferin (from *Mangifera indica*) in rat plasma with UPLC–MS/MS were calculated by Han et al. (2010). Another LC–MS-based method was developed for simultaneous estimation of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol in rat plasma (Wang et al., 2009a, b). In this context, Mehta and his coworkers did an extensive review on pharmacokinetic profiles of 50 different therapeutically effective traditional

TABLE 11.4 Selected LC–MS-Based Pharmacokinetics Studies of Phytoconstituents

Chemical Class	Analyte	Matrix	MS Analyzer	References
Isoquinoline alkaloids	Tetrahydropalmatine, coptisine, palmatine, dehydrocorydaline	Mouse plasma and brain	QqQ	Gao et al. (2014)
	Magnoflorin	Rat urine, feces, plasma, and bile	Orbitrap	Xue et al. (2015)
Steroidal alkaloids	Aconitine and five others Aconitum-alkaloids	Rat plasma	QqQ	Liu et al. (2014)
	Solasodine (p.o.) solasodine	Rat plasma	IT	Ma et al. (2014)
Indol alkaloids	(Iso-)corynoxeine, (iso-)rhynchophylline, hirsutine, hirsuteine, geissoschizine methyl ether	Rrat plasma and brain	QqQ	Kushida et al. (2013)
Flavones	Baicalein, wogonin, oroxylin A and their glucuronides baicalin, wogonoside, and oroxyloside	Rat plasma	Q-Trap	Fong et al. (2014)
Flavonoids	Rutin, vitexin-4″-*O*-glucoside, vitexin-2″-*O*-rhamnoside, hyperoside, vitexin, shanyenoside A	Rat plasma	QqQ-MRM	Zhu et al. (2015)
Flavonoids	Apigenin, quercetin, apigenin-7-*O*-β-D-glucoside, quercetin-3-*O*-β-D-glucoside, 3′-methoxyluteolin-7-*O*-β-D-glucoside, and tricin-7-*O*-β-D-glucopyranoside	Rat plasma	Triple quadrupole electrospray ionization (ESI)	Dai et al. (2015)
Phenolics	Xanthones *C*-glycosides Mangiferin Neomangiferin plasma	Rat plasma	TOF–ESI	Xie et al. (2016)
Terpenoids	Andrographolide (1), 14-deoxy-11,12-didehydroandrographolide (2), neoandrographolide (3), and 14-deoxyandrographolide	Rat plasma	QqQ-MRM	Pholphana et al. (2016)

Continued

TABLE 11.4 Selected LC–MS-Based Pharmacokinetics Studies of Phytoconstituents—cont'd

Chemical Class	Analyte	Matrix	MS Analyzer	References
	Ginkgolide A and B	Urine	QqQ-MRM	Dew et al. (2014)
Secoiridoid and flavonoid glycosides	Swertiamarin, gentiopicroside, sweroside, mangiferin, isoorientin, and isovitexin	Rat plasma, bile, urine, and feces	Ion trap–MRM	Sheng et al. (2014)
Coumarin	Glycycoumarin	Rat plasma and urine	DAD/ESI	Wang et al. (2014)
	Oxypeucedanin, marmesin, byakangelicin, columbianetin, psoralen, xanthotoxin, neobyakangelicol, isoimpinellin, bergapten, heraclenin, oxypeucedanin, ethanolate, imperatorin (14), phellopterin, isoimperatorin	Rat plasma	QqQ–MRM	Zhao et al. (2016)

medicinal plants (Mehta et al., 2015). All of these data suggested that LC–MS technology offers a high-throughput analytical platform for the pharmacokinetics study of herbal medicines. Several LC–MS-based pharmacokinetics studies of phytoconstituents are shown in Table 11.4.

Herbal medicine contains a mixture of bioactive chemical compounds that exert their bioactivity in agonistic, synergistic, or complementary ways. This makes a bioequivalence study very challenging due to the lack of knowledge of the active ingredients present. The variation in the quality of herbal medicine depends on several parameters: plant collection and/or cultivation, harvesting, primary processing, washing, cutting, fumigation, freezing, distillation, drying, GAP (good agricultural practice), and extraction solvents, among others. There are major problems in a bioequivalence study of herbal medicine because of the complex variation of the metabolites with their different physiochemical properties. Recent advances in chromatographic and hyphenated analytical techniques enable researchers to identify and quantify the active constituents present in herbal medicine, as well as in human plasma after therapeutic administration. These techniques have been applied to both the bioavailability and pharmacokinetics study of herbal medicine, as mentioned in the previous section. After absorption of herbal medicine in the body, the effective plasma concentration is found to be very low, at the microgram/nanogram level (Loew and Kaszkin, 2002). The higher sensitivity and selectivity of an LC–MS instrument enables it to measure the drug concentration in plasma at the microgram level without any false-positive data. In addition, the generation of several chemically diverse compounds on metabolism and biotransformation of HM make it very challenging to measure all analytes in a single dose study. Several reports have been found on LC–MS-based bioequivalence studies of herbal medicine/phytopharmaceuticals. A bioequivalence study of ginkgolides A, B, and bilobalide was carried out by LC–MS in healthy subjects after oral administration of 160 mg extract. The bioavailability of Ginkgold tablets with reference extract and *G. biloba* capsules was reported in healthy volunteers in an open, single dose, crossover design (Bhattaram et al., 2002). Another LC–MS-based bioequivalence study of hypericin and pseudohypericin present in Hypericum extract was reported in human volunteers. The bioavailability of Hypericum extracts was also tested with different formulations containing hyperforin and 0.3% hypericin (Kerb et al., 1996). The fruit of *Aesculus hippocastanum* (horse chestnut tree) is a well-known medicinal plant possessing a mixture of triterpenic saponins called escin. This plant is used to treat edema by raising the vascular tone and influence on microcirculation. Several randomized, blind or double-blind, two-period crossover bioequivalence studies of horse chestnut formulation have been reported. A bioequivalence study of standardized Willow bark (Salix species) was carried out in an LC–MS-based platform. This study suggested that salicin is responsible for the analgesic and antirheumatic activity of Willow bark (Chrubasik et al., 2000). A sensitive, rapid LC–MS-based method was reported in a bioequivalence study of quercetin and its glycosides (Wittig et al., 2001). The bioavailability and pharmacokinetics of thymol after oral administration in 12 healthy male volunteers was reported (Kohlert et al., 2002). Thus, it can be concluded that LC–MS can be utilized for bioequivalence studies of several herbal preparations due to its higher sensitivity.

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Chapter 12

Chemoprofiling and Marker Analysis for Quality Evaluation of Herbal Drugs

Chapter Outline

12.1 IMPORTANCE OF CHEMOPROFILING AND MARKER ANALYSIS

Natural products derived from botanicals are mostly available from wild sources and face challenges for ensuring consistent product quality. Extrinsic factors, such as soil conditions, availability of light and water, temperature variations, nutrients, and geographical location, affect the accumulation or percentage of phytochemicals or phytoconstituents present in plants. Directives on the analytical control of plant materials must consider that the material to be examined has complex and inconsistent composition; therefore, the analytical limits are not as precise as for the single chemical entity based on chemoprofiling through marker analysis. Further, cultivation and harvesting techniques, postharvest processing, and storage methods also influence the physical appearance of the plant and its chemical qualities. According to the Natural Health Product Directorate of Canada, "marker compounds are a constituent that occurs naturally in the material and that is selected for special attention (ex: for identification or standardization) by a researcher or manufacturer" (e.g., markers used in commercial products include hypericins and stabilized hyperforins from St. John's Wort, silymarin in milk thistle, ginsenosides from ginseng). Marker selection may be based upon a variety of different factors, including stability, ease of analysis, time and cost of analysis, relevance to therapeutic effect, indicator of product quality or stability, or previous use by other manufacturers or researchers. To identify or authenticate the source of the material, markers play an important role. They can be used in several ways to evaluate quality, so as to ensure the efficacy and safety of the natural health products (NHPs). There are several contexts for defining the concept of markers.

Marker compounds are not necessarily pharmacologically active all the time, but their presence is well established in products with characteristic chemical features. Marker components may be classified as active principles, active markers, analytical makers, and negative markers, while biomarkers may be defined as those markers with known pharmacological activity (Mukherjee et al., 2011).

Draft guidelines stated by the USFDA describe a marker compound as a chemical constituent of a botanical raw material, drug substance, or drug product that is used for identification and/or quality control purposes, especially when the

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active constituents are not known or identified. The active constituent is responsible for the intended pharmacological activity or therapeutic effects. Chemical standardization often involves chemical identification by spectroscopic or chromatographic fingerprint and chemical assay for active constituents or marker compounds, if available. The key challenges in the development of analytical methods for botanicals and herbal preparations are:

(i) analysis of marker or active compounds in a complex and sometimes unknown environment,
(ii) target analytes may be polar and thermolabile,
(iii) lack of chemical reference substances (CRSs) and certified reference materials,
(iv) selection of extraction method, and
(v) batch-to-batch variation of the composition of the plant materials obtained.

Botanicals are standardized based on the presence of a known active ingredient or specific markers when the active markers are not yet recognized. This can help in establishing the quality of the product, depending on the characteristic fingerprints. With the chemical fingerprint obtained, the method should be able to perform composition analysis and monitor the batch-to-batch variation of the plant materials obtained for use (Ong, 2004). Herbs are known to contain several components and, in many cases, the absolute compound responsible for the pharmacological activity is unknown. Therefore, there is a need to establish a chemical fingerprint or chemoprofile to evaluate the various groups of phytoconstituents present therein. Chemoprofiling can thus be used to authenticate plant material, identify the quantification of active compounds and relate the chemical composition to biological activity for product standardization and validation (Mukherjee et al., 2013). Chemoprofiling and marker compound analyses are gaining momentum for use in standardizing traditional medicinal formulations. However, marker testing is in no way a substitute for tests, such as physicochemical, chemical, macroscopic, and microscopic tests. But it is an efficient procedure to ensure the identity and purity limits of herbal drugs (Mukherjee, 2002). Different analytical techniques, such as HPLC, HPTLC, LC–MS, are used in marker profiling of herbal drugs. Several aspects in this context has been described in Fig. 12.1.

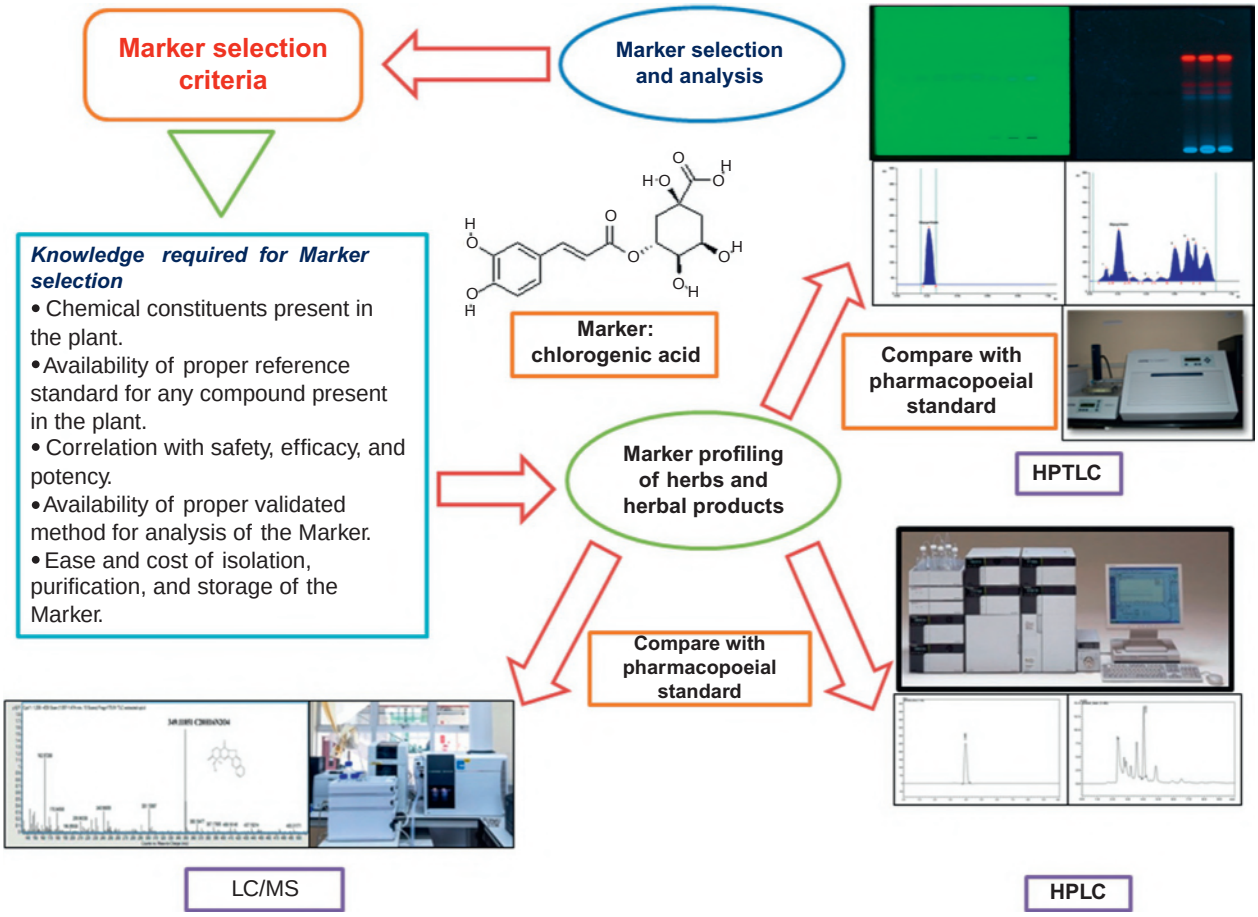


FIG. 12.1 Different analytical techniques used for marker profiling.

12.2 CLASSIFICATION OF MARKERS PRESENT IN HERBAL DRUGS

Botanical raw material and their extracts usually contain complex mixtures of several chemical constituents. For a large majority of botanical plant materials and their extracts used as dietary supplements, it is not known with certainty which of the various components is responsible for the reported pharmacological effect. It is generally believed that several constituents act synergistically to provide the reported effect. Constituents of herbal drugs can be classified into four categories as follows (Srinivasan, 2006).

12.2.1 Active Principles

Constituents that may have known clinical activity and thus are known by the name of the active principle(s).

12.2.2 Active Marker(s)

These constituents have some known pharmacological activity, contributing to some extent to the efficacy. For example, alliin present in garlic comes under this category. These constituents may or may not have clinically proven efficacy. A minimum content or range for active markers is usually specified in pharmacopoeial monographs. A quantitative determination of the active marker(s) during stability studies of botanical dosage forms provides the necessary information.

12.2.3 Analytical Markers

Certain constituents of the botanical raw materials and their extracts are chosen as candidates for quantitative determination in cases in which neither defined active principles nor active markers are known. These markers aid in the positive identification of the article under test. Further, maintaining a minimum content or a specified range of the analytical markers helps to achieve standardization of the plant extract and to arrive at a suitable expiration date during stability studies.

12.2.4 Negative Markers

Some constituents may have allergenic or toxic properties that render their presence in the botanical extract undesirable. A stringent limit for these negative markers may be specified in compendial articles.

The European Medicines Agency (EMA) defines chemical markers as chemically defined constituents or groups of constituents of herbal medicinal products, which are of interest for quality control purposes in spite of whether they possess any therapeutic activity. Different chemical markers can be classified as follows:

- Therapeutic components.
- Bioactive components.
- Synergistic components.
- Characteristic components.
- Main component.
- Correlative components.
- Toxic components.
- General components.

A total of 282 chemical markers are listed in the Chinese Pharmacopeia (2005 edition) for the quality control of Chinese herbal medicines. As discussed in the monographs of the American Herbal Pharmacopeia (AHP), the use of single or multiple chemical markers is important to quality control (Mukherjee et al., 2014). The classification and importance of marker compounds has been further explained in Fig. 12.2. Some medicinal plants, along with their phytomarkers, are shown in Table 12.1.

12.3 BOTANICAL REFERENCE STANDARD (BRS) AND PHYTOCHEMICAL REFERENCE STANDARD (PRS)

Apart from the challenges of marker selection and method development for herbal preparations, appropriate characterization of reference standards is absolutely essential. There are official, often country-specific stipulations, which define the requirements that must be met by a compound in order to qualify as a reference standard. Furthermore, the term “reference

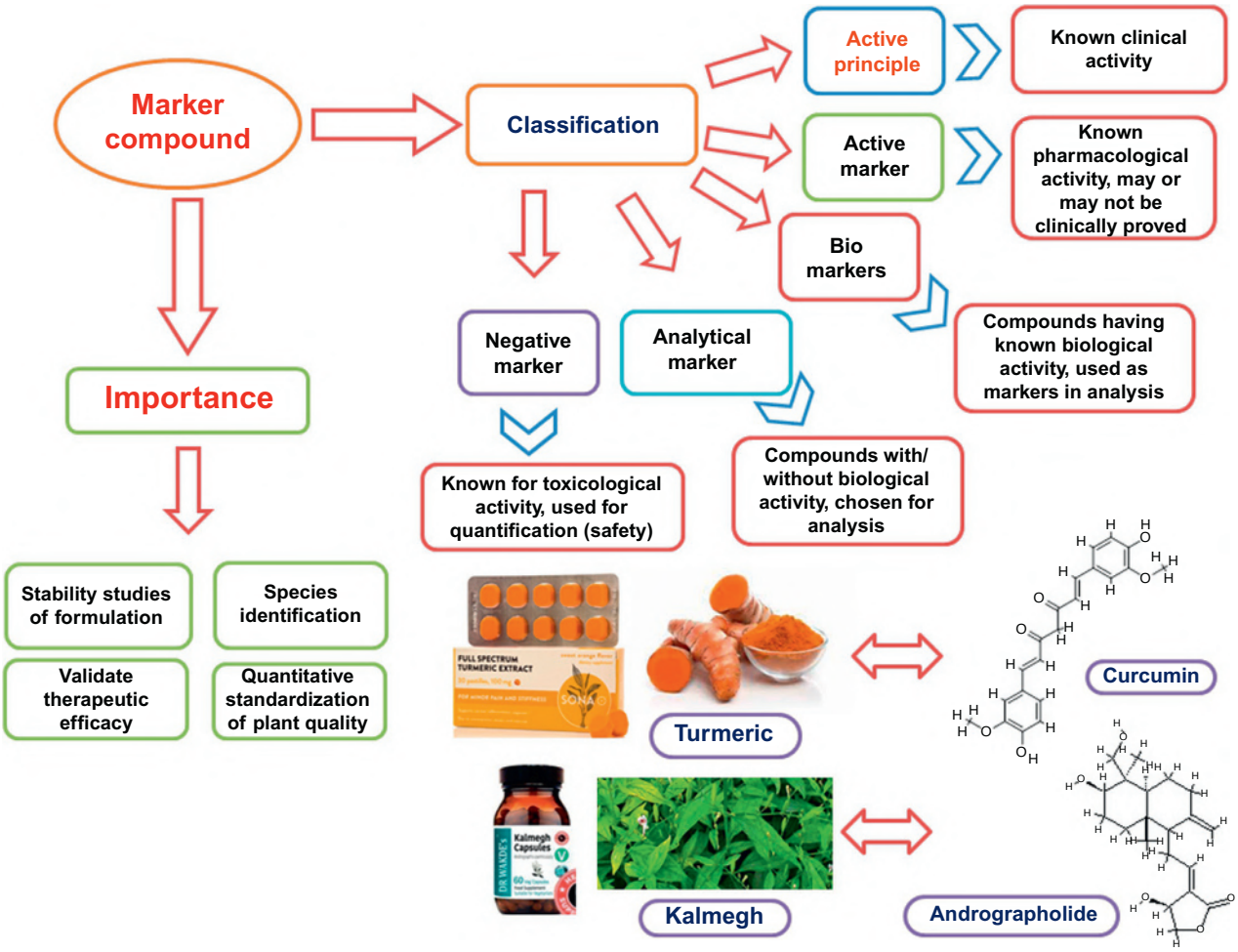


FIG. 12.2 Classification and importance of marker compounds.

standard" is also used in the context of reference preparations and reference spectra within the European Pharmacopeia. CRS offered by the European Directorate on the Quality of Medicines & Health Care (EDQM), are primary reference standards by definition. A primary standard is defined as being, "A standard shown to have suitable properties for the intended use, the demonstration of suitability being made without comparison to an existing standard" (Zöllner and Schwarz, 2013). Compounds that are to be used as reference standards are characterized by means of identity testing and exactly defined purity testing to obtain a content assignment (Schwarz et al., 2009). In the context of the Indian Pharmacopeia, reference substances, especially botanical reference standards (BRS), and also phytochemical reference standard (PRS) are required for comparison of the quality of herbal drugs. The Indian Pharmacopeia Commission has initiated the process of providing Indian Pharmacopeia reference substances to the stakeholders. The pharmacopoeial standards provide a reliable mechanism for independent assessment of the quality of medicines. The pharmacopoeial standards are enforced by the central, state, and union territory drug regulatory authorities of India in accordance with the Drugs and Cosmetics Act 1940 and Rules 1945 thereunder, as amended from time to time. The Indian Pharmacopeia (2014) incorporated the general requirements of herbs and herbal products standards and monographs with respective reference standards; these are described in Table 12.2.

Reference substances (Indian Pharmacopeia Reference Substances) are required to fulfill the requirement of the monograph in the IP. The phytochemical reference standard (PRS) are highly characterized authentic compounds used whenever available and feasible for the purpose of quality control of herbal samples. A botanical reference standard (BRS) is an authentic standard whose botanical identity and genuineness has been well established to both genus and species levels. When a PRS is not available, a BRS serves as a standard for maintaining the quality of herbal samples under examination. The India pharmacopoeia Commission (IPC) has initiated the work for providing BRSs and PRSs to the stakeholders. After acquisition, a candidate reference material is verified by the IPC or by an authorized laboratory and, if found suitable on the basis of the analytical tests, is labeled, stored, and distributed to the stakeholders on demand (Prakash et al., 2017)

TABLE 12.1 Phytomarkers From Medicinal Plants Used in Therapy

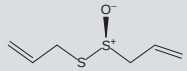
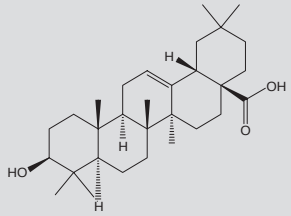
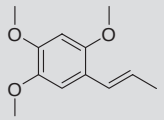
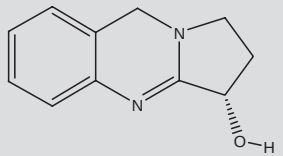
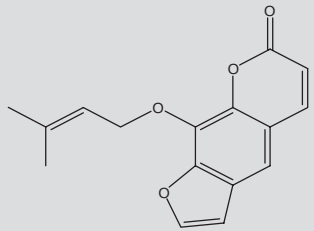
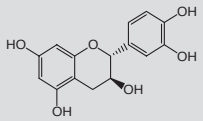
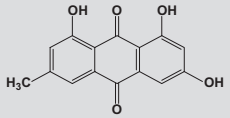
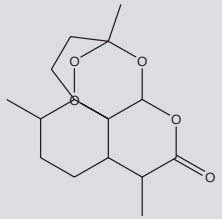
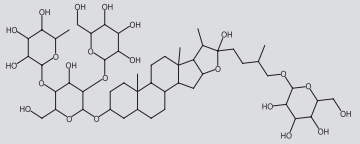
Medicinal Herbs	Common Name	Parts Used	Major Phytoconstituents/Phytomarkers Used	Therapeutic Activity	References
Allium sativum L. (Liliaceae)	Lahasun	Bulb	Alliin, methiin, isoalliin, allicin [1]  Allicin [1]	Antidiabetic, cardiotonic, hypolipidemic	Suleria et al. (2015)
Achyranthes aspera L. (Amaranthaceae)	Apamarga	Root	Oleanolic acid [2], β-sitosterol, spinasterol, ecdysterone, betalaine  Oleanolic acid [2]	Antiviral (HSV), antibacterial, antiinflammatory, antitumor, analgesic	Mukherjee et al. (2013)
Acorus calamus L. (Acoraceae)	Vacha	Rhizome	α-Asarone [3], eugenol, dipentene, asaronaldehyde, terpinolene  α-Asarone [3]	Memory disorder, learning performance, stomachache, antiaging, anticholinergic	Mukherjee et al. (2007)
Adhatoda vasica Nees (Acanthaceae)	Vasaka	Fresh, dried mature leaves	Adhatodic acid, vasicine [4], adhatodine, anisotine  Vasicine [4]	Antibacterial, bronchodilatory, antiasthmatic, antiulcer, anticancer	Singh et al. (2011a, b)
Aegle marmelos Carr. (Rutaceae)	Bel	Fruit pulp	Marmelosin [5], alloimperatorin, psoralen, aegeline, egelenine, marmelin, limonene, β-phellandrene, p-cymene  Marmelosin [5]	Cardiotonic, gastroprotective, antivirus, anticancer	Baliga et al. (2011)

TABLE 12.1 Phytomarkers From Medicinal Plants Used in Therapy—cont'd

Medicinal Herbs	Common Name	Parts Used	Major Phytoconstituents/Phytomarkers Used	Therapeutic Activity	References
Albizia lebbeck L. (Leguminosae)	Shirisha	Stem bark	Catechin [6], kaempferol, albizinin  Catechin [6]	Anti-allergic, antihistaminic	Venkatesh et al. (2010)
Aloe vera L. (Liliaceae)	Ghritkumari	Leave (gel)	Aloe emodin [7], chrysophanol, aloesin, aloin  Aloe emodin [7]	Wound healing, anti-inflammatory, antifungal, hypoglycemic, gastroprotective, hepatoprotective, immunomodulatory	Mukherjee et al. (2014)
Artemisia annua L. (Asteraceae)	Qinghaosu	Leaves	Artemisinin [8], artemisitene, arteannuin B, dihydroartemisinic acid, artemisinic acid  Artemisinin [8]	Antimalarial, antimicrobial, antipyretic etc.	Suberu et al. (2016)
Asparagus racemosus Willd (Liliaceae)	Satamul	Root	Shatavarin I [9], asparagamine, racemosol  Shatavarin I [9]	Phytoestrogenic activity, adaptogenic, antidyspepsia, cardioprotective, antitussive	Bopana and Saxena (2007)

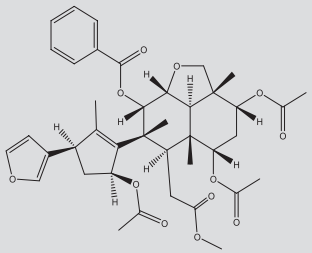
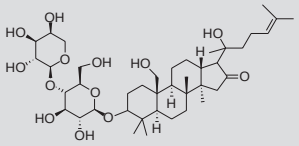
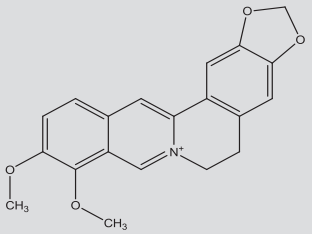
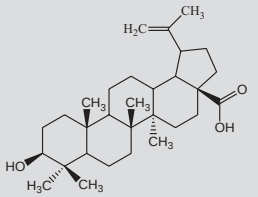
Azadirachta indica A. (Meliaceae)	Nim	Dried leaf	Nimbidin-A [10], nimbolide, azadirachtin, gallic acid, epicatechin, catechin, margolone, margolonone  Nimbidin-A [10]	Antiinflammatory, immunostimulant, hypoglycemic, antiulcer, antibacterial, hepatoprotective	Biswas et al. (2002)
Bacopa monnieri L. (Scrophulariaceae)	Mandukaparni	Dried whole plant	Bacoside [11], bacopasaponins, brahmine, nicotine, herpestine, apigenin, monnierasides  Bacoside [11]	Memory enhancer, antiamnesic	Allan et al. (2007)
Berberis aristata D.C. (Berberidaceae)	Daruharidra	Dried stem	Berberine [12], karachine, aromoline, berbamine  Berberine [12]	Hepatoprotective, antidiarrheal, cardiotonic, antidiabetic	Potdar et al. (2012)
Carissa spinarum L. (Apocynaceae)	Karanda	Root	Lupeol, β-sitosterol, naringenin, betulinic acid [13], ursolic acid  Betulinic acid [13]	Anthelmintic, antipyretic, purgative	Chanda et al. (2014)

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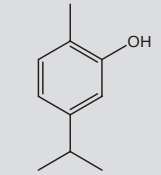
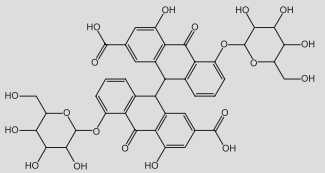
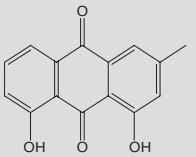
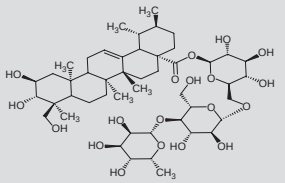
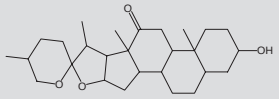
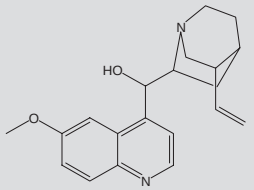
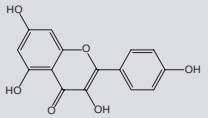
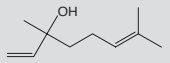
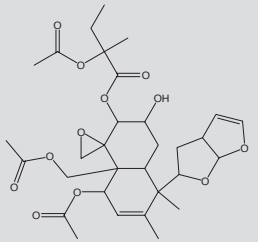
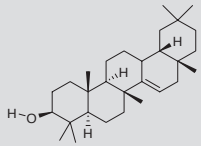
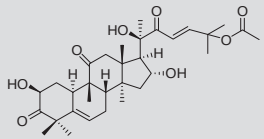
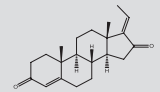
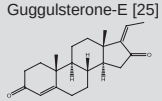
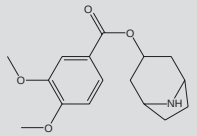
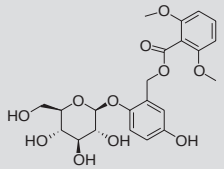
TABLE 12.1 Phytomarkers From Medicinal Plants Used in Therapy—cont'd

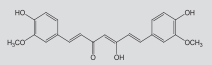
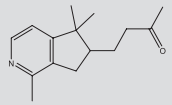
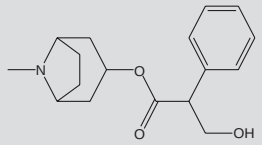
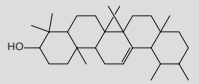
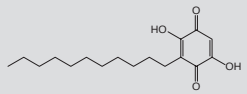
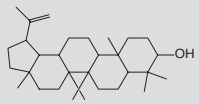
Medicinal Herbs	Common Name	Parts Used	Major Phytoconstituents/Phytomarkers Used	Therapeutic Activity	References
Carum carvi L. (Umbelliferae)	Kalajira	Dried ripe fruits	Carvacrol [14], carvone, α-pinene, limonene, γ-terpinene, linalool, carvenone, p-cymene  Carvacrol [14]	Antimicrobial, anticancer, antidiabetic, diuretic	Johri (2011)
Cassia angustifolia Vahl. (Leguminosae)	Sanaya	Dried leaves	Sennoside [15], aloe-emodin, rhein, chrysophanol  Sennoside A [15]	Purgative, hemostatic, scabicide	Dave and Ledwani (2012)
Cassia tora L. (Fabaceae)	Pavand	Dried seed	Chrysophanol [16], chrysoobtusin, aurantio-obtusin, physcion, emodin, obtusifolin  Chrysophanol [16]	Antilipidemic, antiseptic, diuretic, antimutagen	Dave and Ledwani (2012)
Centella asiatica L. Urban (Apiaceae)	Brahmi	Whole plant	Madecassoside, madasiatic acid, brahmoside, brahminoside, asiaticoside [17]  Asiaticoside [17]	Anxiolytic, antidepressant, antiepileptic, cognition enhancing, antiulcer, antinociceptive	Gohil et al. (2010)

Chlorophytum borivilianum Santapau (Asparagaceae)	Safed musli	Tuber	Borivillianosides, stigmasterol, hecogenin [18]  Hecogenin [18]	Aphrodisiac, immunomodulatory, anticancer	Khanam et al. (2013)
Cinchona officinalis L. (Rubiaceae)	Cinchona	Bark	Quinine [19], quinidine, cinchonine, and cinchonidine  Quinine [19]	Used for malaria fever, indigestion, mouth and throat diseases, cancer	Achan et al. (2011)
Cinnamomum tamala Nees (Lauraceae)	Tejpatra	Dried mature leaves	Kaempferol [20], quercetin, myricetin  Kaempferol [20]	Antidiabetic, anticancer, antiinflammatory, hepatoprotective, antimicrobial	Sharma and Rao (2014)
Cinnamomum zeylanicum Blume (Lauraceae)	Dalchini	Dried inner bark	Cinnamaldehyde, eugenol, α-terpineol, β-caryophyllene, linalool [21], limonene, myrcene, β-pinene  Linalool [21]	Antidiabetic, antihyperlipidemic	Ranasinghe et al. (2013) and Sharafeldin and Rizvi (2015)
Clerodendrum phlomidis L. (Verbenaceae)	Agnimantha	Dried mature root	Pectolinaringenin, scutellarin, clerodin, clerosterol, clerodendrin A [22]  Clerodendrin A [22]	Analgesic, antiinflammatory, antiamnesic, antimicrobial, antiasthmatic, hypoglycemic, immunomodulatory	Kumaradoss et al. (2010)

Continued

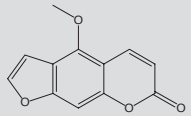
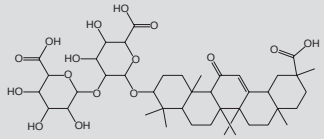
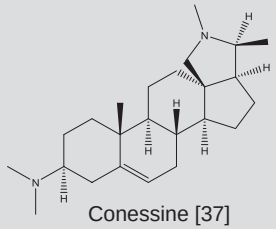
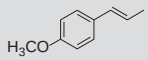
TABLE 12.1 Phytomarkers From Medicinal Plants Used in Therapy—cont'd

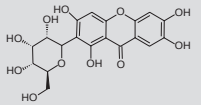
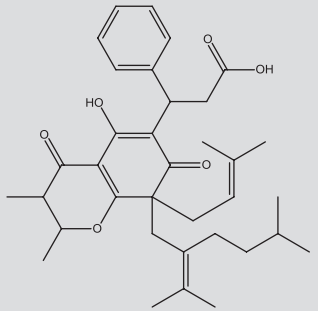
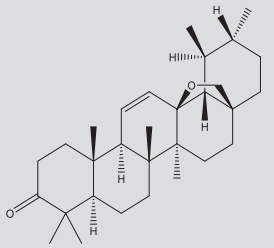
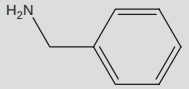
Medicinal Herbs	Common Name	Parts Used	Major Phytoconstituents/Phytomarkers Used	Therapeutic Activity	References
Clitoria ternatea L. (Fabaceae)	Aparajita	Root, flower, leave	Taraxerol [23], taraxerone, kaempferol, rutin, quercetin, myricetin, β-sitosterol  Taraxerol [23]	Antimicrobial, antipyretic, antiinflammatory, analgesic, diuretic, local anesthetic, antidiabetic, antiplatelet	Mukherjee et al. (2008)
Coccinia grandis L. (Cucurbitaceae)	Kunduru	Aerial parts	Taraxerol, β-amyrin, lupeol, cucurbitacin-B [24]  Cucurbitacin-B [24]	Skin diseases, bronchial catarrh, bronchitis, carminative, antipyretic, galactagogue, antiemetic, antispasmodic, expectorant	Gantait et al. (2010a)
Commiphora mukul Hook. (Burseraceae)	Guggul	Exudates of stocks	Guggulsterol I, guggulstertol II, guggulsterone-E [25], guggulsterone-Z [26], sesamin, camphorene  Guggulsterone-E [25]  Guggulsterone-Z [26]	Antihyperlipidemic, cardioprotective, antiwrinkle, antimicrobial	Anurekha and Gupta (2006)
Convolvulus pluricaulis Choisy (Convolvulaceae)	Shankhapushpi	Whole plant	Shankhapushpine, convolamine, convoline, convolidine, convolvine [27], confoline, convosine, kampferol, scopoletin  Convolvine [27]	Anticonvulsant, antidepressant, anxiolytic, antithyroid, memory enhancing	Agarwal et al. (2014)
Curculigo orchioides Gaertn. (Amaryllidaceae)	Kalimusli	Rhizome	Curculigoside A [28], curculigines, orchiosides  Curculigoside A [28]	Antihistaminic, immunostimulant, antiinflammatory	Venkatesh et al. (2009)

Curcuma longa L. (Zingiberaceae)	Haldi	Rhizome	Curcumin [29], demethoxycurcumin, bisdemethoxycurcumin  Curcumin [29]	Antitumor, antioxidant, antiarthritic, antiamyloid, antiischemic, antiinflammatory	Gantait et al. (2011)
Cyperus rotundus L. (Cyperaceae)	Nagarmotha	Dried rhizome	α-Cyperone, α-rotunol, β-cyperone, β-pinene, β-rotunol, β-selinene, cyperene, cyperenone, cyperol, rotundine A [30], rotundosides  Rotundine A [30]	Analgesic, antiandrogenic, antimicrobial, anticonvulsant, antidiabetic, hypolipidemic, neuroprotective, nootropic, lactogenic, hepatoprotective	Pirzada et al. (2015)
Datura metel/stramonium L. (Solanaceae)	Dhatura	Dried seed, whole plant	Hyoscyamine [31], scopolamine, tigloidin, aposcopolamine, apoatropin  Hyoscyamine [31]	Anticholinergic, antiasthmatic, anticancer, antiinflammatory	Soni et al. (2012)
Eclipta alba Hassk (Asteraceae)	Bhringaraj	Whole plant	Eclalbatin, α-amyrin [32], ursolic acid, oleanolic acid, ecliptasaponin, daucosterol  α-Amyrin [32]	Antihepatotoxic, antihyperlipidemic, immunomodulatory, antiinflammatory	Thakur and Mengi (2005) and Nm (2011)
Embelia ribes Burm. (Myrsinaceae)	Vidang	Dried mature fruits	Embelin [33], embolic acid, rapanone, daucosterol, embelinol, embeliol, vilangin, christenbine  Embelin [33]	Antifertility, analgesic, antibacterial, antiinflammatory, antidiabetic, cardioprotective	Mhaskar et al. (2011)
Ficus racemosa L. (Moraceae)	Gular	Dried bark	Leucocyanidin, lupeol [34], stigmasterol  Lupeol [34]	Hypoglycemic, hepatoprotective, antitussive, anthelmintic, analgesic, antipyretic	Paarakh (2009)

Continued

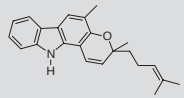
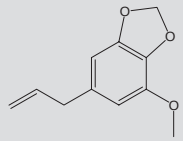
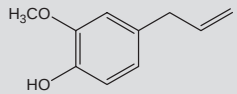
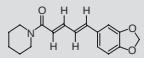
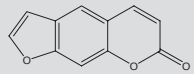
TABLE 12.1 Phytomarkers From Medicinal Plants Used in Therapy—cont'd

Medicinal Herbs	Common Name	Parts Used	Major Phytoconstituents/Phytomarkers Used	Therapeutic Activity	References
Ficus religiosa L. (Moraceae)	Pipal	Bark	Lanosterol, β-sitosterol, stigmasterol, bergapten [35], bergaptol  Bergapten [35]	Antidiabetic, nootropic, wound healing, anticonvulsant, antiinflammatory	Singh et al. (2011a, b)
Glycyrrhiza glabra L. (Leguminosae)	Mulethi	Root and rhizome	Glycyrrhizin [36], liquiritin, isoliquiritin, liquiritigenin, isoliquiritigenin, licochalcone, rhamnose  Glycyrrhizin [36]	Antiinflammatory, antiulcer, hepatoprotective, anti-allergic, antiarthritic, antiarrhythmic, antibacterial, antiviral, antiasthmatic	Gantait et al. (2010b)
Holarrhena antidysenterica Roth (Apocynaceae)	Kurchi	Dried stem bark	Conessine [37], isoconessine, conarrhimine, holarrifine, kurchamide, kurcholessine,7 trimethylconkurchine, conessidine, holarrhidine, kurchenine, holarrhessimine  Conessine [37]	Anthelminthic, antimicrobial activity, antimutagenic, antihypertensive, antimalarial	Sinha et al. (2013)
Illicium verum Hook. (Magnoliaceae)	Ansafal	Fruit	Anethole [38]  Anethole [38]	Anticholinesterase, treatment of dyspeptic complaints, catarrhs of the respiratory tract, infant colic, stomachache, neurotropic, hypothermic, analgesic	Bhadra et al. (2011)

Mangifera indica L. (Anacardiaceae)	Aam	Leaf, bark, fruits	Catechin, mangiferin [39], myrcene, linalool, gallic acid, protocatechuic acid, kaempferol, quercetin, hyperin, lupeol  Mangiferin [39]	Antioxidant, hypolipidemic, antidiabetic, antiatherogenic, hepatoprotective	Rai et al. (2007)
Mesua ferrea L. (Guttiferae)	Nagkesara	Dried stamen	Mesuaferrone-A and -B, mesuaferrol, mesuanic acid [40]  Mesuanic acid [40]	Antiasthmatic, anti-inflammatory, estrogenic, antiplasmodial, antimicrobial	Chanda et al. (2013)
Momordica charantia L. (Cucurbitaceae)	Karela	Fresh fruit	Momorcharins, momordicin [41], momordicinin, momordin, charantin, charine, cryptoxanthin, cucurbitins, cucurbitacins, cycloartenols, diosgenin, erythrodiol, galacturonic acids, gentisic acid  Momordicin [41]	Antidiabetic, antiviral, antibacterial, anticancer	Grover and Yadav (2004)
Moringa oleifera Lam (Moringaceae)	Sahajan	Root bark, seed, stem bark	Moringine [42], moringinine, vanillin, campesterol, stigmasterol  Moringine [42]	Antihypertensive, antispasmodic, antiulcer, anticancer, antimicrobial	Anwar et al. (2007)

Continued

TABLE 12.1 Phytomarkers From Medicinal Plants Used in Therapy—cont'd

Medicinal Herbs	Common Name	Parts Used	Major Phytoconstituents/Phytomarkers Used	Therapeutic Activity	References
Murraya koenigii L. Spreng (Rutaceae)	Kadhi Patta	Leaf	Mahanine, mahanimbine [43], isomahanimbine, koenigine, koenine, girinimbine, koenimbidine, koenidine, murrayanine  Mahanimbine [43]	Antiepileptic, CNS stimulant, antiamnesic, antiinflammatory, antioxidant, immunomodulator	Pandit et al. (2011)
Myristica fragrans Houtt. (Myristacaceae)	Jaiphal	Endosperm of dried seeds	Pinene, camphene, eugenol, myristicin [44]  Myristicin [44]	Aphrodisiac, antibacterial, antifungal	Gupta et al. (2013)
Piper betle L. (Piperaceae)	Pan	Leaf	Anethole, ciscaryophyllene, eugenol [45], α-thujene, trans-β-ocimene, terpinolene, geraniol  Eugenol [45]	Vasorelaxatory effect, antioxidant, antiplatelet, antifertility, anticarcinogenic, antimutagenic, antimicrobial, antispasmodic, carminative, antispasmodic	Rai et al. (2005)
Piper longum L. and *Piper nigrum* L. (Piperaceae)	Pipar and Kalimirch	Fruit, dried stem	Piperine [46], pipene, pieramides, piperamine  Piperine [46]	Antiaging, revitalizing, memory enhancing, adaptogenic, antidiarrheal, antispasmodic, immunomodulatory, remedies for cough, cold, fever, asthma and other respiratory problems, anticancer, antithyroid, hepatoprotective, antihypertensive	Ahmad et al. (2012) and Harwansh et al. (2014)
Psoralea corylifolia L. (Leguminosae)	Bavachi	Fruit	Psoralen [47], isopsoralen, corylifolin, corylin, psoralidin, bakuchiol, corylidin, corylin, glucose, corylifolinin, neobavaisoflavone  Psoralen [47]	Antimicrobial, estrogenic, antitumor	Chopra et al. (2013)

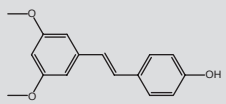
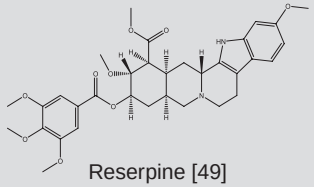
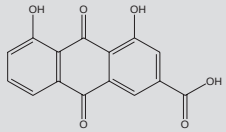
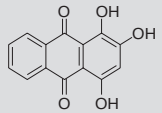
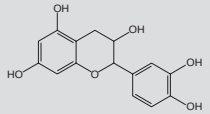
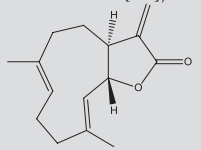
Pterocarpus marsupium Roxb. (Leguminosae)	Piyasala	Heart wood	Pterostilbene [48], isoliquiritigenin, liquiritigenin  Pterostilbene [48]	Antidiabetic, antihyperglycemic, hepatoprotective, cardiotonic	Devgun et al. (2009)
Rauwolfia serpentina L. (Apocyanaceae)	Sarpagandha	Root	Reserpine [49], sarpagine, raupine, rauhimbine  Reserpine [49]	Antidepressant, anxiolytic	Vakil (1955)
Rheum emodi Wall.	Rhubarb	Leaves, rhizomes	Emodin, aloe-emodin, chrysophanol, rhein [50], physcion  Rhein [50]	Antioxidant, cytotoxic, antimicrobial, antifungal, antitumor, antidiabetic, antiproliferative, immunomodulator	Pandith et al. (2014)
Rubia cordifolia L. (Rubiaceae)	Manjistha	Root	Purpurin [51], mollugin, alizarin, lucidin pimeveroside, ruberythric acid, rubiadin  Purpurin [51]	Used in skin ailments, wounds, ulcers, acne, antioxidant, radioprotective, hepato-protective, tyrosinase inhibitor	Biswas et al. (2015)
Saraca asoca Rose. (Leguminosae)	Ashoka	Stem bark	Catechin, epicatechin [52], epigallocatechin  Epicatechin [52]	Antimenorrhagial, antiestrogenic, antimicrobial, anticancer	Shirolkar et al. (2013)
Saussurea lappa C.B. (Compositae)	Kutha	Dried roots	Costunolide [53], arbusculin A  Costunolide [53]	Anticancer, hepatoprotective, antiulcer, immunomodulator, anticonvulsant	Zahara et al. (2014)

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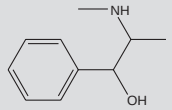
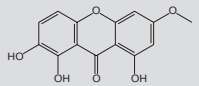
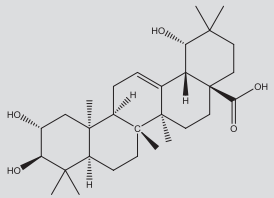
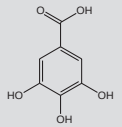
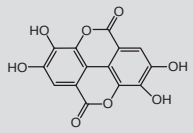
TABLE 12.1 Phytomarkers From Medicinal Plants Used in Therapy—cont'd

Medicinal Herbs	Common Name	Parts Used	Major Phytoconstituents/Phytomarkers Used	Therapeutic Activity	References
Sida cordifolia rhombifolia L. (Malvaceae)	Pitabala	Dried roots	Ephedrine [54], vasicinone, hypaphorine  Ephedrine [54]	Antimicrobial, antiulcer, cytotoxic, analgesic, antiinflammatory, antipyretic, cardioprotective	Dinda et al. (2015)
Swertia chirata Buch. (Gentianaceae)	Chirayata	Whole plant	Amarogentin, swertianin [55], amaroswerin, chiratanin, chiratenol, decussatin, enicoflavine, erythrodiol, gentianine, gentiocrucine, swertanone, swertenol  Swertianin [55]	Anthelmintic, antileishmanial, anticholinergic, anticonvulsant, antiinflammatory, antimalarial, antipyretic	Joshi and Dhawan (2005)
Terminalia arjuna W. (Combretaceae)	Arjuna	stem bark	Arjunin, arjunic acid [56], arjunolic acid, arjuntin, arjunosides, punicalin, punicallagin, terchebulin  Arjunic acid [56]	Cardioprotective, antihyperlipidemic, antiatherogenic	Dwivedi and Chopra (2014)
Terminalia chebula Retz. (Combretaceae)	Haritaki	Fruit	Gallic acid [57], chebulagic acid, punicalagin, chebulanin, corilagin, terchebulin  Gallic acid [57]	Anticarcinogenic, hepatoprotective, cytoprotective, cardioprotective, hypolipidemic, antiviral, purgative, immunomodulatory	Bag et al. (2013)
Terminalia belerica Roxb. (Combretaceae)	Bahera	Fruit	Gallic acid, gallotannic acid, ellagic acid [58], termilignin, thannilignin, chebulaginic acid, phenyllemblin, β-sitosterol, bellaricanin  Ellagic acid [58]	Antiatherosclerotic, hepatoprotective, cardioprotective, cytoprotective, cardiotonic, antimutagenic, antifungal	Ponnusankar et al. (2011)

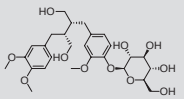
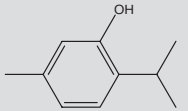
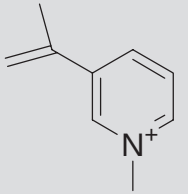
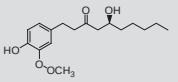
Tinospora cordifolia Willd. (Menispermaceae)	Guduchi	Dried stem	Tinosporide, furanolactone clerodane diterpene, furanoid diterpene, tinosporoside A [59]  Tinosporoside A [59]	Anticancer, antidiabetic, antiinflammatory, hypolipidemic	Sinha et al. (2004)
Trachyspermum ammi L. (Umbelliferae)	Ajwain	Dried fruits	Thymol [60], p-cymene, pinene, carvone, limonene, dillapiole  Thymol [60]	Antidiarrheal, gastroprotective, antihypertensive, antiinflammatory, antihyperlipidemic	Asif et al. (2014)
Trigonella foenum-graecum L. (Fabaceae)	Methi	Fruit (seed)	Trigonelline [61], trigocoumarin, trigomethyl coumarin, gitogenin, trigogenin, tannic acid  Trigonelline [61]	Anticholinesterase, antidiabetic, antiulcer, wound healing, CNS stimulant, antiinflammatory, antipyretic immunomodulatory, antioxidant	Kumar et al. (2010)
Zingiber officinale Roxb. (Zingiberaceae)	Sonth	Rhizome	6-Gingerol [62], shogaol, cineole, geraniol, curcumene, citral, terpineol, borneol  6-Gingerol [62]	Antipyretic, antitussive, hypotensive, cardiotonic, antiplatelet, antiviral (hRSV), anticancer, antioxidant, hepatoprotective, antiinflammatory, bronchitis, other respiratory tract infections	Rai et al. (2006) and Harwansh et al. (2014)

TABLE 12.2 Reference Substances of Some Plants Used in the Indian Pharmacopeia

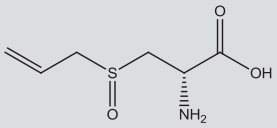
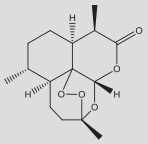
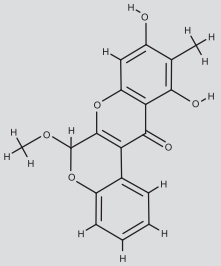
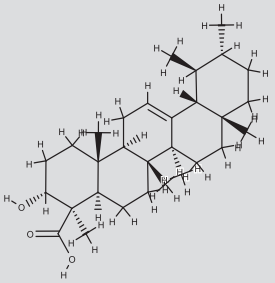
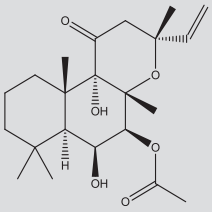
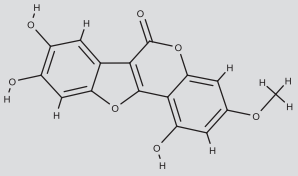
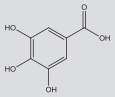
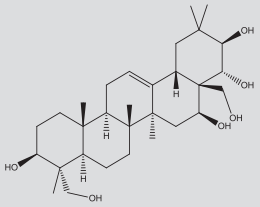
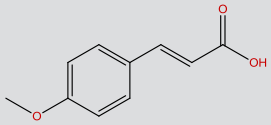
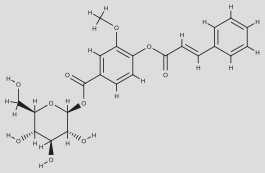
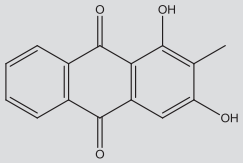
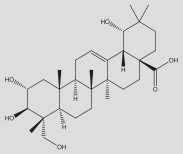
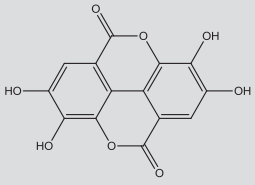
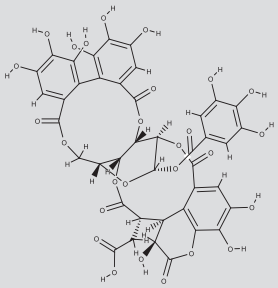
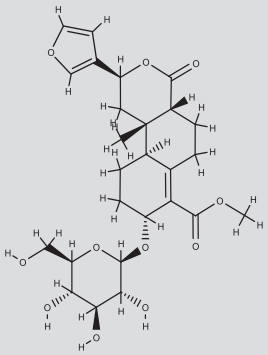
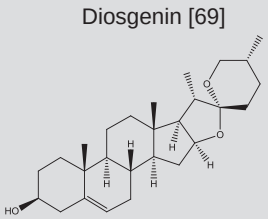
Plant Name	Reference Standard Used
Allium sativum (Lasuna)	Alliin [75] 
Artemisia annua (Artemisia)	Artemisinin [65] 
Asparagus racemosus (Shatavari)	Shatavarin [78]
Boerhavia diffusa (Punarnava)	Boeravinone [77] 
Boswellia serrata (Kunduru)	11 keto β-boswellic acid [73] 
Coleus forskohlii	Forskolin [68] 
Eclipta alba (Bhringraj)	Wedelolactone [67] 

TABLE 12.2 Reference Substances of Some Plants Used in the Indian Pharmacopeia—cont'd

Plant Name	Reference Standard Used
Emblica officinalis (Amalaki)	Gallic acid [63] 
Gymnema sylvestre (Gudmar)	Gymnemagenin [70] 
Hedychium spicatum (Shati)	P-methoxy cinnamic acid ethyl ester [79] 
Mangifera indica (Amra)	Mangiferin [39]
Ocimum sanctum (Tulasi)	Eugenol [45]
Picrorhiza kurroa (Kutki)	Kutkin [74] 
Piper longum (Pippali Large)	Piperine [46]
P. longum (Pippali small)	Piperine [46]
P. nigrum (Maricha)	Piperine [46]
Rauwolfia serpentina (Sarpagandha)	Reserpine [49]
Rubia cordifolia (Manjistha)	Rubiadin [76] 
Terminalia arjuna (Arjuna)	Arjungenin [64] 
Terminalia bellirica (Bhibhitaki)	Ellagic acid [66] 

Continued

TABLE 12.2 Reference Substances of Some Plants Used in the Indian Pharmacopeia—cont'd

Plant Name	Reference Standard Used
Terminalia chebula (Haritaki)	Chebulagic acid [72] 
Tinospora cordifolia (Guduchi)	Cordifolioside A [71] 
Tribulus terrestris (Gokhru)	Diosgenin [69] 

12.4 DNA-BASED MARKER ANALYSIS

Organoleptic characteristics for documentation of herbs are one of the criteria for identification of medicinal plants. However, it may not be accurate all of the time due to variations. The herbs are documented, examined, and analyzed and the content of various plant cells is observed under a microscope. These studies, supported by organoleptic markers or anatomical characters, are many times inaccurate. Further, analytical chromatographic techniques were used for authentication of herbs. However, during the last 20 years, molecular markers have quickly complemented the classical ways. Molecular markers are usually cited as biochemical constituents, together with primary and secondary metabolites in plants and macromolecules, namely, proteins and deoxyribonucleic acids (DNA). Secondary metabolites as markers are extensively employed in quality control and standardization of herbal medicine, however, they also have a few limitations. The focus is currently on the development of markers supported by genetic composition, which are thus distinctive, stable, and omnipresent to the plants. Age, state, or environmental factors do not affect DNA-based markers (Chan, 2003). Various kinds of DNA-based markers, specifically, RFLP (restriction fragment length polymorphism), ISSR (intersimple sequence repeat), SSR (simple sequence repeat), RAPD (random amplified polymorphic DNA), and AFLP (amplified fragment length polymorphism) are used for plant species discrimination coupled with methods of plant identification, including taxonomy, physiology, and embryology (Joshi et al., 2004).

12.5 DNA BARCODING FOR IDENTIFICATION OF HERBS

Species-specific variation in DNA of different plants can be determined by DNA barcoding. It has gained wide interest in the area of medicinal plant research due to its use in the analysis of herbs based on genetic characters (Blaxter, 2004; Hebert et al., 2003). DNA barcoding is not restricted by morphological characteristics and physiological conditions and permits authentication of different plant species. The method is also standardized to specific DNA barcodes and universal primers, which are favorable for building databases and establishing a universal common place for identification. DNA barcoding is able to perform fast, accurate, and automatic identification of species from a wide range and quality of raw materials (Marshall, 2005). This addresses the difficulties concerned in classifying herbs and might lead to a taxonomical renaissance in their identification (Hajibabaei et al., 2005; Hebert and Gregory, 2005). This is a very effective tool to enrich the authentication of plants and to find adulteration with closely connected species that are indistinguishable due to their physical, microscopic, and anatomical characteristics (Chen et al., 2014).

DNA is often recovered from recent, dried, processed biological material with very small sample amounts, as required. A variety of DNA fingerprinting techniques have been developed to judge DNA polymorphism for authentication of plant species. The different ways presently in use involve polymerase chain reaction (PCR) for DNA amplification. Recently, DNA sequencing has been used either along with, or as a replacement for conventional DNA techniques (Pferschy-Wenzing and Bauer, 2015). PCR-based DNA fingerprinting techniques are often classified in line with the sort of genetic markers used, as follows:

- AFLP technique.
- RAPD technique.
- Intersimple sequence repeat (ISSR) method.
- Cleaved amplified polymorphic sequence, originally named PCR–RFLP.

Nevertheless, these strategies have been used for the authentication of various medicinal plant species. A few examples are as follow:

- Discrimination of *Illicium verum* from its toxin adulterant *Illicium anisatum*. This method was capable of detecting trace amounts of *I. anisatum* admixed with *I. verum* (ratio: 1:100–1:500) (Chen et al., 2014).
- Single-locus strategies involve sequence-based PCR-amplified markers, specifically single-locus microsatellite markers, also called SSRs. SSRs are short DNA sequences that occur as tandem repeats of mono-, di-, tetra-, penta-, and hexanucleotides. Usually, a pair of primers that flank the microsatellite marker is employed for PCR amplification of the targeted locus. These amplification products are then separated by electrophoretic methods and patterns and read out with the help of visualization (Chen et al., 2014).

Single-nucleotide polymorphisms (SNPs) represent a single nucleotide difference between two individuals at a defined location. These markers are notably helpful once applied as microarrays that permit the synchronous detection of high numbers of SNP sites or in conjunction with multiplex PCR techniques. SNPs are known by the direct sequencing of multiple copies of constant genomic region, or existing knowledge may be derived from databases. DNA sequencing is being combined with or even replaced by the well-established DNA fingerprinting technique.

One single region has not been found sufficient to distinguish between plant species. Hence, the Consortium of the Barcode of Life has provided a uniform protocol for the barcoding of plants and has advised a mixture of the two chloroplast regions matK and rbcL as applicable barcodes. Different sequencing methods used for the sequencing of DNA are listed below:

- Traditional Sanger method.
- 454 pyrosequencing.
- SOLID.
- Illumina.

These techniques have a powerful impact on DNA fingerprinting and barcoding: standard sequence-based markers, such as SSRs and SNPs, will be quicker alternatives with reduced price and energy. On the other hand, because of the time and value efficiency of those techniques, larger genomic regions, compared with those presently applied in DNA barcoding, are simply amenable to sequencing. Owing to the simplicity and price efficiency of this approach, the entire plastid genome is used as a barcode for the identification of plant species (Chen et al., 2014). Some limitations for the uses of DNA-based methods are:

- Low quality of the extracted DNA from plants.
- High concentrations of certain secondary metabolites, such as polysaccharides, tannins, essential oils, phenolics, or alkaloids, may interfere with DNA extraction or PCR.

Discrimination between plant parts is not possible by DNA-related methods. Therefore, adulteration with other plant parts of the same plant cannot be ruled out with these techniques (Pferschy-Wenzing and Bauer, 2015).

12.5.1 Development Steps for Building a DNA Barcoding of Herbal Materials

Establishing a DNA barcoding database of herbal materials involves sample collection, verification of voucher herbarium specimens, DNA extraction, PCR amplification, sequencing, sequence assembly, species identification, and morphological verification. A reliable barcoding plays a significant role for identification of the source plants. A minimum of three duplicate assortments per species are required; every collection (uniquely numbered) has to embody a little plant sample for DNA extraction and voucher herbarium specimens of the entire plant (ideally flowering or fruiting) and be sourced from the same plant or population of plants. These collections should be presented in the form of photos and elaborate field notes describing any identification characters not evident from the herbarium specimens, for example, collector's name and phone data, GPS location, habitat, and plant height (Bridson and Forman, 1992; Chen et al., 2014).

12.5.1.1 DNA Extraction

High-quality genomic DNA is a vital requirement for correct identification in DNA barcoding. As such, DNA extraction of herbal materials should be performed rigorously and quickly, with the use of smart sterile techniques to avoid DNA degradation and contamination between samples. Herbal materials are normally harvested long before they are used.

(a) Root, rhizome, stem, and cortex material

Generally, before being ground to a fine powder in liquid nitrogen for the purpose of DNA extraction, the surface of herbal material is cleaned with 75% alcohol. In most cases, root and rhizome-based herbs have high levels of polysaccharides and polyphenols. These should be removed, utilizing polyvinyl pyrrolidone (PVP) and β-mercaptoethanol in the first stages of DNA extraction. Root- and rhizome-based herbs are a rich source in fiber plant energy stores and starches, and often, the number of fabric and corresponding extraction chemicals should be exaggerated. Some herbs contain a large amount of parenchyma and fiber; therefore, the amount of plant starting material, PVP, and β-mercaptoethanol should be increased accordingly for DNA extraction (Chen et al., 2014).

(b) Leaf and flower material

The leaves and flowers of plants have low fibrous and secondary metabolite content and, as such, tend to be the best tissues to extract high-quality DNA. For younger, healthy leaves, the direct PCR methodology is strong enough to get the target DNA barcode. However, for several other materials, such as dry roots, stems, and fruits, the tactic is not effective as these materials contain several secondary metabolites that disrupt PCR amplification. In our opinion, with respect to the direct PCR methodology, time is really saved by conducting DNA extraction using proper plant tissues (Chen et al., 2014).

(c) Fruit and seed material

Fruit- and seed-based herbs typically have high oil content, which might hamper grinding and lead to low DNA yields. The amount of starting material should hence be increased. A solvent, such as acetone, may also be used in extraction to eliminate phenolic resin compounds (Chen et al., 2014).

12.5.1.2 PCR Amplification and Sequencing

ITS2 and psbA–trnH are the standard and supplementary DNA barcodes for characteristic herbal materials, respectively. The universal primers for the ITS2 barcode are S2F (5′-ATGCGATACTTGGTGTGAAT-3′) and S3R (5′-GACGCTTCTCCAGACTACAAT-3′) (Chen et al., 2010). The universal primers for the psbA–trnH barcode area unit are fwd PA (5′-GTTA TGCATGAACGTAATGCTC-3′) and rev TH (5′-CGCGCATGGTGGATTCAC AATCC-3′) (Chen et al., 2010; Kress et al., 2005). The reaction conditions and elements used for PCR amplification can be found in the preceding study. Purified PCR products have to be to be sequenced in both directions (Chen et al., 2014).

12.5.1.3 Sequence Assembly

The original forward and reverse sequences have to be assembled using programs for sequence assembly, and quality control can be performed as shown in a previous study (Chen et al., 2010). All complete ITS2 or "psbA–trnH" sequences are retrieved in accordance with the GenBank annotations.

12.5.1.4 Species Identification

Users can question their sequences on the DNA barcoding information for ancient medicines, such as Traditional Chinese Medicines (http://www.tcmbarcode.cn), to simply verify the species identity of the questioned sequences. The procedures only require the pasting of the sequences queried into the text box of ITS2 or psbA–trnH and pressing the "submit" button. The results area is displayed, and the best-hit sequence is the closest match to the question (Chen et al., 2014).

12.5.2 Significance of DNA Barcoding

- DNA barcode identification can be applied to a large variety of herbal materials. If the result indicates the species to diverge from the species anticipated, then the species may be an accidental or intentional substitute species, which may or may not be harmful. By concluding that the item is not the right species, the employment of that sample (and presumably the entire batch) will be avoided along with a possible adverse reaction.
- To resolve causes of adverse reaction and such applications of this technology, particularly once employed in combination with morphological and/or chemical authentication strategies (Chen et al., 2014).

12.5.3 Restriction Fragment Length Polymorphism (RFLP)

RFLPs are thought of as the primary development within the field of genetic markers and are responsible for initiating the sphere of molecular genetics. The technique relies on the principle of variation that occurs because of the incidence of mutations in restriction nuclease binding and cleavage sites; in addition, any reorganization within the genomic region flanked by restriction sites that conjointly disrupts their distribution, and therefore causes polymorphism, contributes to RFLP. The digested fragments vary in size and are separated using Southern blot analysis. Alternatives of Southern analysis in RFLPs are enzyme chain reactions. Like other primarily DNA-based markers, there is ample literature (mainly PCR–RFLP) that indicates the use of this method in the identification of medicinal plants. For example, six plant species—*Desmodium giganicum*, *Aegle marmelos*, *Solanum xanthocarpum*, *Solanum indicum*, *Tribulus terrestris*, *Oroxylum indicum*—were identified through PCR–RFLP and therefore the regions amplified were internal transcribed spacer (ITS) with the help of ITS1 (F) and ITS4 (R) primers (Biswas and Biswas, 2013). An equivalent technique has been applied to *Boerhavia diffusa* L., during which 700 bp of ITS region was obtained via enzyme chain reaction and once this region was restricted with Msp I, provided four distinctive fragments that differentiated this species from *Trianthema portulacastrum* and *Trianthema monogyna* (Biswas et al., 2013; Feng et al., 2010) and completely differentiated *Angelica sinensis* from its seven different adulterant Angelica species; here a combination of primers specific for the ITS region were designed that amplified 520 bp from the adulterants and no product was amplified with the DNA of *A. sinensis* (Ganie et al., 2015a, b).

12.5.4 Simple Sequence Repeats (SSRs)

SSRs are comprised of 2–5 bp DNA monomeric units that are recurrent multiple times at a particular locus. Such markers are fully codominant and are used for the fingerprinting procedure, marker-assisted choice, kinship, breeding behavior, such as selfing and outcrossing, and establishing population structure. Microsatellites are regionally tandemly duplicated and are found distributed throughout. Therefore, it is vital to amplify a selected microsatellite in a locus-specific manner using locus-specific primers. Hon et al. (2003) applied 16 microsatellites to investigate 150 and 40 *Panax quinquefolius* and Oriental Ginseng roots, respectively (Hon et al., 2003). Of the 16 microsatellites, nine could distinguish between Chinese samples and *P. quinquefolius*. A study was undertaken of the Capsicum species, usually used as spices, which are analyzed through microsatellites. The study involved 800 lines collected from totally different locations in Central and South America. SSR primers (5751 pairs) were designed and searched for similarity with the tomato genome (Shirasawa et al., 2013; Ganie et al., 2015a, b).

12.5.5 Random Amplification of Polymorphic Deoxyribonucleic Acid (RAPD)

In random amplification of polymorphic DNA (RAPD) technology, random short artificial oligonucleotide primers (10–12 base pairs) are required to amplify the genomic DNA through PCR under low annealing temperature. The amplicons generated are separated on agarose gels according to size. The primer size is short, thus the annealing temperature varies in the

range 28–38°C. In this temperature range, primers anneal when they find complementary sequences from the genome and therefore the profile of amplified DNA varies in size according to the nucleotide sequence homology and the primer at the tip of every amplified product (Ganie et al., 2015a, b). RAPDs are widely used for authentication of plant species of medicinal value. Two varieties of *Silybum marianum* that are quite difficult to distinguish in dried conditions may be differentiated with RAPD. The banding pattern amplified with primer OPP-10 contained 2 (600 and a 1000 bp) characteristic bands; primers OPG-03, OPC-17 generated 2 distinctive bands (1000 and 300 bp) (Thormann et al., 1994). This profile, specific for milk thistle var. album, might differentiate it from milk thistle var. purple (Abouzid, 2014). Ali et al. (2013), while characterizing *Clitoria ternatea* at the interzonal level, found complete monomorphism with primer OPN-02, Hence, the identification of this herb with OPN-02 is a sensible choice (Ali et al., 2013). A molecular characterization of *Convolvulus pluricaulis* indicated that primer OPN-09 is the species-specific primer for the herb (Ganie et al., 2015a, b). A typical band of 2.2 kB amplified by Primer OPN-05 was found once different accessions of *Evolvulus alsinoides* were studied through RAPD analysis (Ganie and Sharma, 2014). Laboratories with restricted budget prefer RAPDs because the entire method depends only on the thermal cycler and gel electrophoresis unit. RAPD scans with efficiency and differentiates taxa below the species level (Choo et al., 2009 because it deals with both the coding and noncoding regions of the genome.

12.5.6 Amplified Fragment Length Polymorphism (AFLP)

This technique, which is a combination of each RFLP and RAPD, relies on the detection of restriction fragments by PCR amplification and may be used for DNA of any origin, complexity, or quality (Vos et al., 1995). The fingerprints are made with no previous information of the sequence, employing a restricted set of primers. Fragments are generated with two different end sequences that form the template meant for adapter ligation. Such adapters then lay the foundation for PCR, which is performed using end-labeled nested primers with selective nucleotides. The amplified product is electrophoresed in 6% denaturing polyacrylamide gel and autoradiographed or detected through fluorescent detection. AFLPs are usually considered extremely valued markers for analyzing the genetic diversity for each animal and plant systems compared with authentication reports (Ganie et al., 2015a, b). Seven specific fragments known with the primer combinations E-ATC + M-noble metal and E-GCA + M-GT might function as the genetic markers for *Aloe vera* (Tripathi et al., 2011). Though the three species of the Echinacea may be easily distinguishable in fresh and intact condition, it becomes very difficult to differentiate them in commercial preparations, which are ground, dry plant elements of genus *Echinacea purpurea* (valuable species for chemotherapeutical properties) mixed with the two other species. Russi et al. (2009) used contemporary material collected from the cultivated genus Echinacea sp. for the discrimination of those species by AFLP. E + CAC/M + AAT and E + CAC/M + AGC generated 13, 9, and 4, and 7, 5, and 5 specific fragments for *E. purpurea*, *Echinacea angustifolia*, and *Echinacea pallida*, respectively (Russi et al., 2009). Passinho-Soares et al. (2006) discovered species-specific bands of various species of the genus Plectranthus. Throughout the study 2, 2, and 1 distinctive bands were confirmed for *Plectranthus barbatus*, *Plectranthus grandis* and *Plectranthus ornatus*, respectively, with the primer combination EcoR 1-CAC + MS 1-GCA (Passinho-Soares et al., 2006). Applying the AFLP technique, P + GC/M + CTA combination, three bands in the range 500–700 bp were discovered in *Embelia ribes*, which is a distinctive feature of the species (Gowda et al., 2010).

12.5.7 Intersimple Sequence Repeat (ISSR)

Intersimple sequence repeat (ISSR) is one of the major markers involved in the amplification of DNA segments found at an amplifiable distance in between two identical microsatellite repeat regions oriented in directions opposite to one another. The system depends on the principle of exploitation of microsatellites as primers that target multiple genomic loci to amplify intersimple sequence repeats of the genomic DNA of various sizes. Microsatellites used as primers for ISSRs are di–penta nucleotides. A survey of the literature reveals that ISSR markers are more dependable than RAPDs (Kojima et al., 1998; Ganie et al., 2015a, b), though it varies with the detection technique used. According to Fang and Roose (1997), more than 99% reproducibility was achieved after performing repeated tests for ISSR markers with the DNA samples of the same cultivar nurtured in several locations, DNA having been extracted from totally different aged leaves of the same, and by performing separate PCR runs (Fang and Roose, 1997). An authentication of the Rheum species was performed with different ISSR primers (Wang, 2011). A successful attempt by Tamhankar et al. (2009) was also made for authentication of samples of Chirayat complex (*Swertia angustifolia* Ham. ex D. Don Nauni, *Swertia chirayita* [Roxb. ex Fleming] Karsten, *Swertia cordata* Wall, *Swertia densifolia* Griseb, *Swertia lurida* Clarke, *Swertia ciliata*, *Swertia paniculata* Wall, *Swertia alata* Clark Mirik, *Swertia bimaculata* Hook f. & Thoms). The DNA of the market samples and the three authentic species was amplified using ISSR primers and the profile thus obtained was compared and the differentiations were obtained with primers 808 and 809 (Tamhankar et al., 2009). The roots of *Cissampelos pareira* L. var. hirsuta (Buch.-Ham. ex. DC.),

usually called Patha in Asian countries, is substituted with two alternative species, *Cyclea peltata* (Lam.) and *Stephania japonica* (Thunb.). ISSR profiles distinguished the real raw drug of "Patha" from its substitutes/adulterants to ensure the standard and legitimacy of this drug within the market (Vijayan et al., 2014).

12.5.8 Sequence Characterized Amplified Region (SCAR)

The most vital marker for authentication of medicinal plants is SCAR. It is a sequence-based mono-locus and a co-dominant marker in which forward and reverse primers are designed from the actual region of a cloned AFLP, RAPD, and ISSR DNA fragment joined to an attribute of interest. SCAR may also be a particular sequence or random DNA fragment within the genome of an organism and the primers for amplification located at any suitable position within, or flanking the unique AFLP, RAPD, and ISSR amplicon. SCAR is a fast, reliable, and extremely duplicable marker used in biological science. The designed primers establish the target species from the pool of related species by the presence of one distinct and bright band within the desired sample. The length and Gc content is an additional concern for SCAR markers and usually 20–25 oligonucleotide bases are sequence specific (Kiran et al., 2010). SCAR markers developed from AFLP and SSR are more reproducible, but such markers are costly, time consuming, and more difficult (particularly in AFLP in which silver staining is needed for the extraction of DNA fragment from polyacrylamide gels). To convert a specific distinctive RAPD, AFLP, ISSR, or SSR band to a SCAR marker, every distinctive band is eluted, cloned, and sequence verified. The nucleotide sequence of the distinctive DNA band is analyzed for uniqueness by comparing with the known DNA sequences available in various databases for synthesizing specific SCAR primers. Yadav et al. (2012) confirmed a 589-bp species-specific band with the RAPD primer OPAA-3 in *Bacopa monnieri* accessions that are not found in alternative adulterant candidates. For further processing, two SCAR primers (between 406 bp of 589 bp sequence of RAPD amplicon) was designed. The PCR confirmation results indicated a definite band from *B. monnieri* and not within the adulterants (Yadav et al., 2012). Seethapathy et al. (2014) attest Ativisha (*Aconitum heterophyllum*) and Musta (*Cyperus rotundus*) through the use of nrDNA ITS sequence-based SCAR markers and once market samples were examined it was found that SCAR primers (Cyr-FP and Cyr-RP) may establish tissue samples containing 750 µg to 4.76 mg/100 mg of Musta in complicated mixtures of DNA extracted from industrial herbal drugs. It was also found that Ativisha was not known through SCAR markers, confirming that this authentic species is not used to prepare herbal drugs despite it being labeled as one of the ingredients in formulations (Seethapathy et al., 2014). RAPD-based SCAR markers for the real herbs, *Cassia angustifolia* and *Cassia acutifolia* and their adulterants, *Cassia sophera* and *Cassia tora* have been developed. An analysis of the results confirmed more than 50% adulteration in the samples (Abdin, 2013). *Lonicera japonica*, a traditionally used medicinal plant, was studied by an improved RAPD analysis and the SCAR markers that were developed differentiated the different accessions/populations (Fu et al., 2013). In a similar manner, while using SCAR markers "SA06 and SB05," *Ophiopogon japonicus* was differentiated with an amplicon of 460- and 553-bp, respectively, from *Liriope platyphylla*; the marker SA12 amplified a 485-bp fragment specific to *L. platyphylla* (Li and Park, 2012). *Phyllanthus amarus* Schum. & Thonn is used as an antipyretic and diuretic, and to treat liver diseases and microorganism infections. These species are differentiated from the less effective species that embrace *Phyllanthus debilis* L. and *Phyllanthus urinaria* (Theerakulpisut et al., 2008).

12.6 CHEMOMETRICS IN MARKER ANALYSIS

In 1971, a Swedish person, Svante Wold, coined the term "kemometri" in Swedish and its English equivalent is "chemometrics" (Kiralj and Ferreira, 2006). In the years 1986 and 1987, two journals were started, named "Chemometrics and Intelligent Laboratory Systems" and "Journal of Chemometrics," which promoted instrumentation and offered new strategies for the development of the latest high-dimensional combined instrumentation (Bansal et al., 2014). This equipment has opened up several new choices for knowledge acquisition in the improvement of analytical techniques. Chemometrics has emerged to have a significant role inside analytical chemistry (Kong et al., 2009). As is understood, HPLC is extensively applied for internal control of herbal drugs due to its sensitivity, superior precision, high resolution, and intensive relevance. LC–MS, GC–MS, and LC–NMR are being progressively utilized in complicated chemical identifications of herbal drugs (Ganzera et al., 2002). This advancement in instrumentation is able to produce a huge amount of data that record the small variations between samples and this has implications for the discrimination of herbal plants. Before analyzing, pretreatment of data is important as unknown elements or unclear interferences cause overlapped peaks and shifted baselines. Thus, we tend to discuss numerous commonly used chemometric techniques in herbal drug standardization, such as principal element analysis (PCA), linear discriminate analysis (LDA), spectral correlative chromatography (SCC), information theory (IT), local least sq. (LLS), heuristic evolving latent projections (HELP), and orthogonal projection analysis (OPA).

12.6.1 Tools for Preprocessing Knowledge

To eliminate or diminish unwanted sources of variations due to instrumental responses from hyphenated techniques and to get more economical results, data preprocessing techniques are applied. Preprocessing techniques that are applied include LLS and normalization. To get the correct quantitative results from a fingerprint, the pretreatment of chromatographic data is important (Bansal et al., 2014).

12.6.1.1 Normalization

A variation in sample concentration may have an effect on the entire chromatographic profile. Hence, the data are examined before performing the statistical procedure. Every recording consists of N peaks, with every peak space C_i of the ith component being used. The total area of all peaks is calculated as the peak area after normalization as follows (Bansal et al., 2014):

$$C = \sum_{i=1}^{N} C_i$$

$$C_i = C_i / C$$

Then, every peak is expressed as a percentage of the sum of the peak areas (Dunn et al., 1984).

12.6.1.2 Local Least Square (LLS) Technique

A major downside in analyzing HDs is the prevalence of signal shift. It has a big influence on the chromatographical profile. To eliminate chromatographical shift, the LLS methodology is employed to correct retention time shift. When the pattern approach is utilized, then it is necessary to correct retention time in all chromatograms unless it causes a significant issue. To match all chromatographical profiles with their retention times, all common constituents should be chosen and used for chromatographic alignment (Li et al., 2004; Bansal et al., 2014).

12.6.2 Tools for Extracting Chemical Data

The aim of these strategies is to observe similarities or extract helpful data from the information obtained from an analytical instrument. These strategies are necessary to seek out the analyte of interest in a complicated mixture of plant fingerprints. Similarity analysis (SA), SCC, and IT strategies are commonly used tools (Bansal et al., 2014).

12.6.2.1 SCC (Spectral Correlative Chromatography)

It is a method to establish the chemical element present in several chromatograms as acquired from a hyphenated instrument. It is based on the fact that the same chemical components should have the same spectra no matter what or how they are eluted through diverse chromatographic columns. The spectral information is utilized to pick up the targeted component from the other two-way chromatograms. There are several procedures for carrying out SCC as follows (Bansal et al., 2014):

- Assess peak purity of targeted element and procure its ultraviolet radiation or VIS-spectrum from the recording.
- To establish the element within the recording of interest through a comparison of a series of spectra at every scan point of alternative recording by their correlation coefficients.
- Get a curve of the parametric statistic vs scan point in the direction of retention time and validate the second step by consideration of the data within the native natural process cluster wherever the target exists.

The result obtained by SCC is very correct in the case of MS because the spectrum is unique compared with the ultraviolet light spectrum (Li et al., 2004; Bansal et al., 2014).

12.6.2.2 Information Theory (IT)

The value of information content depends on the degree of separation of the chromatogram. The more they separate with uniform concentrations, the higher the value of the information content, that is, the more chemical information can be obtained from this chromatogram. By comparing the magnitude of information content, the maximal chemical information under certain chromatographic conditions, which includes all extraction and detection parameters, can be determined (Gong et al., 2003; Bansal et al., 2014).

12.6.3 Tools for Resolution of Mixtures

12.6.3.1 HELP (Heuristic Evolving Latent Projections)

HELP is a multivariate resolution method used to resolve two-way bilinear data into spectra and chromatograms of the pure constituents (Kvalheim and Liang, 1992; Liang et al., 1992). This method employs the feature of visual interface from a latent variable projection graph and also provides information on the local rank of the data matrix.

12.6.3.2 OPA (Orthogonal Projection Analysis)

OPA is a step-wise process and selects one key variable at each step. This method calculates dissimilarity based on the mathematical concept of orthogonalization. OPA compares each spectrum with one or more than one reference spectra and searches for the least correlated spectrum (Sanchez et al., 1996).

12.6.4 Tools for Displaying Data

These tools are helpful to resolve the net analytical signal by discovering the dominant factors, while excluding the relevant interference, therefore giving a correct estimate. These include numerous methods, such as LDA, hierarchical clustering analysis (HCA), SIMCA, and PCA; however, among these PCA and LDA are found to be most typically used (Bansal et al., 2014).

12.6.4.1 PCA (Principal Component Analysis)

The central plan of PCA is to diminish the spatial property of a dataset consisting of a large number of interrelated variables, while keeping most variation within the dataset. This is often accomplished by transforming to latent variables, the principal components (PCs) that are unrelated and ordered so the first few PCs retain most of the variation in all of the first variables. PCA, a multivariable tool, is employed to seek out the primary source of variability present in datasets. It is required to observe cluster formatting and to ascertain the relationship between object and variable (Wold et al., 1987; Bansal et al., 2014).

12.7 CHEMOMETRIC ANALYSIS IN PHYTOCHEMICAL CHARACTERIZATION

An analysis of the fingerprint of 46 Cassia seed samples was performed by applying chemometric methods at two wavelengths of HPLC–UV. Samples were clustered into four groups consistent with the plant sources and preparation procedures. Chemometric tools were effectively applied to predict the class of the four totally different samples within the test set (Lai et al., 2010). Many studies regarding the standard management of *Camellia sinensis* have been reported. The total inhibitor capability of *C. sinensis* extracts is considered to be a very important quality criterion. Various regression models, such as PCA and OPA, were applied for the prediction of total antioxidant capacity using chromatographic fingerprints (Daszykowski et al., 2007; Dumarey et al., 2008; Van Nederkassel et al., 2005).

Ultra-high-performance liquid chromatography–electrospray ionization–mass spectrum analysis (UHPLC–ESI–MS) was used for substance identification of saponins to discriminate *Panax notoginseng* so as to classify them consistently with their phytochemical diversity and saponins accounting for such variations were found through the loading plots of PCA (Dan et al., 2008, 2009). The high-performance liquid chromatography–diode array detector (HPLC–DAD) methodology was utilized to verify triterpenoid acids at the same time.

The HPLC–DAD methodology, combined with chemometrics, was very useful in looking out for *Ziziphus jujuba* resources and presumably also in chemotaxonomic characterization (Guo et al., 2009). Data from the HPLC analyses of *Ocimum americanum*, *Ocimum citriodorum*, and *Ocimum minimum* were used for authentication functions to determine the surface flavonoid of various basil cultivar by applying PCA (Grayer et al., 2004). *Atractylis chinensis* is a medicinal plant used for the institution of HPLC fingerprints in conjunction with a metal profile that assessed the quality procedures and evaluation of data using PCA and LDA. The results obtained showed that the samples were discriminated on the basis of the process methods (Ni et al., 2008).

HPLC–DAD methodology was developed to judge the standard of *Receptaculum nelumbinis* (dried receptacle of genus *Nelumbo nucifera*) by establishing the chromatographic fingerprint and simultaneous determination of five flavonol glycosides, which include hyperoside, isoquercitrin, quercetin-3-*O*-β-D-glucuronide, isorhamnetin-3-*O*-β-D-galactoside, and syringetin-3-*O*-β-D-glucoside (Wu et al., 2013). To judge the standard consistency of economic medicinal herbs,

a straightforward and reliable HPLC methodology with a UV/vis detector was developed, each for fingerprint analysis and quantitation of some pharmacologically active constituents. *Melissa officinalis* L. (lemon balm) was chosen for this study because it is widely used as an aromatic, cookery, and medication remedy (Arceusz and Wesolowski, 2013). Fourier transform infrared spectroscopy (FT-IR) and chemometrics are used to analyze the adulteration in cod liver oil (CLO). This methodology successfully allowed one to form a classification of pure CLO and CLOs adulterated with vegetable oils (Rohman and Che Man, 2011). HPLC fingerprint analysis followed by PCA and linear discriminate analysis (LDA) provided smart discrimination of laurel bark and twig samples and cinnamaldehyde was found to be the most abundant marker component (Ding et al., 2011). A synchronous process analysis of kiwi and pomelo fruits has been performed by HPLC and UV/vis chemical analysis followed by application of chemometric tools, such as cluster analysis (CA), PCA, and LDA for data analysis. The digitized chromatograms provided a stronger classification and discrimination of these fruit samples than the UV/vis spectra (Sârbu et al., 2012).

Herbs, such as mint, thyme, and rosemary, and spices, such as black pepper, chili pepper, cinnamon, cumin, red pepper, and turmeric, were analyzed by atomic spectrum analysis. PCA and HCA classified the samples into five groups and LDA was used to show how these group members were properly classified with respect to the initial group (Karadaş and Kara, 2012). Ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrum analysis (UPLC–QTOF/MS) and multivariate statistical analysis were applied to investigate the process technology of Loquat (*Eriobotrya japonica*) leaf. The variations in samples processed under totally different methods were revealed by unattended PCA (Wu et al., 2013). An HPLC-DAD–MS methodology has been developed and compared the fingerprints of the nonofficial species, *Cistanche* condiment and *Cistanche sinensis*, to the official one, *Cistanche deserticola*. It was found that *C. condiment* has high similarity with the quality and that, whereas *C. sinensis* is usually recommended, *C. condiment* is also used as an alternate species (Jiang et al., 2009). *Notoptergium incium*, having an advanced fingerprint obtained by GC–MS, is resolved by using OPA (Guo et al., 2003). An analogous study was administered for bush capillaries with the help of a set size moving window evolving correlational analysis (FSMWEFA) (Guo et al., 2004). The efficacy of plant medicines is closely associated with their chemical constituents and their concentrations. The consistency may vary slightly with variations in climate, cultivation seasons, handling procedure, and storage. Evaluating the recorded fingerprints, minor variations in concentrations may influence the standard of the herbal plant, whereas tiny variations between the fingerprints will discriminate among species. PCA, SSC, and LDA will classify and discriminate medicinal plant fingerprints effectively (Bansal et al., 2014). A fingerprint analysis of chrysanthemum was developed by combining chemometric methods, such as SA, HCA, and PCA, with ultra-performance liquid chromatography (Liang et al., 2013).

An ultra-performance liquid chromatography with photodiode array detector (UPLC-PAD) methodology for the measurement of five active alkaloids of *Rhizoma coptidis* has been developed. For the classification of samples, activity data was analyzed by SA, PCA, and HCA. This methodology, with chemometrics, is undoubtedly useful to the internal control and analysis of *R. coptidis* (Kong et al., 2009). PCA, SIMCA, and HCA were applied to the HPLC fingerprint of *Epimedium wushanense* so as to find and distinguish the secondary metabolites (Wang et al., 2012; Xu et al., 2009). The differentiation of *Teucrium flavum* was performed by GC–MS, then investigation of their chemical and genetic profiles was performed by using PCA and CA as methods of classification. The results obtained showed that the genetic background was only responsible for variations within the oil composition (Djabou et al., 2011). In another study, differentiation and prediction of cultivation age in ginseng samples cultivated under standardized protocols or recommendations were investigated by NMR-based metabolomic techniques in numerous solvents to develop a differentiation methodology for ginseng cultivation age. The PLS–DA methodology has successfully differentiated 1–6 year ginseng root samples (Yang et al., 2012).

Fourier transform–mid-infrared (FT–MIR) spectra were investigated for discrimination between *Coffea arabica* and *Coffea canephora* and PCA indicated a clear grouping of the spectra consistent with species followed by DA supported by the PCA scores, which yielded a successful discrimination (Kemsley et al., 1995). The combined data matrices of HPLC–DAD and GC–MS were used for the analysis of the advanced traditional Chinese medicine (TCM) plant *Rhizoma Curcumae* and data analysis by the employment of PCA showed an inexpensive separation of the samples for every technique (Ni et al., 2008). Higher-grade fingerprints of various *Artemisia selengensis* by HPLC–DAD were developed, and the results obtained were analyzed by SA, HCA, and PCA and this resulted in the satisfactory discrimination of *Artemisia selengensis* (Peng et al., 2011). Thus, enhanced fingerprinting analysis combined with chemometric methods is a novel, valid, and fast technique, which provides a powerful and meaningful tool to conduct the quality control of herbal drugs comprehensively.

The correct identification and quality control of the starting material is an essential requisite to ensure the reproducibility and quality of herbal medicine, which contributes to its safety and efficacy. The markers have a very vital role in the standardization of herbal drugs. The standardization of herbal medicine includes the authentication of the genuine drug, harvesting the best quality raw material, the assessment of intermediate and finished products, and the detection of harmful

and toxic ingredients. Several markers, such as taxonomic, chemical, genomic and proteomic markers, aid in the identification of herbal drug components. Chemical markers help in the identification of adulterants, confirmation of the collection site, and quality evaluation and diagnosis of herbal intoxication. Chemical markers are pivotal in the current practice of quality control. Various analytical technologies have been developed for quantitative determination of marker compounds in herbal medicines. The advancement of analytical techniques will serve as a rapid and specific tool in herbal research, thereby allowing the manufacturers to set quality standards and specifications so as to seek marketing approval from regulatory authorities for therapeutic efficacy, safety, and shelf life of herbal medicine. Chemical markers are pivotal in the current practice of quality control. Chemical markers should be used at various stages of the development and manufacturing of an herbal medicine, such as authentication and differentiation of species, collecting and harvesting, quality evaluation and stability assessment, diagnosis of intoxication, and discovery of lead compounds. However, a concept of understanding the complex principles of medicinal plants must be developed through marker profiling and related approaches so as to develop evidence-based practice for the validation of herbal medicine.

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Chapter 13

Bioassay-Guided Isolation and Evaluation of Herbal Drugs

Chapter Outline

13.1 BIOASSAY AND ITS IMPORTANCE IN THE EVALUATION OF HERBAL DRUGS

In principle, a bioassay (either in vitro or in vivo system) is used to detect the biological activity of an extract or a pure substance. This may involve testing for antibiotic activity, in vitro inhibition tests, or pharmacological, agricultural, or veterinary screens, which involve both in vitro and in vivo screening models. Bioassay is defined as a measurement of the concentration or potency of a substance by its effect on living cells or tissues by measuring and comparing the magnitude of the response of the test and a standard in a suitable biological system. It is a very useful tool in estimation and discovery of biologically active substances with several pharmacological applications. The specific purposes of bioassay are exploration of pharmacological activity (e.g., antidiabetic, antiinflammatory activity), qualitative and quantitative estimation of drug potency, standardization of drugs to ascertain uniform pharmacological activity, and the determination of the specificity of drug substances (Rahman and Iqbal Choudhary, 2005). In the drug discovery process, the therapeutic utility of a given compound is reflected by its pattern and potency of activity in a variety of bioassays.

Generally, bioassays can be divided into various broad groups, including whole animals, isolated organs of vertebrates, lower organisms (e.g., fungi, bacteria, insects, mollusks, lower plants), cultured cells (such as cancer cells) and tissues of human or animal origin, and isolated subcellular systems, such as enzymes and receptors.

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Based on the particular experimental design, bioassay can be divided into five types:

(i) Graded response assay: drug potency can be estimated by comparing the test responses with the standard response curve. It can be any type of measured response in isolated tissues, but also in whole animals. Examples include contractions of muscle, blood pressure, and blood sugar concentrations.
(ii) Quantal type assay: the threshold dose of the sample required for eliciting a complete or a particular pharmacological effect is determined and compared with a standard, for example, digitalis producing cardiac arrest.
(iii) Matching point or bracketing method: a constant dose of the standard is bracketed by varying the dose of the sample until an exact match between the standard dose responses and the particular dose response of the sample is achieved. This technique is used when the test sample is too small, for example, histamine on guinea pig ileum, posterior pituitary on rat uterus.
(iv) Interpolation assay: conducted by determining the amount of preparation of unknown potency required to produce a definite effect on suitable test animals/organs/tissue under standard conditions.
(v) Multipoint bioassay method: employs the principle of interpolation and bracketing, in which the experiment is repeated multiple times (three or four times) by crossing over of all the samples.

In addition, bioactivity assay can be classified into two major groups, primary and secondary bioassay, based on the sample size. A primary bioassay can be applied to a large number of samples to determine the desired bioactivity (if any). This type of bioassay provides high capacity and is inexpensive and economical. The primary bioassay screens can be used for a large number of samples in order to test their bioactivity. In most cases, they are qualitative, not quantitative. The primary bioassay screening is able to provide reproducible and reliable results and it can be conveyed to the laboratory for routine operations. Another benefit of primary bioassay is that it offers potential tolerance against several impurities available in a crude extract. This assay is also very useful for performing initial pharmacological screening for bioactivity guided fractionation, isolation, and purification of natural product. In general cases, it involves a comparison of the pharmacological response of the unknown preparation with that of the standard; its mode of action and finally their potency are calculated based on the dose–response curve. Secondary testing procedures involve more detailed investigation of lead compounds on a number of model systems in order to select compounds for further preclinical or clinical studies. They are usually low capacity, slow, and costly. Whereas primary screening bioassays can be applied for preliminary screening of drug molecules, secondary screening procedures involve more exhaustive and comprehensive testing of lead compounds on a greater number of system models. These can be lower organisms (e.g., microorganisms, insects, mollusks, protozoa, helminths), isolated subcellular systems (e.g., enzymes, receptors, organelles), isolated intact cells of human or animal origin, isolated organs of vertebrates, or whole animals. Secondary bioassays can correlate with primary assays in respect of exploring specific therapeutic indications. The degree of relevance increases from subcellular systems (molecular assays) to cellular systems (cellular assays) to organs up to conscious animals and human volunteers. A secondary assay related to molecular pharmacology or in isolated subcellular systems is very specific and of higher capacity in a short duration of time. It is very helpful to test a specific hypothesis about the potential of agonists or antagonists of a particular molecular target to demonstrate pharmacological activity (Glaser and Mayer, 2009; Martis and Radhakrishnan, 2011; Montalvão et al., 2014).

In the search for a bioactive compound(s) in medicinal plants and other natural products, during the isolation process, these types of bioassays are performed and this is called bioassay-guided fractionation (isolation). Here, the collected fractions (obtained from column or flash chromatography) undergo several biological screenings to identify the potential bioactive agents and are further processed until the bioactive agent is obtained in a pure form. However, this process often leads to the isolation of known or undesirable metabolites. The process of identifying known compounds responsible for the activity of an extract prior to bioassay-guided isolation is referred to as dereplication. This can mean either full identification of a compound after only partial purification, or partial identification to the level of a class of compounds. Full identification in these cases relies on comparison with a characterized standard. Partial identification serves to:

(a) identify undesirable compounds, such as tannins, polyphenols, and fatty acids,
(b) prioritize samples for extraction,
(c) gather information on the type of compound to facilitate subsequent isolation.

Dereplication strategies generally involve a combination of bioassay, separation science, spectroscopic methods, and database searching, and can be regarded as chemical or biological screening processes (Mammo and Endale, 2015). Extracts from natural sources have served as a valuable source of molecular diversity in many drug discovery programs, and several important drugs have been isolated from natural products. For many years, most natural products chemists were more concerned with the isolation and structural elucidation of secondary metabolites than with their bioactivity. Modern advances in separation and spectroscopic techniques have provided tools for purification and structural analysis that have reached

extraordinary levels of sensitivity and sophistication. The selection of a bioassay method depends on the target disease and chemotaxonomic information of related species in order to identify and characterize a large number of bioactive phytoconstituents from plant sources. Most of the time, bioassay screening or pharmacological evaluation must be used to guide the isolation process in order to obtain pure bioactive components.

Major strategies of bioactivity-guided isolation include preparation of plant extract, screening of different bioactivities (cell based or receptor based), dereplication and lead optimization, isolation, structure elucidation, and finally scale-up for further pharmaceutical development. In this context, different hyphenated instrumental techniques, such as LC/MS and LC–NMR, are successfully applied for the identification and characterization of the bioactive fraction. Also, the establishment of various molecular and cellular bioassays along with high-throughput technologies is very advantageous to increase the probability of discovering new drug candidates from natural products. In this chapter, we will discuss the entire workflow of bioassay-guided isolation and identification of plant secondary metabolites for the evaluation of herbal drugs (Rahman and Iqbal Choudhary, 2005).

13.2 IN VIVO-BASED BIOASSAY

After successful in vitro screening, a drug molecule has to undergo in vivo study to confirm its pharmacological activity at the molecular, cellular, or tissue level. In vivo study is generally employed to explore the pharmacokinetic and pharmacodynamics profile of a drug molecule. A pharmacodynamics study is designed to determine the mechanism of action of drug molecules, whereas a pharmacokinetics study aims to evaluate the absorption, distribution, metabolism, and excretion of drugs in in vivo systems. In combination with proper ethnopharmacological knowledge, botany, and phytochemistry, in vivo-based bioassay studies promise to develop new plant-based therapeutics and also the probable synergistic actions of different plant constituents present in it. In light of the reverse pharmacology approach, in vivo models are widely used to validate the newer plant-derived "hits" capable of interacting with disease-specific protein targets. The major advantages of an in vivo-based assay are based on the high pathophysiological relevance due to similarity of the human genome with rodent and nonrodent animals. The selection of a proper bioassay method should be based on the desired study outcome with good sensitivity, reproducibility, and high statistical significance. Owing to that, there are some major drawbacks of in vivo-based studies, which include some ethical considerations and also low throughput (Atanasov et al., 2015). The aim of this chapter is to provide an overview of different bioassay methods used to detect bioactive compounds prior to isolation of plant-derived phytoconstituents.

13.2.1 Brine Shrimp Model

The Brine shrimp lethality model is rapid, simple, and provides a frontline screening technique for assessing cytotoxicity to identify the potency of anticancer compounds. This model was developed to determine the LD_{50} value of compounds in a brine medium. It is considered as a preliminary tool for natural product assay prior to any specific sophisticated bioassay for confirmation of pharmacological activity. This method is a rapid and inexpensive technique for screening plant extracts and fractions. In this model, the eggs of brine shrimp (*Artemia salina)* are employed. After placing in a brine solution, the eggs are hatched to provide a large number of larvae. The method is based on the percentage of hatching of the cyst, which is incubated in a medium with different concentrations of organic extracts. Toxicity is usually measured by comparing the percentage of hatched nauplii to a control (Carballo et al., 2002; Meyer et al., 1982).

13.2.2 Zebrafish Model

Zebrafish (Scientific name: *Danio rerio*) is a very useful model organism for developmental biology and in research related to oncology and cardiovascular diseases. The zebrafish model was developed to identify novel drug targets within the organism and also for studying chemical mutagenesis, insertional mutagenesis, and high-throughput small-molecule screening. Several behavioral changes, such as sleep/wake patterns or movement response, are studied using the zebrafish model. Various changes in gene expression also can be studied in this model. The zebrafish model offers high screening capacity and a high level of visualization of dynamic physiological processes. The wide application of the zebrafish model in genome analysis, combined with systems-level visualization of dynamic physiological processes and high-volume screening capacity, makes the zebrafish model a potent tool for the future of ethnopharmacological drug discovery.

The embryonic zebrafish is well suited to address this problem as its high fecundity allows screening large numbers of animals and its transparency provides the ability to visualize and quantify physiological, anatomic, biochemical, and gene expression states in a living vertebrate organism. The embryonic zebrafish is reported as a powerful model for determining

the angiogenic-effects-based effectors of hypercholesterolemia, angiogenesis, Parkinson's and Alzheimer's. It has also an emerging role for toxicity screening.

Generally, there are two methods applied in carrying out phytomedical research in zebrafish. In the first case, the plant extract/phytomedicines are dissolved in the water of the zebrafish, which can permeate the dermal layers of zebrafish by the passive diffusion method. Another method is administering the herbal drug mixed with food, mainly applied for lipophilic compounds, which are solubilized in aqueous solution by the addition of dimethyl sulfoxide (DMSO) (Littleton and Hove, 2013). Lipid metabolism in the zebrafish, is to mix the treated materials into food, administering treatments by dietary intervention. Several studies showed that a high degree of conservation exists between mammals and zebrafish, in terms of drug effects and toxicities. During cardiotoxicity screening, Milan and his coworkers reported a high degree of correlation between humans and zebrafish (MacRae and Peterson, 2015). Several reports have been found on the screening of novel neuroactive and psychotropic drugs using the zebrafish model. Moreover, the functional features of the zebrafish model are found to be very suitable for the identification of novel targets in metabolic regulation related to pancreas, liver, and adipose tissue (Kamel and Ninov, 2017) and also for the study of conserved genes associated with the risk of metabolic diseases. It is also employed in the assessment of compound toxicities, chemical alterations by metabolism, pharmacokinetic and pharmacodynamic properties, and modulation of cell niches (Wiley et al., 2017).

The zebrafish model is considered to be extremely useful in angiogenesis assays as it allows rapid screening of treated fish for angiogenesis effects (Parng et al., 2002). In 2011, Liu and his coworkers isolated norviburtinal from the root of *Rehmannia glutinosa* through a bioassay-guided isolation process by employing the zebrafish embryo model (Liu et al., 2011). Several angiogenesis inhibitors were isolated from African medicinal plants by employing the zebrafish model (Crawford et al., 2011). A report has been found that triterpenoid compounds from the ethanol fraction of *Tripterygium wilfordii* inhibit angiogenesis in the zebrafish model (He et al., 2008). A zebrafish model was also developed for studying the hypocholesterolemic activity of plant extracts, as well as other developmental changes at the genetic level. Several reports have been found on the antihypercholesterolemic activity of cinnamon, clove, and turmeric studied in the zebrafish model by measuring decreased blood serum and cholesterol levels (Jin and Cho 2011; Jin et al., 2011). The zebrafish model was employed to explore various anatomical and behavioral changes in neurodegenerative diseases, such as Parkinson's and Alzheimer's disease.

In 2012, Zhang and his group observed the prevention of dopaminergic neuron damage in zebrafish embryos (in Parkinson's disease model) on administration of *Alpina oxyphylla* ethanolic extract. The zebrafish model was employed to study scopolamine-induced memory impairment in which quercetin and rutin were reported to offer significant therapeutic activity in the treatment of Alzheimer's disease (Zhang et al., 2012a,b). The antiinflammatory and proresolution activity of bergapten, isolated from the roots of *Ficus hirta* was reported in an in vivo zebrafish model. Zebrafish is also considered to be a suitable model for experimental neutrophil biology (Henry et al., 2013). Bisabolene sesquiterpenoids isolated from *Curcuma longa* were screened for anticonvulsant activity in zebrafish and mouse-seizure models (Orellana-Paucar et al., 2012). Ismail and his research group carried out a comparative toxicity study on cells and zebrafish embryos (Ismail et al., 2017).

The zebrafish model has also been implemented for studying the antithrombotic and pro-angiogenic effects of *Rubia cordifolia* (Chen et al., 2018). In another study, the antiinflammatory and proresolution activities of bergapten (isolated from *F. hirta* roots) have been reported in the zebrafish model. This study explored the potential of bergapten (a psoralen derivative) as a novel antiinflammatory agent (Yang et al., 2018). Indirubin, a traditional Chinese medicine, has been reported to have a potential antiangiogenic agent in the zebrafish model, which may be responsible for its antileukemic and antipsoriatic activity. Two flavonoid compounds, quercetin and rutin, were reported to prevent scopolamine-induced memory deficits in zebrafish, which may further offer a potential therapy against AD (Richetti et al., 2011).

13.2.3 Drosophila Model

The Common fruit fly (Scientific name: *Drosophila melanogaster*) plays a major role in the genetic analysis of living organisms. There are several advantages of using drosophila over vertebrate models as they are homologous to vertebrates, small and easy to grow in the lab, can be genetically modified, and have a much shorter life cycle. Drosophila serves as a model organism with excellent features and genetic tools that can be utilized to discover the pharmacological properties of various plants or plant-derived components. Different assays available with flies have helped to determine the specific activity (antioxidant, antimutagenic, antigenotoxicity, neuroprotective, life extension, antiapoptotic, etc.) of several plants and their components in a very short time (Panchal and Tiwari, 2017). The drosophila model has contributed significantly in several key research areas, such as genetics, embryonic development, behavior, and disease-related signaling studies. It is considered to be a potential invertebrate model that is highly similar to a human disease model. The pharmacological

effects of plant-derived compounds can be studied in the drosophila fly based on phenotypic, developmental, and behavioral changes. The identification of novel gene functions in disease-related signaling pathways can be explored through this model. Phenotypic changes associated with several disease conditions can be studied with tissue-specific overexpression and downregulation of the drosophila gene (Panchal and Tiwari, 2017). Drosophila is emerging as a valuable system for use in the clinical drug discovery process (Jennings, 2011). The genotoxic potential of some medicinal plants was studied by detecting mutation induction, clastogenic and aneugenic effects, and structural chromosome abnormalities in the drosophila model and thus ensuring safer uses of medicinal plants (Sponchiado et al., 2016). Another major application of drosophila-based research involves the study of embryonic development, neurodegenerative disorders, diabetes, aging, drug abuse, and cancer. This model can also be implemented in exploration of insecticidal secondary metabolites of plant origin that are useful in insect pest management in agriculture (Rattan, 2010). The important uses of the drosophila model are as follows:

- Research related to regenerative biology
- Study of several cellular activities for tissue regeneration, such as cell division, differentiation, and movement.
- High-throughput screening (HTS)
- Study of safety profiles
- Development of disease models by mimicking genetic fingerprints to test for drug efficacy
- The model serves as a valuable system for studying the biological safety of bioengineering technologies
- Production of artificial tissues by precise manipulation of cells and growth factors.

The drosophila has long been used as a vulnerable model organism for developmental and genetics studies. The drosophila fly characterizes with several features of phenotypic plasticity, which is found very effective in studying overexpression and downregulation of neurodegenerative disease-related genes.

Various motor dysfunctions related to neurological diseases can be studied through the larval and adult behavior of drosophila. Antioxidant and antiaging properties can be found in different enzymes, for example, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S transferase (GST), and malonaldehyde (MDA) assays, present in the drosophila system. Drosophila is also used as a model organism for identifying and characterizing of apoptosis related to cell death signaling pathways; thus, the antiapoptotic activity of medicinal plants can be explored. Additionally, an immune-cytochemistry assay can be studied by observing the expression of protein in diseased flies (Panchal and Tiwari, 2017). Reports have been found that *C. longa* and *Emblica officinalis* increase the life span in *D. melanogaster* (Rawal et al., 2014). In this study, a mass culture of *D. melanogaster* was used, which was maintained in the laboratory at constant temperature and humidity on standard media. For the control lines, the food media used were without addition of any supplement. In the case of treatments, the regular food media were mixed with either turmeric powder or with amla at different concentrations. For each experimental condition and control, 36 vials were set up with 6 flies (3 males and 3 females) in each (total 216 drosophila flies) and were kept inside a BOD incubator with constant temperature ($24 \pm 1°C$) and other environmental conditions. The life span and the body weight of single fly were recorded at 10, 20, and 30 day intervals during their life span in both sexes separately in case of both control and treated flies. Further, SOD activity assay and catalase activity assay were carried out by using drosophila fly crude protein extract. The protein extract was prepared by homogenizing six flies in a lysis buffer. The lysate was then centrifuged and the supernatant was collected as crude extract (Rawal et al., 2014). It was also reported that citrus juices modulate cytotoxicity, genotoxicity, antigenotoxicity and longevity in drosophila (Fernandez-Bedmar et al., 2011).

The leaf extract of *Centella asiatica* has a potential role in the treatment of Parkinson's disease in the transgenic drosophila model (Siddique et al., 2014). Both the green and black tea polyphenols have been reported to extend the lifespan of drosophila, which is considered to be a useful model of developmental and reproduction biology (Peng et al., 2009). The toxicity assays were performed by preparing larval food as described above at 0, 2.5, 5, and 10 mg/mL concentrations of GTP. For each concentration, 6 flies per sex were placed in each vial ($n = 120$ per treatment) for egg laying. After 24 h, flies were removed and the number of eggs laid was recorded. Larval development was checked every 24 h, and the number of pupae and emerged offspring, including dates of occurrence, were recorded (Lopez et al., 2016). Suppression of reactive oxygen species by *Acorus calamus* was reported in drosophila under stress full conditions (Fathima et al., 2014). Some naturally occurring *insecticides* were isolated from rhizomes of *Nuphar japonicum*, in which the *insecticidal* activity was confirmed *against* the larvae of *D. melanogaster* (Miyazawa et al., 1998). The quantitative genotoxicity and mutagenicity of several medicinal plants was studied in the drosophila model reported by Sponchiado et al. (Sponchiado et al., 2016). The mutagenic potential of *Luehea divaricata* was studied in a drosophila cell line (Felício et al., 2011). The genotoxicity and antigenotoxicity effect of some essential oils was studied in the *D. melanogaster* model (Idaomar et al., 2002).

13.2.4 Animal Model

In vivo animal studies are mostly used in the development of disease models for understanding different biochemical and disease pathways involved in disease pathophysiology. They can be utilized in the study of several pharmacodynamics and pharmacokinetics parameters, for example, absorption, distribution, metabolism, excretion, and toxicity (ADMET) of drug molecules. Animal models can be divided into three categories based on how well they reproduce the human disease or condition, homologous, isomorphic, and predictive (Conn, 2013).

(i) Homologous animal models are the most desirable, as they have the same causes, symptoms, and treatment options available for humans.
(ii) Isomorphic animal models have the same symptomology as the human conditions and treatment options are generally the same.
(iii) The predictive animal models are often used in diseases or conditions that are poorly understood or simply do not occur in animals (Zak and Sande, 1999).

The animal models that are widely reported in in vivo pharmacological experiments include guinea pigs, rabbits, dogs, cats, and other nonhuman primates, such as rhesus monkeys, the common marmoset, the capuchin monkey, baboon, and the spider monkey. It is notable that the clinical efficacy largely depends on number of animals involved in a study because there is always a chance of error related to it. It has to be noted that, in order to minimize the error, the number of animals involved in the study should be statistically significant. The outcome of any assay must exceed the margin of error inherent to the model itself. If the expected outcome is higher than the margin of error, then fewer animals are required, whereas more animals are required as the margin of error becomes large (Blass, 2015). Various animal models used in in vivo studies are presented in Table 13.1.

TABLE 13.1 Different Animal Models Used in In Vivo Studies

Disease State	Animal Model	References
Diabetes	Effects on normal rats	Hultman (1959)
	Effect of glucose tolerance in rats	Chandran et al. (2015)
	Potentiation of the action of exogenous insulin	Mukherjee et al. (1997)
	Study on streptozotocin (STZ) induced diabetic rats	Dufrane et al. (2006)
	Alloxan induced diabetes models in rabbits	Sharma et al. (2003)
	Adrenaline-induced hyperglycemia in rabbits	Krusteva (1992)
	Dithizone induced diabetes	Kadota (1950)
	Monosodium glutamate induced diabetes	Nagata et al. (2006)
	Insulin antibodies induced diabetes	Jin et al. (2010)
	Gold thioglucose obese diabetic mouse model	Karasawa et al. (2011)
	Virus induced diabetes	Yoon et al. (1980)
	Hormone induced diabetes	Campbell and Rastogi (1966)
	Spontaneous diabetic obese rodent models	Wang et al. (2013)
	Spontaneous diabetic non obese rodent models	Wang et al. (2013)
	Surgical model of diabetes mellitus	Choi et al. (2004)
	Type I diabetes model	Van Belle et al. (2009), Leiter and Schile (2013)
Diuretic activity	Lipschitz test	Sayana et al. (2014)
	Saluretic activity in rats	Gadge and Jalalpure (2011)

TABLE 13.1 Different Animal Models Used in In Vivo Studies—cont'd

Disease State	Animal Model	References
Antidiarrheal activity	Castor oil-induced diarrhea in rats	Awouters et al. (1975)
	Charcoal meal-induced diarrhea	Sahoo et al. (2016)
	Gastrointestinal motility tests	Sahoo et al. (2016)
	Pge2-induced enteropooling model	Sahoo et al. (2016)
Depression	The forced swimming test	Petit-Demouliere et al. (2005)
	The learned helplessness model	Sherman et al. (1982)
	Tail suspension test	Can et al. (2012)
	Muricide behavior in rats	Ueki et al. (1972)
	Reserpine induced hypothermia	Danielson et al. (1985)
Anxiety	The elevated plus maze model	Walf and Frye (2007)
	Light dark model	Arrant et al. (2013)
	Social interaction study	Varlinskaya and Spear (2008)
	Vogel lick conflict test	Liao et al. (2003)
	Marble burying test	Kedia and Chattarji (2014)
Memory and cognition	Novel object recognition test	Antunes and Biala (2012)
	Amyloid-β model	Choi et al. (2014)
	Passive avoidance	Sarkaki et al. (2013)
	Contextual fear conditioning model	Wehner et al. (2004)
	Morris water maze model	D'Hooge and De Deyn (2001)
	Scopolamine induced amnesia	Saraf et al. (2011)
	MPTP model	Porras et al. (2012)
Hypertension	Constriction of the renal artery in mice, rats, rabbits, dogs, pigs, and nonhuman primates	Lerman and Zeiher (2005)
	Prolonged exposure to mineralocorticoids	Lerman and Zeiher (2005)
	Doca-induced hypertension	Lyer et al. (2010)
	Stress-induced hypertension	Bayorh et al. (2003)
Analgesic/ antinociceptive activity	Formalin-induced pain	Segawa et al. (2013)
	Acetic acid induced analgesic model	Ganeshpurkar and Rai (2013)
	Mouse abdominal constriction test (writhing test)	Ganeshpurkar and Rai (2013)
	Hot-plate test	Ganeshpurkar and Rai (2013)
Antiinflammatory activity	Carrageenan induced rat paw edema	Meckes et al. (2004)
	Croton oil-induced ear edema model	Zanini et al. (1992)
	Cotton pellet-induced granuloma model	Kumar et al. (2016)
	Histamine induced inflammation	Koh et al. (2002)
	Antiinflammatory activity in guinea pigs sensitized by ovalbumin	Boskabady et al. (2012)

Continued

TABLE 13.1 Different Animal Models Used in In Vivo Studies—cont'd

Disease State	Animal Model	References
Hypercholesterolemia	High fat fed diet in rat	Meier and Gressner (2004)
	Triton Wistar rat model	Shankar et al. (2008)
	Hypolipidemic activity in rats	Anreddy et al. (2010)
	Cholesterol-diet induced hypolipidemic evaluation in rabbits	Fani et al. (1988)
	Hereditary hyperlipidemia in rabbits	Toru et al. (1987)
	Hereditary hypercholesterolemia in rat	Kovari et al. (2009)
	Transgenic animal model	Noriyuki et al. (2008)
	Fructose induced hypertriglyceridemia in rat	Masamichi et al. (2005)
	Hypolipidemic activity in Syrian hamsters	Dhanya and Hema (2008)
	Effect of HMG-COA reductase inhibitors	Jeffery et al. (1996)
	Lymph fistula model for cholesterol absorption	Iritani and Nogi (1972)
Cardiovascular activity	Heart failure induced by myocardial infarction	Wu et al. (2007)
	Heart failure induced by doca (rat model)	Syed et al. (2016)
	Heart failure induced by doca (guinea pig model)	Tiritilli (2001)
	Pulmonary hypertension	Wang et al. (2018)
	Coronary artery ligation	Gao et al. (2010)
Wound healing activity	Excision wound model	Mukherjee et al. (2000), Mukherjee and Suresh (2000)
	Incision wound model	Udupa et al. (1994)
	Dead space wound model	Saha et al. (1997)

13.3 BIOASSAY IN ISOLATED ORGANS

Organ-based assays are found to be very significant as secondary screens to confirm the pharmacological activity of active extracts or compounds. Consequently, they represent an essential connection between the high technology of the primary screens and the realities of pharmacological effectiveness. Organ-based assays are used to screen the pharmacological activity of active extracts or compounds. Due to their nonspecific nature, they are used in the initial stage of a drug screening program to confirm the presence or absence of certain pharmacological activities of any drug substances. This method involves the removal of an organ or a portion of an organ from a freshly killed animal and its suspension in a tissue bath.

Many different buffers, such as Krebs' solution, Tyrode's solution, and Locke's solution, are used to support the activity of isolated organs. An organ-based assay is often employed to check the effect of the drug without considering other factors, such as absorption, metabolism, excretion, or interference. Generally, the most common sources of isolated tissues are guinea pigs, rats, rabbits, cats, and dogs. For example, segments of the gastro-intestinal tract or spirally cut strips of vascular tissue are mainly used in the organ bath method for isolated organs. The isolated guinea pig ileum has been used extensively for drug screening as it offers contractions to many agonists, including acetylcholine, angiotensin, arachidonic acid, bradykinin, histamine, prostaglandins, serotonin, and substance P for nonspecific antispasmodic activity and for parasympathicomimetic or parasympathicolytic activity.

The isolated rabbit jejunum is suitable for studying adrenergic mechanisms, whereas rat phrenic nerve-diaphragm preparation is used for the study of the action of muscle relaxants. The tracheal lung parenchyma strips from sensitized guinea-pigs is often used as a model system for studying bronchial disease. The vasodilating effects of a test drug can be studied using the rabbit central ear artery perfusion model, in which the perfusion system is connected to a suitable device for detecting changes in perfusion pressure to indicate vasoconstriction or vasodilation (Montalvão et al., 2014). Several isolated organs have been reported by Vane (1997). Some of the isolated organs used in in vivo studies are described in Table 13.2.

TABLE 13.2 Some Examples of Isolated Organ Assays

Drug	Activity Assayed	Preparation
Histamine	Contractile effect	Isolated, atropinized terminal ilium of guinea pig
	Fall in blood pressure	Anesthetized and atropinized cat
5 Hydroxytryptamine	Contractile effect	Isolated atropinized tar uterus, isolated fundus strip of rat stomach
Acetyl choline	Contractile effect	Isolated rectum abdominus muscle of frog, rat ileum
	Inhibition of cardiac contraction	Isolated mouse heart
Noradrenaline	Rise in blood pressure	Blood pressure of the pithed cat
Adrenaline	Inhibition of the tone	Isolated rabbit duodenum Isolated rat uterus Isolated cecum of fowl
Insulin	Increase in glycogen content	Isolated rat diaphragm
Oxytocin	Contractile effect	Isolated rat uterus
Histamine	Contractile effect	Isolated, atropinized terminal ilium of guinea pig
	Fall in blood pressure	Anesthetized and atropinized cat
5-Hydroxytryptamine	Contractile effect	Isolated atropinized tar uterus, isolated fundus strip of rat stomach
Acetyl choline	Contractile effect	Isolated rectum abdominus muscle of frog, rat ileum
	Inhibition of cardiac contraction	Isolated mouse heart
Noradrenaline	Rise in blood pressure	Blood pressure of the pithed cat
Adrenaline	Inhibition of the tone	Isolated rabbit duodenum Isolated rat uterus Isolated cecum of fowl
Insulin	Increase in glycogen content	Isolated rat diaphragm
Oxytocin	Contractile effect	Isolated rat uterus

13.4 CELL-BASED BIOASSAY

Cell-based assays are considered to be a fundamental strategy in HTS for identifying the functional changes of target proteins in response to a drug molecule. In this assay, the targets may involve specific molecules or a particular signaling pathway, even the whole cell, but not very specific. This assay offers higher relevance in the drug discovery process for both pharmacological characterization and target identification. A cell-based assay is very advantageous in the detection of cytotoxicity, permeability, and effects on cell growth simultaneously (Zhang et al., 2012a,b). Various types and sources of cells have been used in cell-based assays, for example, immortalized cell lines, primary cells, human cancer cell lines, cancer stem cells, mesenchymal stem cells, embryonic stem cells, and induced pluripotent stem cells (Zhang et al., 2012a,b).

The selection of cell types is found to be very important in HTS for meaningful selection of the assay design, for example, endothelial cells are selected to study angiogenesis; epithelial cells might be used for dermatological research (Atanasov et al., 2015). As reported by Atanasov and his coworkers, cell-based assays can be classified into two types, target-oriented or phenotypic. A target-oriented assay is based on the functional identification of a specific protein or pathway, whereas phenotypic models explore some cellular changes with a complex regulation (e.g., cell proliferation). Phenotypic cell-based assays can be used to verify the activity of compounds identified by protein-based in vitro assays at the cellular level. Moreover, cell-based phenotypic models might also be used to study the underlying molecular mechanisms of certain biological effects, possibly leading to the discovery of new target molecules or pathways affecting the respective phenotype (Atanasov et al., 2015).

There are several techniques used in cell-based assays, such as immuno-cytochemistry, FACS-analysis, real-time PCR, Western blotting, immunoprecipitation, or "omics"-techniques (e.g., genomics, transcriptomics, proteomics and metabolomics techniques), for characterization of global changes in gene expression or metabolite quantities (Lee and Bogyo, 2013; Schenone et al., 2013; Ziegler et al., 2013). Other detection methods in cell-based assays are the FRET and BRET techniques, label-free methods, flow cytometry, and atomic force microscopy in HTS assay to detect more biologically relevant characteristics of compounds in living systems (Zhang et al., 2012a,b). Besides mammalian cells, yeasts have also been employed for the establishment of whole-cell phenotypic assays with applications in drug discovery, for example, for high-throughput functional screening based on the activation of caspases and other proteases involved in cell death and inflammation (Hayashi et al., 2009) and as a screening tool for pharmacological modulation of GPCRs (Minic et al., 2005), but also to understand the mechanism of the action of drugs or to identify novel drug targets and target pathways (Hoon et al., 2008). The spectrum of cell-based assays has been widened by the engineering of cells enabled to act as reporters (e.g., luciferase- or fluorescence-based) of a specific intracellular response influenced by the tested substance (Baird et al., 2014; Fakhrudin et al., 2014). Aside from the use of reporter systems, a variety of phenotypic parameters have been successfully selected as readout, for example, changes in cell morphology, cell adhesion, cell proliferation, migration, differentiation status, metabolic status, redox status, and cell apoptosis or senescence.

The antimicrobial activity against *Escherichia coli*, ATCC 25922, and *Staphylococcus aureus* ATCC 25923 of several medicinal plants has been shown through bioluminescent whole-cell reporter gene assays (Nybond et al., 2015). A fluorescence-based cellular assay was employed in the exploration of some natural product–based quinic acid derivatives as histone deacetylase inhibitors (Son et al., 2016). Cell-based assays, including luciferase reporter assay, MTT-based cytotoxicity assay, and fluorescence polarization competition assay, were found effective in studying the inhibition of colon cancer cell growth by targeting RNA-binding protein (Lan et al., 2015). Table 13.3 describes different enzyme assay principles (Wildey et al., 2017).

There are several research studies based on cell lines, which have been performed for the screening of pharmacological activity in medicinal plants. For example, the anticancer and antioxidant activity of several medicinal plants from Morocco were studied in human embryonal rhabdomyosarcoma cancerous cell lines, rat embryonal rhabdomyosarcoma cancerous cell lines, and Monkey kidney cancerous cell lines (Bouyahya et al., 2018). In this study, an evaluation of the cytotoxicity activity of *Myrtus communis*, *Arbutus unedo*, *Origanum compactum*, *Cistus crispus*, and *Centaurium erythraea* was performed (Bouyahya et al., 2018). The leaves of *Olinia usambarensis* were assayed for their potential cytotoxicity activity on human ovarian cancer cell lines (Deyou et al., 2017). A screening of the cytotoxic activity of several medicinal plants from Iranian traditional medicine was performed in the MCF-7 cell line, reported to treat cancer and related disorders (Naghibi et al., 2014). A cell line assay was also developed for evaluating the tumoricidal activity of andrographolide in the NSCLC cell line (Gong et al., 2015).

13.4.1 Cell Viability Assays

A cell viability assay is performed based on the ratio of live and dead cells. This assay is based on an analysis of cell viability in cell culture for evaluating in vitro drug effects in cell-mediated cytotoxicity assays for monitoring cell proliferation. Various methods are involved in performing a cell viability assay, including the dilution method, surface viable count, roll tube technique, nalidixic acid method, fluorogenic dye assay, and the Trypan Blue Cell Viability Assay. Another major assay procedure is the MTT assay, which is very sensitive and very applicable to measure cytotoxicity (loss of viable cells) as well as cytostatic activity (shift from proliferation to quiescence) (An and Tolliday, 2010). Cell viability assays can determine the effect of drug candidates on cells and be used to optimize the cell culture conditions. The parameters that define

TABLE 13.3 Types and Principles of Cell Viability Assays

Category of Cell Viability Assay	Principles
Membrane integrity assay	Determination of membrane integrity via dye exclusion from living cells
Functional assay	Examining metabolic components that are necessary for cell growth
DNA labeling assay	Cell selection and viability assay
Morphological mechanism based assay	Determination of morphological changes
Reproductive assay	Determination of the growth rate

cell viability can be as diverse as the redox potential of the cell population, the integrity of cell membranes, or the activity of cellular enzymes. Table 13.3 describes the categories and principles of cell viability assays.

13.4.2 Cell Proliferation Assays

Cell proliferation is a biological process by which the number of cells increases by a large number over time through cell division. It plays a vital role in regular tissue and cellular homoeostasis for the proper growth, development, and maintenance of an organism. This type of assay monitors the growth rate of cell populations. It is useful to test the effects of pharmacological agents or growth factors, assessing cytotoxicity, or investigating the circumstances of cell activation. Four types of cell proliferation assays related to DNA synthesis, metabolic activity, antigens associated with cell proliferation, and ATP concentration are reported (Romar et al., 2016).

13.4.3 Cytotoxicity Assays

Cytotoxicity is defined as the toxicity caused due to the action of chemotherapeutic agents on living cells. Cytotoxicity tests are very important in nanoparticles as they help in the determination of the proposed biomedical use. The method for determination of cytotoxicity and cell viability involves dyes, such as Trypan Blue, Alamar Blue, neutral red, and Coomassie Blue. The method differentiates the various cells in terms of colors. The cells are differentiated based on the ratio of the color uptake of both living and dead cells. The other methods for assaying cytotoxicity include tritium-labeled thymidine uptake assay, the MTT method, WST assay, and dehydrogenase-based assay (Li et al., 2012).

13.4.4 Second Messenger Assay

Second messengers are molecules that relay signals from receptors on the cell surface to target molecules inside the cell. They greatly amplify the strength of the signal and cause some kind of change in the activity of the cell. They are a component of cell signaling pathways. There are several second messenger assays reported to have potential application in the cell-based assay platform. The major examples of second messengers are guanine nucleotide binding assays, cAMP assays, inositol phosphate accumulation assays, and intracellular calcium assays. Among them, cAMP has been found to be one of the most important second messengers widely measured in HTS. The basic principle of cAMP assays lies in fluorescence- or luminescence-based homogeneous assays or time-resolved fluorescence resonance energy transfer technology. Inositol phosphate (IP) accumulation assays have been used to develop functional screens for Gq-coupled receptors, as well as β2-adrenergic, histamine H2, melanocortin MC4, CGRP, and dopamine D1 receptors. Cellular Ca is considered to be another second messenger involved in embryonic development with muscle contraction. The intracellular calcium assay is involved in the homogeneous measurement of intracellular calcium changes caused by the activation of G-protein coupled receptors or calcium channels (Thomsen et al., 2005). There are several Ca indicators used to assay calcium mobilization related to essential physiological process (Zhang et al., 2012a,b).

13.4.5 Reporter Gene Assay

Reporter genes encode protein products that can be rapidly and sensitively assayed as surrogate markers when fused to regulatory regions of genes of interest. The reporter gene analyzes the promoter gene regions to regulate gene expression and conveys signal transduction from extracellular signaling molecules to the nucleus to stimulate gene transcription. In functional genomics, the reporter gene assay is very useful for the identification of a promoter gene to study its expression pattern and strength, other gene expression, and transformation and transfection assay. Reporter gene transfection has become an important area of small-animal imaging. In this system, genes are transferred into the animal tissue using a vehicle, such as an adenovirus, adeno-associated virus, retrovirus, or liposome, or as naked DNA. Both the reporter gene and the therapy gene are simultaneously inserted into the cell. The reporter gene uses a specific probe to track the expression of the therapy gene. As a reporter technology, bioluminescence finds its greatest potential by helping to characterize the enormous complexity of living systems. The commonly used reporter genes are green fluorescent protein (GFP), β-galactosidase, firefly luciferase, Renilla luciferase, and β-lactamase (Zhang et al., 2012a,b).

Luciferase-based genetic reporter assays provide sensitive methods for assaying gene expression, enabling the accurate quantification of small changes in transcription resulting from subtle changes in biology. It is commonly used as a tool to study gene expression at the transcriptional level. This gene technology has been used to create tools for screening entire genetic pathways in living cells (Smith-Jones, 2007; Thorne et al., 2010). The major advantages of this technique involve greater sensitivity and extraordinary accuracy for signal detection.

13.5 ENZYME-BASED BIOASSAY

An enzyme-based assay is based on measuring enzyme activity and its kinetics based on the change in concentration of a substrate or product. It mainly reflects the catalytic properties of an enzyme. There are four major types of enzyme inhibition assay reported, direct, indirect, continuous, and discontinuous. In direct continuous assay, the difference in the spectroscopic and fluorometric properties of substrate and product is measured directly. This assay is based on a change in the absorbance, fluorescence, pH, and viscosity of the substrate and product involved in an enzymatic reaction. The turbidimetric enzyme assay is based on light scattering of the substances, for example, a bacterial lysozyme assay. A fluorescence assay depends on the change in electronic transition of the absorbing molecule to an excited state. In this assay, the fluorescent molecule emits part of the absorbed energy as light with lower energy but higher wavelength, and thus it is considered to be more sensitive than absorbance assays. This method is highly sensitive, safe, and can detect in small quantities. Another direct assay involves potentiometric techniques, in which a change in pH is measured due to the progress of a chemical reaction in which protons are liberated or taken up.

One major example of a pH-based assay is the hydratase assay involved in a carbonic anhydrase inhibition assay. Some enzyme-catalyzed reactions are also measured by the polarimetric method in which one isomer is converted to another due to the enzymatic reaction involved. Here, the enzyme converts one optically active molecule to an inactive one or vice versa; for example, isomerase catalyzes the conversion of L-alanine into its isomeric (mirror-image) form, D-alanine. Another enzymatic analysis involves enthalpimetry (microcalorimetry) in which an enzyme-ligand reaction is carried out under controlled conditions in a calorimeter and the temperature change is monitored. The change is very small; hence, an extremely accurate thermostating or insulation is required. For example, serum cholinesterase activity can be monitored by kinetic direct injection enthalpimetry by the action of hydrolases.

Enzymatic activity, such as catalase, penicillinase, and glucose oxidase, can be assayed by enthalpimetry. Enthalpimetric assays have also been shown to be applicable to enzyme immunoassays, TELISA (thermometric enzyme immunosorbent assay). In the radiochemical assay method, a radioactively labeled substrate is used. The isotopes most commonly used for labeling purposes are ^{3}H (tritium), ^{14}C (carbon), ^{32}P (phosphorous), ^{35}S (sulfur), and ^{131}I (iodine). All of these isotopes emit beta radiation (electrons) as they decay. The substrate is then separated from the product, usually by chromatography or electrophoresis, and the product concentration is determined indirectly by measuring the radioactivity of the product fraction. A typical example of enzymatic analysis by a radiochemical procedure is that involving the cholinesterase-catalyzed hydrolysis of [^{14}C]-acetylcholine.

A radiochemical assay has been developed to measure the activity of dihydropyrimidinase (DHP) in human liver homogenates. The method is based on the separation of radiolabeled dihydrouracil from *N*-carbamoyl-β-alanine by HPLC with on-line detection of radioactivity combined with detection of CO by a liquid scintillation counting (Mattiasson, 1977; Palmer and Bonner, 2007; Van Kuilenburga et al., 1999). There are several methods for evaluating the induction or inhibition of the respective enzymes, which helps in screening of herbal drugs; details are described in Chapter 14.

13.6 RECEPTOR-BASED BIOASSAY

Receptor-based assays are used to detect the binding of ligands or, conversely, ligands used to detect receptors. The receptor molecules combine with the effector systems to evaluate the agonistic or antagonistic properties of natural or synthetic ligands. A novel receptor-based bioassay for the quantitative measurement of taxol was reported in which the active metabolites bind reversibly to the receptor protein tubulin, a process similar to antibody and antigen interaction. The receptor-based bioassay method was developed to detect the binding capacity of some beta agonist drugs. The aromatic hydrocarbon receptor (AhR) assay utilizes an activated transcription factor that mediates many of the biologic and toxicologic effects of halogenated aromatic hydrocarbons, benzoflavones, digoxin, and others. Several other purinergic and adenine receptors were found to have potential for searching for novel ligands in the drug discovery process (Boyd et al., 2009; Seidel et al., 2000; Strosberg and Leysen, 1991).

13.7 HIGH-THROUGHPUT SCREENING

HTS is required to simultaneously test the compounds in functional or binding assays. In the drug discovery process, HTS is employed for rapid screening of biochemical activity of a large number of drug-like compounds. The heart of the HTS system is a plate, or tray, which consists of tiny wells in which assay reagents and samples are deposited, and their reactions are monitored. The configuration of the plate has changed from 96 wells (in a matrix of 8 rows by 12 columns) to 384, and now to a high-density, 1536-well format, which enables large-scale screening. The assay reagents are coated onto the

plates or deposited in liquid form together with test samples into the wells. There are several detection methods in HTS, for example, spectroscopy, mass spectrometry, chromatography, calorimetry, X-ray diffraction, microscopy, and radioactive methods. The major advantages of an HTS assay are high sensitivity (single molecule detection), high speed (automation), minimization of sample (microtiter plate assay), and finally the ability to detect low background signals. It mainly uses automation and large dataset processing to quickly assay the biological or biochemical activity of large numbers of small molecule libraries, protein libraries, siRNA/shRNA libraries, and cDNA libraries. HTS is considered to be a starting point for different drug discovery programs in the field of medicinal chemistry, ADMET/PK, pharmacology, chemical biology programs, target identification, target validation, and mechanism of action. There are several detection techniques employed in HTS assays, as follows (Wildey et al., 2017):

- Absorbance
- Fluorescence intensity
- Fluorescence resonance energy transfer
- Time resolved fluorescence
- Fluorescence confocal spectroscopy
- Luminescence
- Bioluminescence

An HTS assay can be classified based on the capacity of sample screening; for example, low-throughput screening deals with 1–500 samples per day, with the sample size increasing as the number of samples is increased. For medium-throughput screening, the sample size generally lies between 500 and 10,000, whereas in case of high throughput and ultra-high throughput, the sample size goes up to 10,000–100,000 and >100,000, respectively (Wildey et al., 2017).

Virtual screening is an in silico-based technology that has an increasing role in the lead identification stage in drug discovery (Weber 2002). It is regarded as a complementary approach to HTS, and when coupled with structural biology, enhances the chances of identification of the lead. Virtual high-throughput screening (vHTS) applies in silico approaches in order to yield lead structures from large virtual molecular databases to explore biologically active compounds (Szymański et al., 2012). vHTS utilizes computational algorithms and biophysical knowledge to recognize lead molecules by which "hit" rates can be improved.

13.8 DEREPLICATION AND OTHER APPROACHES FOR BIOASSAY-GUIDED ISOLATION

In the search for bioactive metabolites and new lead compounds, the usual procedure involves biological screening followed by bioassay-guided isolation. However, this often leads to the isolation of known or undesirable metabolites. The process of identifying known compounds responsible for the activity of an extract prior to bioassay-guided isolation is referred to as dereplication. This can mean either full identification of a compound after only partial purification, or partial identification to the level of a class of compounds. Full identification in these cases relies upon comparison with a characterized standard. Partial identification serves to:

(a) Identify undesirable compounds, such as tannins, polyphenols, and fatty acids.
(b) Prioritize samples for extraction.
(c) Gather information on the type of compound to facilitate subsequent isolation.

Dereplication strategies generally involve a combination of bioassay, separation science, spectroscopic methods, and database searching, and can be regarded as chemical or biological screening processes (Colegate and Molyneux, 2007). Conventional natural products discovery programs rely on bioactivity-directed fractionation methodology for the isolation, purification, and structural elucidation of bioactive lead compounds from crude extracts. This process is often tedious, expensive, and time consuming; moreover, it may end up with disappointing outputs when isolating well-known previously characterized compounds. This can be simplified and the process becomes more effective if it is supported by rapid dereplication techniques, which are capable of identifying the known compounds efficiently. Dereplication is of great importance at an early stage of the natural products drug discovery process prior to a large-scale isolation process. In particular, hyphenated techniques, namely, MS, MS–MS networking, IMS, and NMR, as well as genomics, bioinformatics, and metabolomics approaches, have played a major role in dereplication. In addition, UV spectroscopy and mass spectrometry are of prime importance in the field of dereplication procedures.

When hyphenated with HPLC, LC-UV/PDA and LC–MS provide effective separation techniques in natural product-based research. The UV profile of compounds in an extract, obtained using LC-UV/PDA, serves as a fingerprint that is characteristic of certain structural classes. LC–MS is considered to be a selective, sensitive, and powerful tool as the molecular weight

provides an entry point into the molecular formula of the compounds. The introduction of the X-hitting algorithm, capillary-scale NMR probes, MALDI-TOF imaging, de novo sequencing techniques, in silico dereplication, computer assisted numerical analysis, bioinformatics, genomics, proteomics, and metabolomics have all been reported for dereplication of natural products in the drug discovery process (Mammo and Endale, 2015; Smyth et al., 2012). The process of dereplication can be classified into two major classes: separation techniques and hyphenated techniques (Gaudencio and Pereira, 2015).

13.8.1 Separation Methods

Medicinal plants are reservoirs of naturally occurring chemical compounds with different polarities and with structural diversity. There is a huge challenge of identification and characterization of these bioactive compounds from a complex mixture. A complex mixture must be separated prior to characterization of the compounds. There are a variety of separation techniques involved in dereplication of natural products. Among them, chromatography is the most important technique. It enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis. The importance of different separation techniques is described in subsequent sections.

13.8.1.1 Column Chromatography

Column chromatography (CC) is one of the most useful methods for the separation and purification of both solids and liquids. This is a solid–liquid technique in which the stationary phase is a solid and the mobile phase is a liquid. The principle behind column chromatography is adsorption, in which a mixture of components dissolved in the mobile phase is introduced in to the column and the components move depending on their relative affinities. The choice of the solvent depends on the solubility characteristics of the mixture. The solvents should also have sufficiently low boiling points to permit ready recovery of eluted material. In CC, different mobile phases (in increasing order of polarity) can be used, for example, petroleum ether, hexane, chloroform, and ethyl acetate. However, the polarity of both the stationary and mobile phases is the most important factor in adsorption chromatography. It is found to be very useful in the separation of mixtures of compounds, purification processes, the isolation of active constituents, and the separation of diastereomers (Gaudencio and Pereira, 2015).

13.8.1.2 Vacuum Liquid Chromatography

In vacuum liquid chromatography (VLC), vacuum is applied in the column instead of forced flow to speed up the fractionation process. In VLC, column beds usually consist of silica of 40–60 mm particle size or reversed-phase silica. The open end of the column is easily accessible for the sample (as liquid or adsorbed to inactivated silica or diatomaceous earth) and the mobile phase, which is frequently a stepwise gradient with increasing elution power (e.g., hexane to methanol for silica columns). VLC is a popular method for fractionation of crude extracts due to its ease of use and high sample capacity. Eluted fractions are usually analyzed by TLC for their composition (Bucar et al., 2013).

13.8.1.3 Flash Column Chromatography

Flash chromatography, also known as medium pressure chromatography, involves an air pressure driven hybrid of medium and short column chromatography that is optimized for rapid separation of the mixtures of compounds present in plant extracts. In flash column chromatography (FCC), the eluent is rapidly pushed through a short column under gas pressure (normally nitrogen or compressed air). The glass column is packed with silica gel of defined particle size with large inner diameter. The major advantages of FC over CC is that it is faster and economic methods, ideal for the separation of compounds up to gram quantities and the automated changes between normal phase and reversed phase chromatography. In comparison to open-column chromatography, a smaller particle size (around 40 μm in the case of silica) can be used, hence increasing peak resolution. Online peak detection is possible, usually by coupling to a UV detector. Excellent separations of compounds from *Curcuma xanthorrhiza* (curcumin, xanthorrhizol), *Piper nigrum* (amides), and *Salvia miltiorrhiza* (tanshinones) were obtained by FC on prepacked RP 18 cartridges based on empirical rules involving HPLC separations. Some recent examples of FC as part of the isolation strategy include acylphloroglucinols from *Hypericum empetrifolium*, which were isolated by FC on silica, RP-18 and a final purification on RP-HPLC, antiplasmodial aporphine alkaloids, and sesquiterpene lactones from *Liriodendron tulipifera* (Bucar et al., 2013).

13.8.1.4 Solid Phase Extraction

Solid phase extraction (SPE) consists of a liquid or gaseous test sample in contact with a solid phase, whereby the analyte is selectively adsorbed on the surface of the solid phase. The basic principle of SPE is the partitioning of compounds between two phases of solid and liquid and there must be greater affinity for the solid phase than for the sample matrix.

The compounds retained on the solid phase can be removed by eluting solvent with a greater affinity for the analytes. In a modern SPE system, the adsorbent is packed between two flitted disks in a polypropylene cartridge and liquid phases are passed through the cartridge either by suction or by positive pressure.

13.8.1.5 Thin-Layer Chromatography and High-Performance Thin-Layer Chromatography

One basic separation technique for plant extracts is thin-layer chromatography (TLC), which is based on the principle of adsorption chromatography or partition chromatography or combinations of both, depending on the adsorbent and solvents employed. TLC is suitable for nonvolatile, or low-volatility, or strongly polar analytes, which could damage or destroy LC or GC columns, or would not be detectable by LC or GC. HPTLC is considered to be a more sophisticated form of thin-layer chromatography with better analytical precision and accuracy, lower analysis time, and lower cost per analysis. The detailed methodologies of TLC and HPTLC are described in Chapters 8 and 9, respectively.

13.8.1.6 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a form of liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase at high pressure to give fast and improved separation. Chromatography is a technique in which the mixtures of components are separated on a stationary phase under the influence of a mobile phase. There have been various forms of the stationary phase during the course of time. These include paper, silica on glass plates, immobilized liquids, gels, and solid particles. Along with the development of different adsorbent materials, the focus has increased on analyzing particular classes of analytes by quicker and more competent systems. HPLC is one of the most popular, modern, powerful, and versatile chromatographic separation techniques that have been routinely used to separate the components in an herbal extract or product. It helps to identify each component, to quantify separated components, and to obtain the chemical profile or fingerprint of a crude mixture (Weston and Brown, 1997). HPLC has also been used in many different ways to determine known or unknown substances in herbal products in order to assure their quality in the industry, as well as by the competent authorities. Food, beverages, perfumes, and also herbal drugs are among the herbal products that contain several naturally present secondary metabolites in their raw materials and some that form during processing. HPLC is extensively used for the analysis of all these herbal products. The details of the application of HPLC for the evaluation of herbal drugs are discussed in Chapter 10.

13.8.1.7 Ultra-High-Performance Liquid Chromatography

The ultra-high-performance liquid chromatography (UHPLC) technique for separations is increasing compared with the HPLC technique because it eliminates complications resulting from the use of larger sample volumes and larger particle sizes, as well as the complications resulting from increased generated wastes and increased analysis time. The UHPLC technique is superior compared with HPLC due to its increased separation efficiency, improved resolution, and faster analysis time. It generally involves smaller particle size (<2 μm) and high pressure (>350 bar). It is an emerging technique in the drug discovery process for its ability to perform rapid separations without sacrificing high-quality results in terms of resolution, accuracy, and reliability. In recent years, several hyphenated techniques, including UHPLC-MS, SFE-UHPLC, and UHPLC-DADESI-MS, have been introduced in natural product-based research. This technique is a very versatile and powerful tool for the separation of natural products from crude extracts for selective detection and general profiling. The technique is precise, robust, faster, and sensitive and relies upon smaller volumes of organic solvents than HPLC. The advantages of introducing UHPLC are a decrease in sample turnaround time for manufacturing and product development, the use of less organic solvents, and a reduction in generated waste (Singh and Singh, 2012).

13.8.1.8 Gas Chromatography

Gas chromatography (GC) is applied to separate and analyze the volatile chemical compounds that vaporize without decomposing. It possesses very high chromatographic resolution. In GC, the mobile phase (carrier gas) is a gas (usually N_2, He, Ar, or H_2) and requires the analyte to be either naturally volatile or one that can be converted to a volatile derivative. GC is useful in the separation of small organic and inorganic compounds with low polarity, low boiling point, or that are volatile after being derivatized (Gaudencio and Pereira, 2015). GC plays a role in separation and introduces target substances into an MS system by directly injecting analytes into a chromatographic column or introducing analytes into a chromatographic column after injecting and heating. The chromatographic column is heated thermostatically or is program-controlled. Each component is separated by the difference of thermodynamic properties (the difference of boiling points and the difference

of selective absorption in the stationary phase) and the different distributions in the stationary phase and mobile phase (carrier gas). The major applications of GC in herbal drug research are as follows:

- Identify most volatile compounds
- Quantification of fixed and volatile oils
- Identification of adulterants and contaminants
- Quality control of essential, volatile, and fixed oils
- Characterization of volatile compounds
- Chemical fingerprinting of volatile compounds

13.8.1.9 Capillary Electrophoresis

Capillary electrophoresis (CE) is the separation of solutes based on different rates of migration though an electric field through a background electrolyte in which anions move toward the anode and vice versa. The charge and size influence the movement of the charged particles in opposite ways. Various separation modes for CE are capillary zone electrophoresis, nonaqueous capillary electrophoresis (NACE), capillary gel electrophoresis (CGE), capillary electro-kinetic chromatography (CEKC)/capillary electro-chromatography (CEC), micellar electro-kinetic chromatography (MEKC), and microemulsion electro-kinetic chromatography (MEEKC) (Unger, 2009). Different classes of chemical compounds, for example, alkaloids, anthraquinones, anthocyanins, carbohydrates, catechins, coumarins, flavonoids, glucosinolates, phenolic acids, proanthocyanidins, red wine pigments, saponins, and xanthones have been reported by Unger (2009). For example, optimization of the separation of Vinca alkaloids was carried out by nonaqueous capillary electrophoresis (Barthe et al., 2002). The nonaqueous capillary electrophoresis method was used to separate anthraquinones in *Xanthophytum attopvensis* Pierre (Li et al., 2005a,b). The separation method was developed for simultaneous determination of fraxin, esculin, and esculetin in *Cortex fraxini* by the nonaqueous capillary electrophoresis method.

13.8.2 Hyphenated Techniques

13.8.2.1 Liquid Chromatography–Mass Spectrometry

Liquid chromatography–mass spectrometry (LC–MS) is becoming a method of choice for profiling metabolites in complex plant matrices/herbal mixtures. Most metabolomics studies utilize the chromatographic separation method before mass spectrometric analysis, by which it can reduce sample complexity and alleviate matrix effects during ionization. The details of the methodology of LC–MS required for the separation, quality evaluation, and validation of herbal drugs are described in Chapters 11 and 17.

13.8.2.2 Gas Chromatography–Mass spectrometry

Gas chromatography–mass spectrometry (GC/MS) is one of the earliest techniques in metabolomics research. It is the most versatile tandem mass spectrometry technology. The major principles of ionization in GC–MS techniques are the electron impact (EI) and chemical ionization (CI) techniques. Here, the target substances enter into MS through GC, are ionized into gaseous ions in the ionization source, and then enter into the mass analyzer. Ions with different mass-to-charge ratios are sequentially separated and reach the electron multiplier, generating an electrical signal, in order to give information on the target substances, making qualitative analysis more accurate by using information on the ion fragments (Lisec et al., 2006; Xue et al., 2015). The details of the methodology of GC–MS required for separation, quality evaluation, and validation of herbal drugs are described in Chapter 17.

13.8.2.3 Liquid Chromatography–Nuclear Magnetic Resonance

Another major analytical platform available for plant metabolomics study is nuclear magnetic resonance (NMR) spectroscopy. It offers several advantages over mass spectrometry by offering high reproducibility, quantitative analysis in a wide dynamic range, and the ability to determine the structures of unknowns. NMR is a very suitable method to carry out such analyses because it allows the simultaneous detection of diverse groups of secondary metabolites (flavonoids, alkaloids, terpenoids, and so on) besides abundant primary metabolites (sugars, organic acids, amino acids, and so on). The details of the methodology of GC–MS required for separation, quality evaluation, and validation of herbal drugs are described in Chapter 17.

13.8.3 X-Ray Crystallography

X-ray crystallography enables the identification of the atomic and molecular structure of a crystal. The determination of the absolute configuration of chiral compounds is one of the most difficult analyses of molecular structures. NMR and

spectrometric methods can determine, in principle, only relative stereochemistry. X-ray crystallography is the only method that can determine the absolute configuration of chiral molecules, on the basis of the anomalous scattering effects of heavy atoms. The X-ray technique provides direct structural information on molecules at the atomic level and is recognized as a reliable structure determination method (Gaudencio and Pereira, 2015).

13.8.4 "Omics"-Guided Approach

Several developments and scientific advances have improved the analysis of biological systems. Rapidly expanding important tools and research fields include:

(i) Genomics: DNA sequencing and its related research. Genetic fingerprinting and DNA microarray.
(ii) Proteomics: protein concentrations and modifications analysis, especially in response to various parameters.
(iii) Metabolomics: analogous to proteomics, but dealing with metabolites. The major applications of omics techniques in medicinal plant research are botanical identification and authentication, isolation and characterization of active components, screening of target molecules, mechanism of effect of phytopreparations, identification of synergistic activity of plant extracts, and several drug metabolism and toxicity studies. The omics techniques are classified into three basic groups: genomics, proteomics, and metabolomics.

13.8.4.1 Genomics

Plants synthesize an abundance of metabolites with potential therapeutic applications. Plants produce diverse secondary metabolites through various biochemical pathways. These secondary metabolites are subdivided into a number of distinct groups on the basis of their chemical structure and synthetic pathways and those groups are the alkaloids, terpenoids, and the phenolic compounds (Chakraborty, 2018). Several metabolic tools, such as recombinant technology combining enzyme engineering and plant genetic engineering, are involved to identify the major metabolic pathways in the drug development process. The genome sequences encompass essential information on the plant origin, evolution, development, physiology, inheritable traits, and epigenomic regulation, which are the premise and foundation of deciphering genome diversity and chemodiversity (especially various secondary metabolites with potential bioactivities) at the molecular level (Hao and Xiao, 2015). There are some major applications of genomics in herbal medicine research that have been reported. Genomics studies are linked to investigate some major biosynthetic pathways through the omics-guided approach for exploration of the biosynthetic pathways of medicinal plants. The development of genomics has led to the elucidation of the secondary metabolism pathways and has helped researchers to gain a better understanding of the regulation of these processes. Functional gene expression analysis controls several pathways related to plant development, disease, and defense mechanisms, which further improve the breeding efficiency and accelerate better germplasm findings. Genomics studies can be used to develop complete genome information and gene mutant libraries for the discovery of functional genes related to secondary metabolite biosynthesis.

13.8.4.2 Proteomics

The characterization of the proteins involved in the biosynthesis of bioactive compounds is an important step in the drug discovery process. Various biological changes related to plant growth and environmental stresses can be determined through proteomic techniques. This can be considered as an important determinant for controlling some metabolic pathways, which can accelerate the development of new cultivars. The study of proteomics is also very helpful in determining specific compounds responsible for intra- and interspecies variation. Proteomic techniques can measure several systemic changes during cellular metabolism to analyze the bioactive compounds derived from medicinal plants. A comparative proteomics study of different parts of plants is used to identify the specific tissue protein that produces secondary metabolites. The content variation of secondary metabolites present in different cultivars can be revealed through proteomic study. Proteomics studies are widely used to identify molecular mechanisms underlying several biochemical pathways (Hashiguchi et al., 2017). In combination with metabolomics, proteomics offers a novel strategy to explore the mechanism of action of several herbal drugs by interpreting metabolite–disease and protein–disease associations. The application of proteomics research may also be applicable for therapeutic monitoring of herbal drugs. Several reports are available on the physiological variation in metabolic phenotyping and functional genomic studies of medicinal plants (Gavaghan et al., 2002; Mumtaz et al., 2017).

13.8.4.3 Metabolomics

Metabolomics aims at the identification and quantitation of small molecules involved in metabolic reactions. LC–MS has offered a growing platform for metabolomic studies due to its high throughput, soft ionization, and good coverage of metabolites. The role of LC–MS in plant metabolomics studies is described in detail in Chapter 17.

13.8.5 In Silico Dereplication

In silico dereplication is a very useful approach to identify some of the central criteria in designing compound libraries to modulate the functions of proteins based on their diversity, drug-likeness, and biological relevance. The ADME prediction and drug likeness evaluation provide an insight into the pharmacokinetic properties of all synthetic, as well as natural, compounds. Generally, ADMET-related properties are calculated by using a PreADMET server. PreADMET generates physically relevant descriptors and uses them to perform ADMET predictions. These ADME predictors are Plasma Protein Binding (PPB), Blood Brain Barrier Penetration (BBB), Human Intestinal Absorption (HIA), and Caco2 and MDCK cell permeability. The drug-likeness properties of compounds (obtained from mass data) are assessed using the same server, on the basis of property-based filters/rules, such as the Rule of Five, the CMC rule, and the MDDR rule, as they define acceptable boundaries for certain molecular physicochemical properties of the druggable candidates. The drug-likeness properties can be calculated from DruLito software, an open-source java-based virtual drug-likeness tool. Here, several physiochemical properties, such as molecular weight (MW), number of hydrogen bond donors (nHBDon), number of hydrogen bond acceptors (nHBAcc), octanol and water partition coefficient (AlogP), number of rotatable bonds (nRotBt), and the number of aromatic rings (nAR), were considered as the major molecular attributes (Tian et al., 2015).

The molecular docking method is very useful to investigate the binding mode of small molecules into protein pockets and a large number of algorithms and scoring functions to assess the protein–ligand interactions exist (Drwal and Griffith, 2013). It can be used for virtual ligand screening and virtual affinity profiling. Two distinct but complementary computer-driven drug discovery approaches can be applied: ligand-based methodology and structure-based methodology. The ligand-based method, based on QSAR modeling, provides an insight into the correlation between the physicochemical and structural properties of the ligands and their biological function and potency. The structure-based method relies on pharmacophores, which are derived from the structure of the protein target. The translation of interaction sites into pharmacophores takes place using energy-based or geometry-based methods. In combination with both methods, the selection of compounds with promising biological activity can be derived by several qualities, such as docking score, ligand conformation and orientation, interactions between ligand and protein, and pharmacophore fit, and are combined with chemical intuition and literature-based knowledge (Drwal and Griffith, 2013). In silico dereplication employs several molecular descriptors, which are processed by various chemoinformatics tools to predict the physiochemical and biological properties of drug molecules (Balaban, 2014). For example, topological descriptor MDEO-12, the electronic descriptor TopoPSA, the quantum-chemical descriptor, and the energy of the highest occupied molecular orbital (3HOMO) have a remarkable performance in discriminating antitumor, antibiotic, and overall biological lead-like compounds, respectively. Some other 2D descriptors, namely, DRAGON, SYBYL, and CODESSA have also been reported to have several applications in descriptors in the drug design process (Helguera et al., 2008).

These approaches can be used in virtual screening, prioritization of compound libraries, and the design of building blocks toward the lead-like libraries of NPs. One of the most widely used virtual screening approaches is quantitative structure–activity relationship (QSAR) modeling. Another extended ligand–protein docking method, INVDOCK, is able to study the virtual interaction between receptor active sites and small molecules to identify suitable ligands. This is generally done by studying ligands and binding conformations at a receptor site. This will act as a potential tool in structural genomics, proteomics, protein function, pharmacokinetics, and drug metabolism. For example, INVDOCK 3 was used for prediction of therapeutic targets of nine medicinal plant ingredients (genistein, ginsenoside Rg1, quercetin, acronycine, baicalin, emodin, allicin, catechin, and camptothecin) (Gaudencio and Pereira, 2015).

Throughout the ages, medicinal plants have been considered as an effective alternative source for procuring novel chemical constituents with excellent therapeutic activity. As plants produce diverse secondary metabolites, their identification and characterization is very important in the drug discovery process. The ethnopharmacological relevance of several plants provides an ideal background in the natural product-based drug discovery process. Several approaches for the separation and identification of active chemical constituents from plant extracts have been applied in the early drug discovery process. In the search for potentially active compounds, the single or multiple bioassay techniques have been widely employed to uncover potential biological activity. Recent advancements in HTS provide a rapid screening method for the evaluation of pharmacological activity by identifying the most bioactive principles. This approach is also helpful for the structural determination of bioactive compounds and thus facilitates the development of new compounds with desirable bioactivities.

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Chapter 14

Therapeutic Evaluation of Herbs With Enzyme Inhibition Studies

Chapter Outline

14.1 IMPORTANCE OF ENZYME INHIBITION STUDIES IN THE EVALUATION OF HERBAL MEDICINE

Herbal medicines (HMs) are the richest source for traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical products, and new chemical entities for synthetic drugs. Since long ago, herbal medicines have been used by human civilizations throughout the world because of their many therapeutic benefits. This applies generally to those medical and healthcare systems that are practiced in a traditional manner and not presently considered to be part of modern Western medicine (Cordell and Colvard, 2012). Herbal medicines are quite popular among people because of their practical benefits, traditional beliefs, economic advantage, easy access and other reasons having a regional, religious, and social basis. HMs are time honored and are being used by people for their own healthcare, so these systems are well rooted with an extensive clinical basis, in which scientific validation is sometimes the major constraint for their development. In spite of all these setbacks, HMs have shown consistent growth in the global market. This discipline has evolved over many years by exploiting natural products useful for numerous diseases from indigenous resources (Houghton and Mukherjee, 2009).

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In the past, most new drugs have been discovered as secondary metabolites and derived molecules from natural resources. Interest in natural products research continues due to the failure of alternative drug discovery techniques to produce many lead molecules in several therapeutic areas, such as immunosuppression, antiinfectives, and metabolic disorders. Natural products (NPs) have been exploited as a big source of new lead structures that might be used as models for the development of new molecules or drugs in the area of pharmaceuticals. It is obvious from natural product research that NPs have been, and will be an important source of new drugs for the pharmaceutical industry. Newer techniques have been introduced to improve and accelerate natural product drug discovery and development in order to find drug targets, lead molecules, and structure elucidation (Patwardhan, 2007). Drug discovery leading to robust and viable drug candidates is a challenging scientific task. This is a transition from a screening hit to a drug molecule, which requires expertise and experience. There are various important techniques, including automated separation techniques, high-throughput screening (HTS), and combinatorial chemistry, which are used for enhancing drug discovery from NPs. These methods can be used for minimizing the inherent limitations of natural products and offer a unique opportunity to re-establish NPs as a major source for drug discovery (Lahlou, 2013).

Enzymes are often the primary molecular target for their essential catalytic roles in many physiological processes that may be altered in disease states. Enzymes generally act through their catalytic activity, which usually takes place by binding with low-molecular-weight, drug-like molecules. In the drug discovery and development process, there is a large and growing interest in the study of enzymes with the aim of identifying inhibitory molecules. Knowledge of medicinal chemistry plays a vital role in studying in vitro evaluation of enzyme–inhibitor interactions. In enzyme inhibition study, drug target affinity is commonly quantified using in vitro-based assays (Copeland, 2000). The development of enzyme inhibitors as therapeutic agents involves the optimization of multiple pharmacological properties beyond the affinity and selectivity of the molecule for its target enzyme. Examples of this include drug absorption from the gastrointestinal tract via active and passive transport mechanisms, metabolic clearance of drugs from systemic circulation, hepatic and renal drug metabolism, and adverse effects mediated by drug interactions with off-target enzymes, ion channels, and receptors (Copeland and Anderson, 2001).

The catalytic power of enzymes is mostly related to the metabolic processes of human health. Enzyme inhibitors are well targeted as an agent for therapeutic benefit against several disease conditions. A detailed understanding of the physiochemical characteristics of enzyme binding sites is very helpful in developing small, drug-like molecules. In this context, the identification of the enzyme target and its inhibitor is of the utmost importance in the early drug discovery process. Initial activity screening is thus required to gather knowledge of the molecular target and to develop an assay design in the high-throughput screening approach. There are several enzymes that are reported to have a potential role in the pathophysiology of several metabolic disorders. In metabolic disorders, the normal metabolic process of converting food to energy on a cellular level is disrupted. The onset of symptoms usually occurs when the body metabolism comes under stress.

Metabolic disease affects the ability of the cell to perform critical biochemical reactions that comprise various processes, such as transport of proteins (amino acids), carbohydrates (sugars and starches), or lipids (fatty acids) (Meisinger et al., 2006). It also increases the risk of various diseases, such as cardiovascular disorder, type-2 diabetes mellitus, obesity, and hypertension. The major pathophysiologies underlying these diseases include insulin resistance, visceral adiposity, atherogenic dyslipidemia, endothelial dysfunction, genetic susceptibility, elevated blood pressure, and hypercoagulable state. There are several enzymes involved in the major biochemical pathways that play a large role in the pathogenesis of disease, such as α-glucosidase, angiotensin converting enzyme (ACE), pancreatic lipase, HMG-CoA reductase (HMGR), carbonic anhydrase, and aldose reductase, the details of which are shown in Table 14.1. The interaction between the components of a clinical phenotype with its biological phenotype (insulin resistance, dyslipidemia, etc.) contributes to the development of these chronic metabolic disorders (Kaur, 2014).

14.2 DIFFERENT STRATEGIES FOR ENZYME INHIBITION STUDIES

Enzymes are considered to be an attractive target for drug development due to their essential catalytic roles in many physiological processes in our body. The enzyme activity may be altered in various disease conditions. In most cases, the enzyme catalysis can be hindered by low-molecular-weight, drug-like molecules, which are known as inhibitors. The in vitro evaluation of enzyme–inhibitor interactions are a key focus for primary screening of new drug molecules. The development of enzyme inhibitors as therapeutic agents involves the optimization of multiple pharmacologic properties beyond the affinity and selectivity of the molecule for its target enzyme. Many of these pharmacological properties have their molecular underpinning in biochemical reactions within the human body, which include drug absorption from the gastrointestinal tract via active and passive transport mechanisms, metabolic clearance of drugs from systemic circulation, hepatic and renal drug metabolism, and adverse effects mediated by drug interactions with off-target enzymes, ion channels and receptors.

TABLE 14.1 Targeted Enzymes in Several Disease Conditions

Target Enzyme	Diseases
Carbonic anhydrase	Glaucoma
Viral DNA polymerase	Herpes
Xanthine oxidase	Gout
Cyclooxygenases	Inflammation
Angiotensin converting enzyme	Hypertension
Dopa decarboxylase	Parkinson's disease
Cyclooxygenase-2	Inflammation
MAP kinase kinase	Cancer
HMG-CoA reductase	Cholesterol lowering
Dehydrogenase	Inflammation
Aldose reductase	Diabetic retinopathy
Phosphodiesterase	Erectile dysfunction
Dihydrofolate reductase	Cancer, immunosuppression
Steroid 5α-reductase	Benign prostate hyperplasia
Sodium, potassium ATPase	Heart disease
Pancreatic lipase	Hyperlipidemia
Acetylcholinesterase	Cognitive disorders

Enzyme assays are based on the measurement of how fast a given (unknown) amount of enzyme will convert substrate to product (the act of measuring a velocity). An enzyme assay is required to determine the concentration of a product or substrate at a given time after starting the reaction. There are several assay procedures to monitor the enzyme–substrate interaction qualitatively as well as quantitatively. These include the spectroscopic, fluorimetric, manometric, and polarimetric methods (Copeland and Anderson, 2001).

- The spectrophotometric method is based on a change of absorbance due to conversion of the substrate to product because of enzyme action, measured in the visible region or in the UV region. This method is mostly used in enzyme assay as it is easy, simple, and sensitive.
- The fluorescence method is applied when the molecule does not contain any UV-absorbing chromophore, but its emission wavelength changes after absorbing light of a different wavelength. This method is applicable when the enzyme-catalyzed reaction follows oxidation and reduction pathway, where change in fluorescence takes place. It is a highly sensitive method to detect small quantities of samples.
- The manometric method is applicable when one of the reaction components is gas. Electrode method is suitable to follow the reactions in which change in pH of the reaction system takes place.
- The polarimetric method is suitable for detection of several isomerases, which can convert optically active isomers to inactive or vice versa. Sampling methods are followed by withdrawing samples at intervals and estimating the substrate or product by chemical methods. This method can be used to study the enzymes that can act on carbohydrates linked with the breakage of a glycosidic link present in the structure.

Enzyme assays are found to be very effective drug targets for their catalytic activity. Most of the pharmaceutical companies are nowadays focusing on low-molecular-weight drug molecules, which can bind to the receptor (enzymes) by altering (inhibition/induction) the disease pathophysiology. This can lead to the development of novel drug candidates that act through inhibition of specific enzyme targets. In fact, most of the drugs marketed by the pharmaceutical companies are small-molecule ones and act as specific enzyme inhibitors. Some examples of these enzymes and the disease pathogenesis

are shown in Table 14.1. Enzyme reactions also play a critical role in the study of drug metabolism and pharmacokinetics. The elimination of xenobiotics, including drug molecules, from systemic circulation is driven by metabolic transformations catalyzed by enzymes. Enzyme-catalyzed reactions are mainly of four types (Copeland and Anderson, 2001).

i. Oxidation reactions, which involve aromatic hydroxylation, aliphatic hydroxylation, N-hydroxylation, N,O,S-dealkylation, deamination, sulfoxidation, N-oxidation, and dehalogenation.
ii. Hydrolytic enzymes, which include ester, amide, and peptide hydrolysis reactions.
iii. A conjugation reaction is another reaction mechanism process by which an enzyme can act through glucuronidation, sulfation, acetylation, peptide conjugation, and glutathione conjugation.
iv. Reductive reactions are another mechanism of enzyme action that play a vital role in enzyme activity.

14.3 INHIBITION STUDY OF ACETYL CHOLINESTERASE AND BUTYL CHOLISTERASE (AChE/BChE)

14.3.1 Importance of Acetyl Cholinesterase and Butyl Cholisterase (AChE/BChE)

Cholinesterases are widespread in cholinergic and noncholinergic tissues, as well as in plasma and other body fluids. AChE has more affinity toward acetylcholine and hydrolyzes acetyl choline faster than BChE. On the contrary, BChE preferentially acts on butyrylcholine, but also hydrolyzes acetylcholine. The principal role of acetylcholinesterase (AChE) is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of acetylcholine (ACh). The inhibition of AChE serves as a strategy for the treatment of Alzheimer's disease (AD), senile dementia, ataxia, myasthenia gravis, and Parkinson's disease. The ChEIs act by inhibiting the degradation of Ach, through reversible and irreversible inhibition of ChEs. AD is one of the most common forms of dementia, affecting many elderly people. Besides the neuropathologic hallmarks of this disease, namely, neurofibrillary tangles and neuritic plaques, it is characterized neurochemically by a consistent deficit in cholinergic neurotransmission, particularly affecting cholinergic neurons in the basal forebrain. The evidence stems from data of several authors who demonstrated a reduction in the activity of enzymes involved in the synthesis of acetylcholine, that is, choline acetyl transferase or excess degradation of Ach by AChE (Mukherjee et al., 2007a,b). Cholinomimetic drugs constitute the first line of treatment for AD, and ChEIs were the first medication approved by the US Food and Drug Administration for the treatment of cognitive deficits and functional symptoms of AD (Rosler et al., 1999; Tariot et al., 2000). ChEIs stop or inhibit enzymes from breaking down acetylcholine when it travels from one cell to another. ChEIs result in increasing the concentration of acetylcholine, leading to increased communication between nerve cells, which in turn, may temporarily improve or stabilize the symptoms of dementia, although very few synthetic drugs are currently approved by regulatory authorities to treat such disease. A search for potent and effective cholinesterase inhibitors that produce minimal side effects in AD patients is a bigger challenge nowadays. As an effective alternative to synthetic drugs, there are a handful of AChE inhibitors from plant sources reported in the literature. Numerous phytoconstituents and promising plant species to serve as AChE inhibitors were reported by Mukherjee et al. (2007a,b).

14.3.2 Assay Procedure for AChE/BChE Inhibition

Acetylcholinesterase hydrolyzes the substrate acetylthiocholine/butyrylthiocholine, resulting in the product thiocholine, which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, which is yellow in color. The color change is detected at 405 nm by using the spectrophotometric method (Ellman's method) (Mukherjee, 2007) (Fig. 14.1). A TLC autobiographic method is also used based on the separation and activity directly detected on the TLC plate. The test relies on the cleavage by acetylcholinesterase of 1-naphthyl acetate to form 1-naphthol, which in turn reacts with Fast Blue B salt to give a purple-colored diazonium dye. The acetylcholinesterase inhibitory activity is detected by observing a white spot against the purple background that appears on the TLC plate (Marston et al., 2002) (Fig. 14.2).

14.3.3 Natural Products as AChE/BChE Inhibitors

Naturally occurring compounds from plants are considered to be a potential source of new inhibitors inhibiting the enzyme AChE. A large number of natural plants extract have been found to be effective inhibitors of AChE and BChE. Several phytoconstituents responsible for the anticholinesterase activity of plants are found to be effective inhibitors of AChE and BChE. Several medicinal plants possessing such activity have been summarized in Table 14.2.

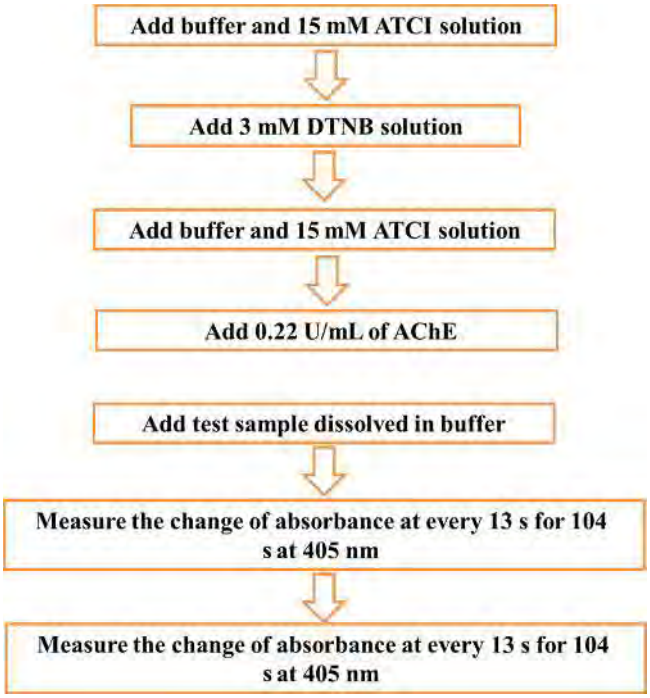

FIG. 14.1 Spectroscopic assay for acetyl cholinesterase (AChE) inhibition (Ellman's method).

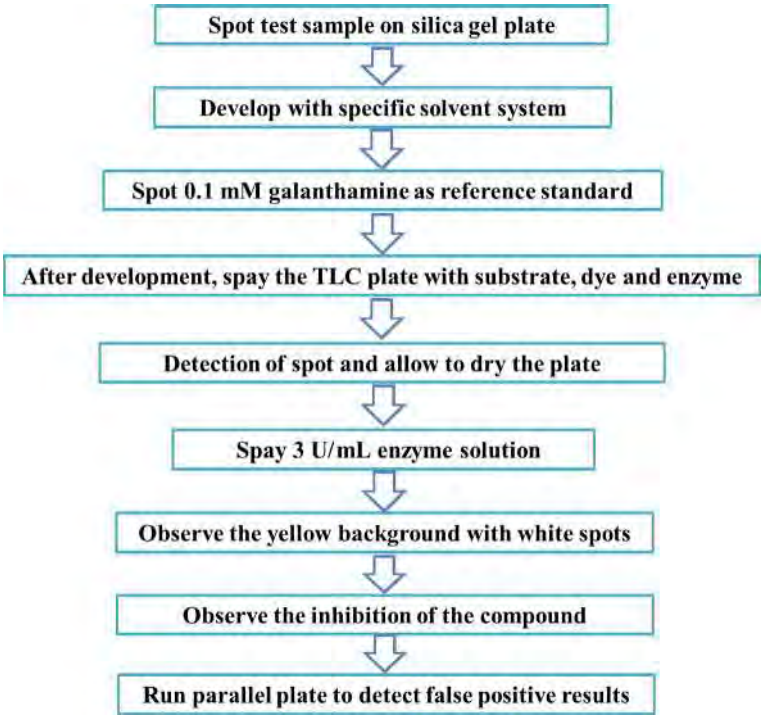

FIG. 14.2 TLC autographic assay for acetyl cholinesterase (AChE) inhibitory activity.

TABLE 14.2 Examples of Medicinal Plants Having AChE/BChE Inhibitory Properties

Name of the Plant	Family	IC_{50} (in % or mg/mL)		References
		AChE	BChE	
Polygonum multiflorum	Polygonaceae	9.11 µg/mL	5.83 µg/mL	Li et al. (2017)
Spatholobus suberectus	Leguminosae	9.27 µg/mL	5.37 µg/mL	Li et al. (2017)
Aristotelia chilensis	Elaeocarpaceae	79.5% at 10.0 mg/mL	89.8% at 20.0 mg/mL	Cespedes et al. (2017)
Mutellina purpurea	Apiaceae	9.30 ± 1.86% at 10.0 mg/mL	91.62 ± 1.53% at 10.0 mg/mL	Orhan et al. (2017)
Salsola grandis	Amaranthaceae	64.90 ± 1.61% at 50 µg/mL	—	Orhan et al. (2017)
Geophila repens	Rubiaceae	68.63 ± 0.45 µg/mL	59.45 ± 0.45 µg/mL	Dash and Sahoo (2017)
Cryptocarya species	Lauraceae	66.83 ± 1.18 µg/mL	30.71 ± 1.93 µg/mL	Othman et al. (2016)
Heinsia crinita	Rubiaceae	32.11 mg/mL	30.4 mg/mL	Oboh et al. (2016)
Caryopteris mongolica	Lamiaceae	27.9 ± 5.2 µM	73.8 ± 1.7 µM	Murata et al. (2016)
Punica granatum	Punicaceae	2.70 ± 0.10 µg/mL	4.5 ± 0.21 µg/mL	Bekir et al. (2016)
Lycopodiella cernua	Lycopodiaceae	0.23 µM	0.62 µM	Hung et al. (2015)
Iris albicans	Iridaceae	—	78.44 ± 0.51%	Hacıbekiroglu and Kolak (2015)
Acorus calamus	Araceae	53.77% at 200 µg/mL	—	Mukherjee et al. (2007a,b)
Cotoneaster horizontalis	Rosaceae	1.54–3.41 mM	5.97–6.84 mM	Khan et al. (2014)
Syzygium aromaticum	Myrtaceae	61.5 ± 1.88 µg/mL	103.53 ± 1.47 µg/mL	Dalai et al. (2014)
Angelica dahurica	Apiaceae	46.3–55.4 µM	42.4–45.2 µM	Seo et al. (2013)
Viburnum tinus	Adoxaceae	66.4 ± 0.65% to 97.7 ± 0.47%	—	Yılmaz et al. (2013)
Murraya koenigii	Rutaceae	0.03 ± 0.09 mg/mL	—	Kumar et al. (2010)
Cistus creticus	Cistaceae	12.9 µg/mL	29.1 µg/mL	Loizzo et al. (2013)
Marsilea quadrifolia	Marsileaceae	51.89 ± 0.24 µg/mL	109.43 ± 2.82 µg/mL	Bhadra et al. (2012)
Illicium verum	Schisandraceae	58.67 ± 0.16 µg/mL	91.84 ± 1.29 µg/mL	Bhadra et al. (2011)
Centella asiatica	Apiaceae	75.0%	>10%	Chaiyana and Okonogi (2012)
Cinnamomum bejolghota	Lauraceae	>25.0%	>50.0%	Chaiyana and Okonogi (2012)
Citrus aurantifolia	Rutaceae	85.8%	82.9%	Chaiyana and Okonogi (2012)
Citrus aurantium	Rutaceae	147.5%	266.6%	Tundis et al. (2012)
Citrus bergamia	Rutaceae	161.6%	243.6%	Tundis et al. (2012)
Anethum graveolens	Apiaceae	100.0%	90.9%	Orhan et al. (2008)

14.4 INHIBITION STUDY OF ANGIOTENSIN CONVERTING ENZYME (ACE)

14.4.1 Importance of ACE

ACE plays a crucial role in the renin-angiotensin system. It is a dipeptidyl carboxypeptidase regulated by chloride and zinc ions. It is present universally in mammalian species. As a principal component of the renin–angiotensin system (RAS) and the kallikrein–kinin system (KKS), ACE plays an important role in regulating blood pressure, fluid and electrolyte balance, and vascular remodeling by hydrolyzing angiotensin I into a potent vasopressor peptide angiotensin II and deactivating the vasodepressor peptide bradykinin (Corvol, Williams, & Soubrier, 1995). In the presence of Zn^{2+}, ACE hydrolyzes the peptide

bond of Phe8-His9 of decapeptide angiotensin I (Ang I) to release octapeptide angiotensin II (Ang II) and C-terminal dipeptide His-Leu. Ang II is one of the most potent vasoconstrictors; upon binding with its type 1 receptor, Ang II contracts vascular smooth muscle, stimulates the secretion of aldosterone and Na^+, K^+-reabsorption in the kidney, and induces water-sodium retention and increases the blood volume. These subsequently result in increasing the blood pressure and the positive inotropic chronotropic effect in the heart. ACE inhibitors (ACEI) inhibit the activity of ACE in the myocardium, kidney, and vessel wall via decreasing the blood pressure and inhibiting myocardial and vascular hypertrophy. It also can improve the autonomic nervous activity of patients with chronic heart failure. As a vasodilator initially, ACEI may be associated with the blockade of endocrine, cardiac tissue, paracrine, and autocrine. ACEI also offers reduction in myocardium hypertrophy and myocardial fibrosis and thus ventricular remodeling is improved. Thus, ACEI is not only used as a hypotensive drug, but is also widely used for the treatment of disease conditions involving the cardiovascular system, urinary system, and endocrine system (Zhao and Xu, 2008).

14.4.2 Assay Procedure for ACE Inhibition

The spectroscopic method is based on the liberation of hippuric acid from hippuryl-His-Leu (HHL) catalyzed by the ACE. The liberation of hippuric acid from hippuryl-L-histidyl-L-leucine is estimated at λ_{max} of 228 nm by the spectrophotometric method. Briefly, a sample solution (at varied concentrations) with ACE (25 mU/mL) is preincubated at 37°C for 10 min. The substrate solution (8.3 mM HHL in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3), along with the test sample, are added to the above mixture and incubated for 30 min at 37°C. Finally, the absorbance of the hippuric acid is measured at 228 nm (Chaudhary et al., 2015) (Fig. 14.3).

14.4.3 Natural Products as ACE Inhibitors

Inhibition of ACE is considered to be an important therapeutic target in the treatment of hypertension. Synthetic ACE inhibitors are believed to have certain side effects, therefore, there is a need to search for safe and therapeutically effective ACE inhibitors from natural sources to serve as alternatives to synthetic drugs. There have been several reports of ACE inhibitors isolated from medicinal plants. So, there is a need to screen for antihypertensive effects in medicinal plants. Several medicinal plants possessing such activity have been summarized in Table 14.3.

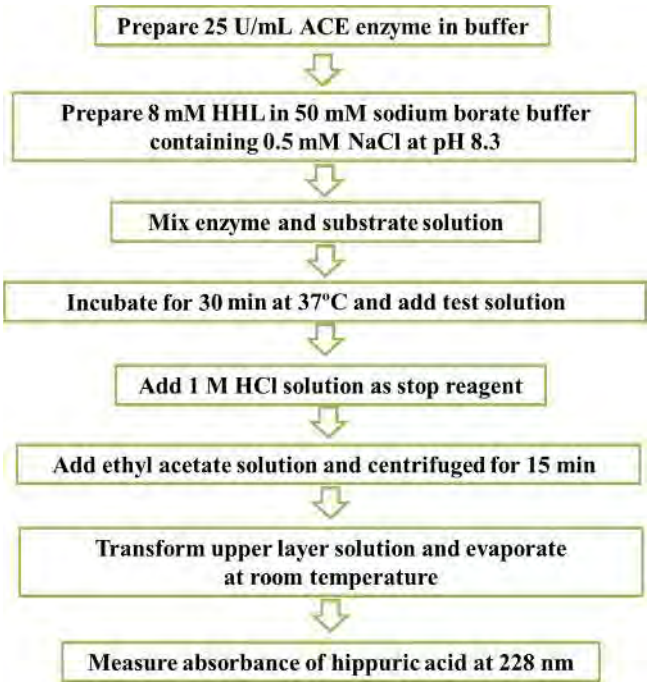

FIG. 14.3 Spectroscopic assay for acetyl cholinesterase (AChE) inhibition.

TABLE 14.3 Medicinal Plants Having ACE Inhibitory Property

Name of the Plant	Family	ACE Inhibition Rate (% or IC_{50})	References
Eleusine indica	Poaceae	32 μg/mL	Tutor and Chichioco-Hernandez (2018)
Parinari curatellifolia	Chrysobalanaceae	13.54 and 39.38 μg/mL	Crown et al. (2017)
Ocimum gratissimum	Lamiaceae	56.3 μg/mL	Shaw et al. (2017)
Piper nigrum	Piperaceae	0.2161–0.04627 mM	Yu et al. (2016)
Ipomoea reniformis	Convolvulaceae	422 ± 21.16 μg/mL	Jabeen and Aslam (2013)
Berberis integerrima	Berberidaceae	88.2 ± 1.7%	Sharifi et al. (2013)
Crataegus microphylla	Rosaceae	80.9 ± 1.3%	Sharifi et al. (2013)
Nymphaea alba	Nymphaeaceae	66.3 ± 1.2%	Sharifi et al. (2013)
Onopordon acanthium	Asteraceae	80.2 ± 2.0%	Sharifi et al. (2013)
Quercus infectoria	Fagaceae	93.9 ± 2.5%	Sharifi et al. (2013)
Rubus sp.	Rosaceae	51.3 ± 1.0%	Sharifi et al. (2013)
Rosa rugosa	Rosaceae	2.5–7.5 mg/mL	Xie and Zhang (2012)
Apium graveolens	Apiaceae	666.26 ± 1.32 μg/mL	Umamaheswari et al. (2012)
Erythroxylum gonocladum	Erythroxylaceae	50% inhibition at 5.86 ± 0.33 μM	Lucas-Filho et al. (2010)
Ailanthus excels	Simaroubaceae	50% inhibition at 280 ± 3.2 μg/mL	Loizzo et al. (2007)
Ligustrum vulgare	Oleaceae	50% inhibition at 25 ± 5 μg/mL	Kiss et al. (2008)
Mucuna pruriens	Fabaceae	52.68 ± 2.02 μg/mL	Chaudhary et al. (2015)
Piper longum	Piperaceae	193.12 μg/mL	Chaudhary et al. (2013)
Hibiscus sabdariffa	Malvaceae	50% inhibition at 68.4 μg/mL	Ojeda et al. (2010)
Cassia tora	Leguminosae	50% inhibition at 30.24 ± 0.20 μg/mL	Hyun et al. (2006)
Rabdosia coetsa	Labiatae	32.42% inhibition at 10 μg/mL	Li et al. (2008)
Ailanthus excels	Simaroubaceae	50% inhibition at 290 ± 2.9 μg/mL	Loizzo et al. (2007)
Sedum sarmentosum	Crassulaceae	408.9 ± 4.6 μg/mL	Hyuncheol et al. (2004)
Abeliophyllum distichum	Oleaceae	278 μg/mL	Hyuncheol et al. (2003)
Clerodendron trichotomum	Verbenaceae	373 ± 9.3 μg/mL	Kang et al. (2003)
Cryptomeria japonica	Cupressaceae	16 μg/mL	Tsutsumi et al. (1998)

14.5 INHIBITION STUDY OF PANCREATIC LIPASE

14.5.1 Importance of Pancreatic Lipase

Lipases or acyl-glycerol acylhydrolases are chemically esterases in nature. Pancreatic lipase plays a key role in the conversion of triglyceride into monoacyl glycerol and free fatty acids. Both the monoacyl glycerol and fatty acid are converted to a mixed micelle in the presence of cholesterol and bile acids. It is then absorbed as monoacyl glycerol and further transformed to triglyceride in which energy is stored (Birari and Bhutani, 2007). The free fatty acid is responsible for the regulation of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein, collectively called the total lipid content. A failure to regulate the total lipid content is considered to be a major causative factor in cardiovascular disease (CVD), as well as obesity and hypertension. An excess disposition of triglyceride leads to hyperlipidemia, which is a major causative factor in the development of CVDs, atherosclerosis, hypertension, diabetes, and other functional depressions of certain organs. Among several causative factors, pancreatic lipase has been proposed as an important target in

developing antiobesity and hypolipidemic agents. By inhibiting the pancreatic lipase action, the conversion of triglyceride is blocked, which further results in decreased triglyceride reabsorption in adipose tissue and finally leads to hypolipidemia (Birari and Bhutani. 2007).

14.5.2 Assay Procedure for Lipase Inhibition

A pancreatic lipase inhibition study is performed through the spectrophotometric method. This method includes *p*-nitrophenol acetate as substrate. On enzyme substrate reaction, *p*-nitrophenol acetate is converted into nitrophenolate ion and the change in color is measured at a λ_{max} of 405 nm (Gonçalves et al., 2010) (Fig. 14.4). Another lipoprotein lipase activity assay involves a fluorogenic triglyceride analog as a lipase substrate. When uncleaved, the substrate remains in a nonfluorescent, quenched state; however, upon hydrolysis by LPL at the sn-1 position, a fluorescent product is produced, which can be measured in a fluorescence microplate reader (Ex. 480–485 nm/Em. 515–525 nm with 495 nm cutoff) (Basu et al., 2011) (Fig. 14.5).

14.5.3 Natural Products as Pancreatic Lipase Inhibitors

Obesity is considered to be a major lifestyle-related disorder associated with dyslipidemia, hypertension, and diabetes. There are several antiobesity agents based on natural products that have been reported to have lipase inhibitory properties. A wide number of plant secondary metabolites, such as alkaloids, terpenoids, and phenolics, have been reported for their pancreatic lipase inhibitory effect. Several medicinal plants possessing such activity are summarized in Table 14.4.

14.6 INHIBITION STUDY OF HMG-COA REDUCTASE

14.6.1 Importance of HMG-COA Reductase

Hyperlipidemia has been ranked as one of the greatest risk factors contributing to the prevalence and severity of coronary heart diseases. It is a condition in which abnormally high levels of lipids, that is, fatty substances, are found in the blood. It is the major causative factor for the progression of such diseases as coronary heart disease, stroke, and atherosclerosis. The elevation of serum total cholesterol and LDL cholesterol has been reported as a primary risk factor for CVD. HMGR is an enzyme that catalyzes the conversion from 3-hydroxy-3-methyl-glutaryl-CoA to mevalonate, which is a major intermediate

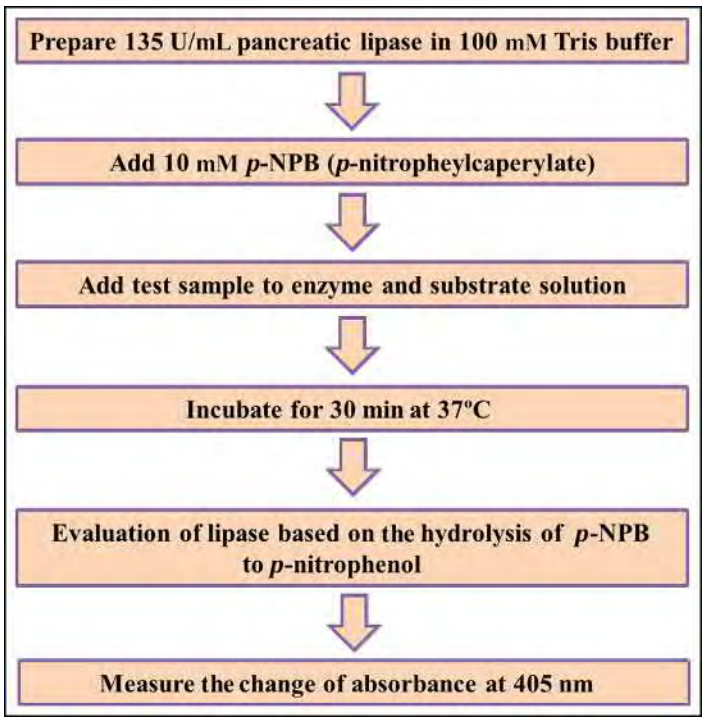

FIG. 14.4 Spectrophotometric assay for lipase inhibition.

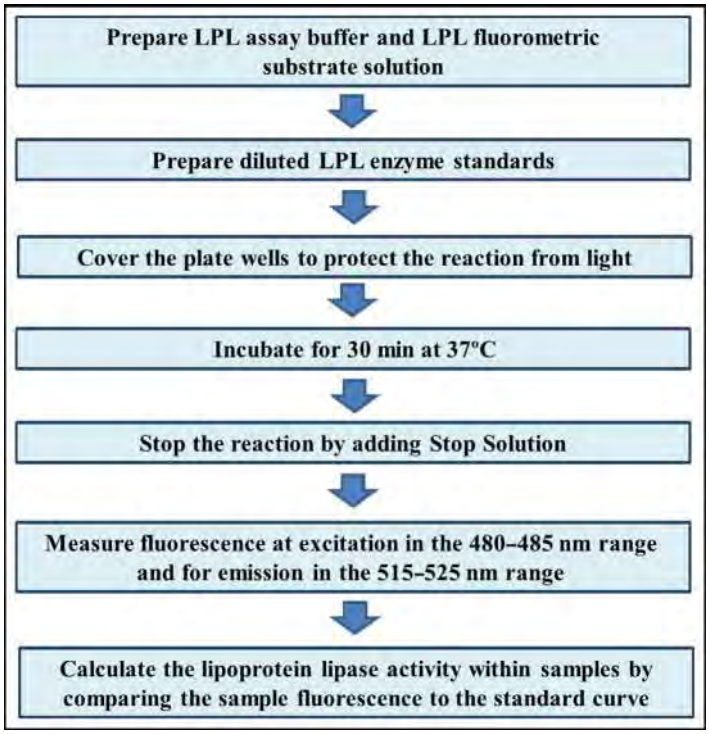

FIG. 14.5 Fluorometric assay for pancreatic lipase inhibition.

TABLE 14.4 Medicinal Plants Having Pancreatic Lipase Inhibitory Activity

Name of the Plants	Family	Inhibition Rate (% or IC_{50})	References
Oroxylum indicum	Bignoniaceae	89.12 ± 6.87% inhibition at 250 µg/mL	Mangal et al. (2017)
Lagenaria siceraria	Cucurbitaceae	157.59 µg/mL	Maqsood et al. (2017)
Alisma orientalis	Alismataceae	64.4 and 45.5 mM	Cang et al. (2017)
Vinca major	Apocynaceae	38.1%	Sukhdev and Shamsher (2018)
Prostanthera rotundifolia	Lamiaceae	390 ± 0.01 µg/mL	Tang et al. (2017)
Achillea phrygia	Asteraceae	72.73	Zengin et al. (2017)
Bupleurum croceum	Apiaceae	23.91	Zengin et al. (2017)
Mentha viridis	Lamiaceae	0.43 and 1.44 mg/mL	Belfeki et al. (2016)
Eucalyptus globulus	Myrtaceae	0.69–1.29 mg/mL	Belfeki et al. (2016)
Tecoma stans	Bignoniaceae	77.58%	Ramirez et al. (2016)
Aloe vera	Asphodelaceae	85%	Taukoorah and Mahomoodally (2016)
Capsicum annuum L.	Solanaceae	31.7%	Shukla et al. (2016)
Tecoma stans	Bignoniaceae	77.58%	Ramirez et al. (2016)
Aloe vera	Asphodelaceae	85%	Taukoorah and Mahomoodally (2016)
Capsicum annuum	Solanaceae	31.7%	Shukla et al. (2016)
Tecoma stans	Bignoniaceae	77.58%	Ramirez et al. (2016)
Colcus forskohlii	Lamiaceae	74.5%	Badmaev et al. (2015)
Salacia reticulata	Celastraceae	53.6%	Badmaev et al. (2015)

TABLE 14.4 Medicinal Plants Having Pancreatic Lipase Inhibitory Activity—cont'd

Name of the Plants	Family	Inhibition Rate (% or IC_{50})	References
Araucaria angustifolia	Araucariaceae	68%	Oliveira et al. (2015)
Colcus forskohlii	Lamiaceae	74.5%	Badmaev et al. (2015)
Salacia reticulata	Celastraceae	53.6%	Badmaev et al. (2015)
Eugenia jambolana	Myrtaceae	23 ± 5.0 μg/mL	Mukherjee and Sengupta (2013)
Azadirachta indica	Myrtaceae	14 ± 3.0 μg/mL	Mukherjee and Sengupta (2013)
Tinospora cordifolia	Myrtaceae	11 ± 2.5 μg/mL	Mukherjee and Sengupta (2013)
Cassia auriculata	Caesalpiniaceae	6.0 μg/mL	Habtemariam (2013)
Rheum palmatum	Polygonaceae	53.8 ± 9.0%	Zheng et al. (2010)
Juglans mandshurica	Juglandaceae	88% at 1 mM	Han et al. (2007)

in the cholesterol synthesis pathway. It is supposed that by lowering serum cholesterol levels, statins may cause regression or stabilization of atherosclerotic plaques. However, recent studies have suggested that these drugs have additional beneficial effects beyond cholesterol reduction. Thus, it is evident now that HMG-CoA inhibitors act against cerebral injury by the selective upregulation of endothelial NO synthase enhancing cerebral blood flow, reducing cerebral infarct size, and improving neurological functions (Endres et al., 1998).

The inhibition of this enzyme is one of the major approaches to restrict the production of cholesterol, as inhibitors interrupt the conversion of HMG-CoA to mevalonate (the rate-limiting step in de novo cholesterol biosynthesis). Reduced synthesis of LDL and enhanced catabolism of LDL mediated through LDL receptors appears to be the principal mechanism for the lipid lowering effects.

14.6.2 Assay Procedure for HMGR Inhibition

HMGR is an enzyme that catalyzes the conversion from 3-hydroxy-3-methyl-glutaryl-CoA to mevalonate, which is a major intermediate in the cholesterol synthesis pathway:

$$\mathrm{HMG-CoA + 2NADPH + 2H^{+} \xrightarrow{HMG-COA\ reductase} mevalonate + 2NADP^{+} + CoA + SH}$$

NADPH is oxidized by the catalytic subunit of HMGR in the presence of the substrate HMG-CoA. As the reaction proceeds, NADPH is converted to NADP and the absorbance of the resultant product (measured at 340 nm) decreases. This change in absorbance is recorded spectrophotometrically. HMG-CoA reductase is the rate-determining enzyme in the mevalonate pathway that catalyzes the conversion of hydroxymethylglutaryl-coenzyme A into mevalonate. Thus, the inhibition of HMGR results in less mevalonate production, which in turn restricts the synthesis of cholesterol (Jung et al., 2005) (Fig. 14.6).

14.6.3 Natural Products as HMG-COA Inhibitors

Cardiovascular diseases (CVDs) arising from atherosclerosis are a foremost cause of death and morbidity worldwide. Atherosclerosis and hypertension are the most common factors responsible for CVDs. The risks of CVD can be controlled by targeting HMGR enzyme. There are several phytochemicals from medicinal plants that are reported to have antiatherosclerosis properties by inhibiting HMGR. Several medicinal plants possessing such activity are summarized in Table 14.5.

14.7 INHIBITION STUDY OF α-GLUCOSIDASE

14.7.1 Importance of α-Glucosidase

α-Glucosidase is an enzyme present at the brush border of the small intestine of the human digestive system. It cleaves the oligosaccharides and disaccharides into simple glucose molecules. Then, it is transported to the basolateral side of the

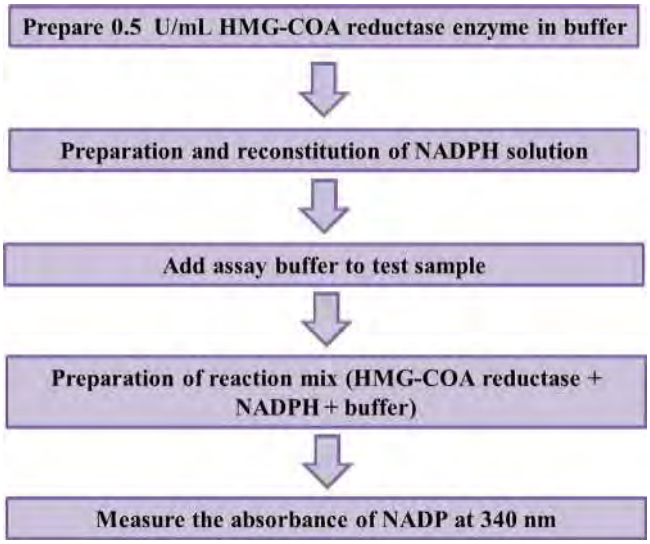

FIG. 14.6 Spectrophotometric assay for HMG COA reductase inhibition.

TABLE 14.5 Medicinal Plants Having HMG-COA Inhibitory Activity

Name of the Plants	Family	IC_{50} (μg/mL)/% Inhibition	References
Aegle marmelos	Rutaceae	22.35 mM	Krushna et al. (2017)
Vernonia condensata	Asteraceae	217 μg/mL	Arantes et al. (2016)
Forsythia suspensa	Oleaceae	13.47%–33.64%	Zhang et al. (2016)
Ficus virens	Moraceae	84 ± 2.8 ng/mL	Iqbal et al. (2015)
Polygonum tinctorium	Polygonaceae	77.5 ± 1.9%	Kimura et al. (2015)
Cyclocarya paliurus	Juglandaceae	—	Jiang et al. (2015)
Basella alba	Basellaceae	74%	Baskaran et al. (2015)
Amaranthus viridis	Amaranthaceae	69.6%	Baskaran et al. (2015)
Muraya koengi	Rutaceae	33.7%	Baskaran et al. (2015)
Piper sarmentosum	Piperaceae	55.1%	Baskaran et al. (2015)
Morus indica	Moraceae	63%–67%	Palvai and Urooj (2014)
Polyalthia longifolia	Annonaceae	20.1 and 30.2 mM	Sashidhara et al. (2009)
Acantholopsis orientalis	Asteraceae	27%	Gholamhoseinian et al. (2010)
Alpinia officinarum	Zingiberaceae	22%	Gholamhoseinian et al. (2010)
Levisitum officinale	Apiaceae	48%	Gholamhoseinian et al. (2010)
Originaum majorana	Lamiaceae	35%	Gholamhoseinian et al. (2010)
Lawsonia innermis	Lythaceae	40.1%	Gholamhoseinian et al. (2010)
Piper nigrum	Piperaceae	40%	Gholamhoseinian et al. (2010)
Quercus infectoria	Fagaceae	84%	Gholamhoseinian et al. (2010)
Rosa damascene	Rosaceae	70%	Gholamhoseinian et al. (2010)
Stachys lavaudulifolia	Lamiaceae	42%	Gholamhoseinian et al. (2010)

GI lumen via GLUT2 and SGLT1 transporters. After a meal, the carbohydrates cannot be absorbed through the intestinal lumen as they need to be metabolized to monosaccharide, that is, glucose, by the action of salivary amylase, and then to pancreatic amylase to produce oligosaccharides and disaccharides. These di- and oligosaccharides are further subjected to α-glucosidase enzyme at the brush border of the intestinal luminal wall, to produce glucose, which is absorbed via specific transporters. This digestion of the larger carbohydrates into simple glucose is a potential target for the development of antihyperglycemic agents. The inhibition of α-glucosidase enzyme leads to inhibition of oligosaccharides into simple glucose and thus prevents post prandial hyperglycemia in Type-II diabetic patients with impaired glucose tolerance. The inhibition of α-glucosidase, a group of key intestinal enzymes, prevented the digestion of carbohydrates (such as starch and table sugar) and thus reduced the amount of carbohydrates in blood sugar. They also decreased both postprandial hyperglycemia and hyperinsulinemia, and thereby they may improve sensitivity to insulin and release the stress on β-cells (Bischoff, 1994; Scheen, 2003).

14.7.2 Assay Procedure for α-Glucosidase Inhibition

The spectroscopic method involves the conversion of *p*-nitrophenyl-α-D-glucopyranoside (PNPG as substrate) into *p*-nitrophenol. The reaction mixture is incubated at 37°C for 30 min and is then allowed to generate *p*-nitrophenol on enzyme–substrate reaction. The amount of the *p*-nitrophenol released is measured spectrophotometrically at 408 nm. The % inhibition and IC_{50} value are calculated (Nair et al., 2013) (Fig. 14.7).

14.7.3 Natural Products as α-Glucosidase Inhibitors

α-Glucosidase inhibitors competitively inhibit glycosidase activity and thus prevent the fast breakdown of sugars and thereby control the blood sugar level. Several α-glucosidase inhibitors have been isolated from medicinal plants. The development of α-glucosidase inhibitors isolated from medicinal plants serve as a source of potential antidiabetic agents. Several medicinal plants have been screened for their α-glucosidase inhibitory activity and are described in Table 14.6.

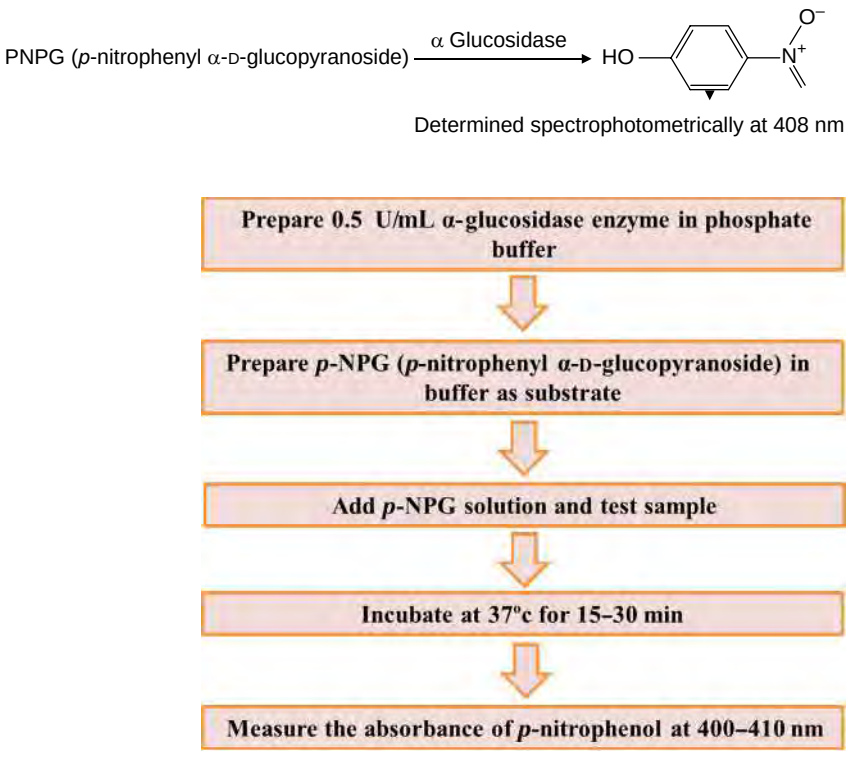

FIG. 14.7 Spectrophotometric assay for α-glucosidase inhibition.

TABLE 14.6 Medicinal Plants Having α-Glucosidase Inhibition Activity

Name of the Plant	Family	IC_{50} Value/% Inhibition	References
Achillea millefollium	Asteraceae	32.07 ± 2.2 μg/mL	Chávez-Silva et al. (2018)
Taraxacum officinale	Asteraceae	39.8–61.2 μM	Choi et al. (2018)
Curcuma zanthorrhiza	Zingiberaceae	39.05–455.01 μg/mL	Nurcholis et al. (2018)
Potentilla astracanica	Rosaceae	1.47 (±0.11) μg/mL	Söhretoglu et al. (2017)
Camellia sinensis	Theaceae	0.09 mg/mL	Shuyuan et al. (2017)
Terminalia chebula	Combretaceae	21.7–64.2 μM	Lee et al. (2017)
Anemarrhena asphodeloides Bunge	Asparagaceae	147.0 ± 11.0 to 466.6 ± 19.2 μg/mL	Nian et al. (2017)
Bidens pilosa	Asteraceae	10.7–74.7 μM	Thien et al. (2017)
Hertia cheirifolia	Compositae	220 μM	Majouli et al. (2017)
Dendrobium formosum	Orchidaceae	26.88 ± 0.66 μM and 69.45 ± 10.14 μM	Inthongkaew et al. (2017)
Hibiscus sabdariffa	Malvaceae	5.21 and 5.2 mmol/L	Shadhan and Bohari (2017)
Phyllanthus niruri	Phyllanthaceae	0.9 ± 0.1 and 1.9 ± 0.02 mM	Beidokhti et al. (2017)
Tribulus terrestris	Zygophyllaceae	6967 ± 343 μg/mL	Ercan and El (2016)
Hypericum scruglii	Hypericaceae	17.25 and 14.05 μg/mL	Mandrone et al. (2017)
Allophylus cominia	Sapindaceae	86 ± 0.3%	Semaan et al. (2017)
Rhynchosia minima	Fabaceae	8.85 mg/mL	Jia et al. (2017)
Nitraria tangutorum	Zygophyllaceae	12.2 ± 1.9 to 30.4 ± 2.7 μg/mL	Zhao et al. (2017)
Centella asiatica	Umbellifers	45.42 and 73.17 μg/mL	Dewi and Maryani (2015)
Andromachia igniaria		0.90–0.08 mM	Saltos et al. (2015)
Rhodiola crenulata	Crassulaceae	3.10 ± 0.09 μM	Chu et al. (2014)
Chrysanthemum morifolium	Asteraceae	229.3 μM	Luyen et al. (2013)

14.8 INHIBITION STUDY FOR RHOKINASE

14.8.1 Importance of Rhokinase

Rhokinase (ROCK), an enzyme related to the serine/threonine kinases family, is an important downstream effector of RhoA. Two isoforms of ROCK are known, ROCK1 (also known as Rho-kinase β) and ROCK2 (Rho-kinase α), the isoforms having different functions. ROCK1 is mainly involved with circulating inflammatory cells and ROCK2 relates to vascular smooth muscle cells. ROCK activity is involved in the expression of PAI-1 mediated by hyperglycemia, indicating that it is a key regulator of cardiovascular injury in patients with diabetes mellitus. Furthermore, the RhoA/ROCK pathway has been reported to be involved in angiogenesis cerebral ischemia, erectile dysfunction (ED) glomerulosclerosis, hypertension, myocardial hypertrophy, myocardial ischemia reperfusion injury, pulmonary hypertension, and vascular remodeling. ROCK inhibitors have shown benefits in Alzheimer's disease, bronchial asthma, cancer, glaucoma, and osteoporosis (Feng et al., 2016).

14.8.2 Assay Procedure for ROCK Inhibition

A ROCK inhibition assay is performed based on the kinase assay method. The kinase reaction is started by the addition of ATP, upon which it phosphorylates the substrate. Subsequently, the detection reagent is added to the reaction mixture. The reagent is linked to the phosphorylated substrate, resulting in a TR-FRET signal, which is proportional to the phosphorylation level (Goswami et al., 2012) (Fig. 14.8).

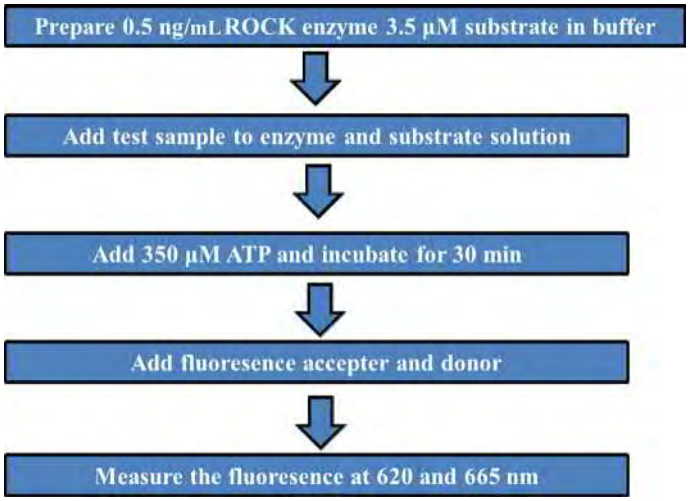

FIG. 14.8 Fluorescence assay for α-glucosidase inhibition.

14.8.3 Natural Products as ROCK Inhibitors

There are several plant extracts capable of inhibiting the ROCK-II enzyme that help in the management of ED. An evaluation of the ROCK inhibition potential of medicinal plants is needed to identify the specific compounds responsible for the inhibition. A large number of medicinal plants have been screened for their ROCK inhibitory activity; they are described in Table 14.7 (Goswami et al., 2012).

TABLE 14.7 Medicinal Plants Having ROCK Inhibitory Activity

Name of the Plant	Family	IC_{50} (% or µg/mL)	References
Albizia lebbeck	Mimosaceae	78.21	Goswami et al. (2012)
Emblica officinalis	Euphorbiacea	77	
Glycyrrhiza glabra	Fabaceae	18.87	
Terminalia chebula	Combretaceae	87.55	
Tribulus terrestris	Zygophyllaceae	20.48	
Tinospora cordifolia	Menispermaceae	26.67	
Pueraria tuberosa	Fabaceae	50.85	
Butea frondosa	Fabaceae	69.3	
Withania somnifera	Solanaceae	25.08	
Cinnamomum cassia	Lauraceae	87.27	
Vitis vinifera	Vitaceae	23.27	
Curculigo orchioides	Hypoxidaceae	73.16	
Syzygium cumini	Myrtaceae	84.01	
Mangifera indica	Anacardiaceae	80.35	
Celastrus paniculatus	Celastraceae	50.36	

14.9 INHIBITION STUDY OF CARBONIC ANHYDRASE

14.9.1 Importance of Carbonic Anhydrase

Carbonic anhydrases are zinc-containing metallo-enzymes found in higher vertebrates, including humans. The major role of the enzyme is the interconversion of carbon dioxide (CO_2) and water to bicarbonate ions and protons (or vice versa). This enzyme family is mainly classified into five distinct subfamilies based on their genetic structure. Among them, α-CA II is one of the most active and cytosolic bound enzymes. It regulates several physiological and pathological processes, including the transportation of CO_2 and bicarbonate ions between metabolizing tissues and lungs, thus maintaining the pH of blood and homeostasis (Supuran, 2008). It also plays a significant role in bone resorption, electrolyte secretion in various tissues and organs, and some other biosynthetic reactions, such as gluconeogenesis, lipogenesis, and ureagenesis. The inhibitory action of different CA isoforms may exhibit several clinical applications, such as diuretics and antiglaucoma, antiobesity, and antiepileptic agents. Sulfonamides, such as acetazolamide, dorzolamide, and brinzolamide, are the most widely used classical carbonic anhydrase inhibitors (mainly CA II mediated). However, they are nonspecific to isoenzymes and associated with several adverse reactions, such as depression, malaise, gastrointestinal irritation, metabolic acidosis, renal calculi, and transient myopia (Supuran, 2010). Several phytomolecules obtained from natural sources, namely, phenols/polyphenols, phenolic acids, and coumarins, have been found to be carbonic anhydrase inhibitors. They can be further explored as novel carbonic anhydrase inhibitors.

14.9.2 Assay Procedure for Carbonic Anhydrase Inhibition

The in vitro esterase activity of carbonic anhydrase activity is estimated by changes in the absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ions over a period of 3 min at 25°C (Verpoorte et al., 1967) (Fig. 14.9). Hydratase activity is assayed based on the time required for a pH change of the nonenzymatic and the enzymatic reactions due to the conversion of CO_2 into bicarbonate in nonenzymatic and enzymatic reactions (Wilbur and Anderson, 1948) (Fig. 14.10).

14.10 INHIBITION STUDY OF HYALURONIDASE

14.10.1 Importance of Hyaluronidase

Hyaluronidases are classes of enzymes that degrade hyaluronic acid (HA), also known as hyaluronan. It is the main component of the extracellular matrix (ECM) with high molecular weight influencing its permeability. The main component of HA is D-glucuronic acid and *N*-acetyl-D-glucosamine, alternately linked by β bonds, which contributes to the viscoelastic properties of the skin (Dunn et al., 2010; Lee et al., 2010; Pierre et al., 2007; Rzany et al., 2009). Hyaluronidase

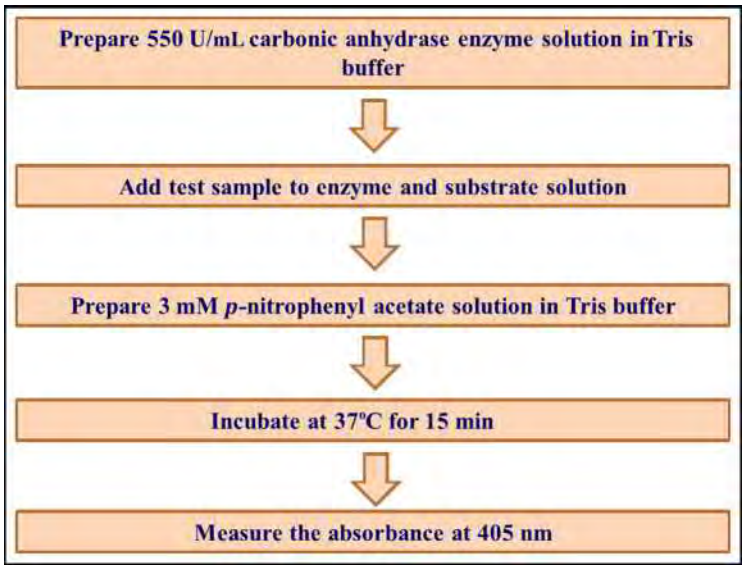

FIG. 14.9 Esterase assay for carbonic anhydrase inhibition.

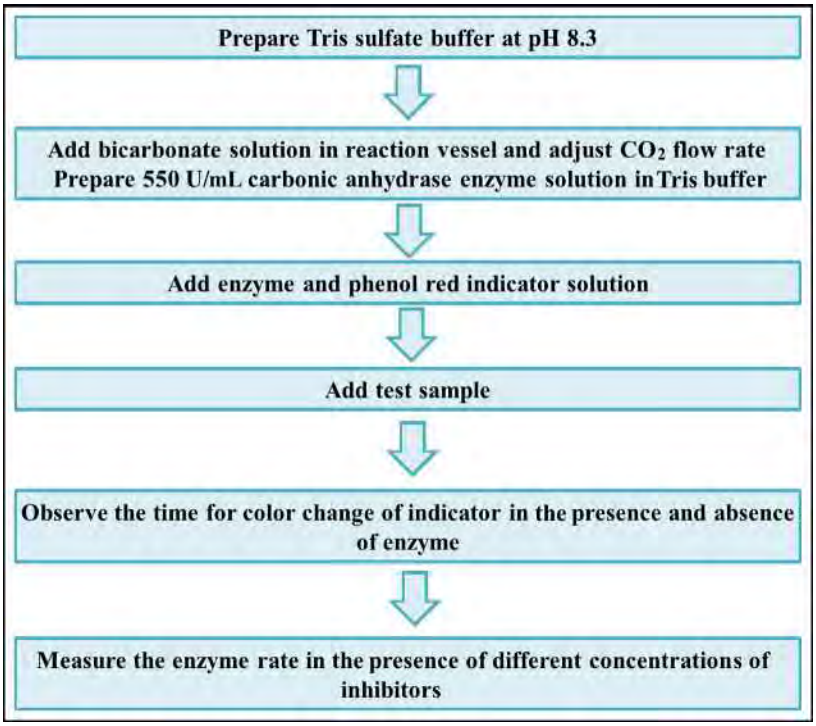

FIG. 14.10 Hydratase assay for carbonic anhydrase inhibition.

degrades HA by cleaving the *N*-acetyl glucosamidic bonds of HA via an elimination process to produce HA oligosaccharides, with chain lengths of 4–25 disaccharides, and which possess angiogenic properties (Guo et al., 1958; Vincent and Lenormand, 2009). The process of HA oligosaccharide formation favors the production of new blood vessels, thus facilitating the development of cancer tumors. The inhibition of HA degradation appears critical and imperative in regulating HA homeostasis, and thus serves as an antiinflammatory, antiaging, antimicrobial, anticancer, antivenom/toxin, and contraceptive agent.

14.10.2 Natural Products as Hyaluronidase Inhibitors

The inhibition of hyaluronidase leads to antiaging, antiwrinkle, antioxidant, antiinflammatory, wound healing, skin whitening, and anticancer activities. There are a number of plant secondary metabolites that have been exploited for their potential antihyaluronidase activity. A list of medicinal plants that have been reported for their hyaluronidase inhibition property is given in Table 14.8.

14.10.3 Assay Procedure for Hyaluronidase Inhibition

Hyaluronidase reacts with the substrate hyaluronic acid to release *N*-acetyl glucosamine. In the presence of any inhibitor, the release of *N*-acetyl glucosamine is reduced and it is monitored by measuring the absorbance at 600 nm. The assay was performed with oleanolic acid as a positive control under exactly the same experimental conditions. An absorbance value of intact undigested hyaluronic acid at 600 nm was considered as 100% (Maity et al., 2011) (Fig. 14.11).

14.11 INHIBITION STUDY OF MATRIX METALLOPROTEINASE (MMP)

14.11.1 Importance of Matrix Metalloproteinase Enzyme

Matrix metalloproteinases (MMPs) are members of the metzincin group of proteases, which share the conserved zinc-binding motif in their catalytic active site. It was originally thought that their main function is to degrade various components of the ECM, yet recent studies have explored their significance as regulators of extracellular tissue signaling networks. Due to the broad spectrum of their substrate specificity, MMPs contribute to the homeostasis of many tissues

TABLE 14.8 Medicinal Plants Having Hyaluronidase Inhibitory Activity

Plant Name	Family	IC_{50} Value or % Inhibition	References
Curucuma aromatica	Zingiberaceae	95.02%	Liyanaarachchi et al. (2018)
Elaeocarpus serratus	Elaeocarpaceae	74.85%	Liyanaarachchi et al. (2018)
Camellia sinensis	Theaceae	34.82%	Liyanaarachchi et al. (2018)
Mesua ferrea	Calophyllaceae	65.64%	Liyanaarachchi et al. (2018)
Azadirachta indica	Meliaceae	70 µg/mL	Kumud and Sanju (2018)
Crocus sativus	Iridaceae	46.7%–64.7%	Muhit et al. (2016)
Pothos scandens	Araceae	18.08 ± 0.46 µg/mL	Maity et al. (2011)
Clitoria ternatea	Fabaceae	19.27 ± 0.37 µg/mL	Nema et al. (2013)
Centella asiatica	Apiaceae	1.6221 mg/mL	Srivastav et al. (2010)
Terminalia chebula	Combretaceae	18.3 ± 5.7 mg/mL	Liu et al. (2015)
Alangium chinense	Cornaceae	8.7 ± 3.1–2.4 ± 0.4 mg/mL	Liu et al. (2015)
Boehmeria nivea	Urticaceae	2.2 ± 0.1 mg/mL	Liu et al. (2015)
Glochidion puberum	Euphorbiaceae	17.0 ± 0.4 mg/mL	Liu et al. (2015)
Hibiscus mutabilis	Malvaceae	27.1 ± 4.4 mg/mL	Liu et al. (2015)
Houttuynia cordata	Saururaceae	34.7 ± 2.6 mg/mL	Liu et al. (2015)
Lespedeza cuneata	Fabaceae	4.8 ± 0.2 mg/mL	Liu et al. (2015)
Melastoma dodecandrum	Melastomataceae	3.0 ± 0.2–43.5 ± 13.3 mg/mL	Liu et al. (2015)
Ocimum basilicum	Lamiaceae	50.1 ± 6.2 mg/mL	Liu et al. (2015)
Oxalis corniculata	Oxalidaceae	1.6 ± 0.1 to 18.6 ± 3.0 mg/mL	Liu et al. (2015)
Phyllanthus urinaria	Phyllanthaceae	14.3 ± 8.1 mg/mL	Liu et al. (2015)
Pinellia ternate	Araceae	1.6 ± 0.6–26.1 ± 2.6 mg/mL	Liu et al. (2015)
Prunella vulgaris	Lamiaceae	44.9 ± 3.4 mg/mL	Liu et al. (2015)

and participate in several physiological processes, such as bone remodeling, angiogenesis, immunity, and wound healing. MMP activity is tightly controlled at the level of transcription and pro-peptide activation by the tissue inhibitors of MMPs. Dysregulated MMP activity leads to pathological conditions, such as arthritis, inflammation, and cancer, thus highlighting MMPs as promising therapeutic targets. Both the hyaluronidase and MMP-1 are the proteolytic enzymes present in dermis, which are responsible for degradation of elastin, hyaluronan, and the collagen fibers network of ECM. It was also reported that repetitive exposure to UV radiation accelerates the synthesis of these enzymes, leading to loss of skin elasticity and decreasing the capacity of the skin to hold water, which are implicated in the formation of the most obvious symptom of aging skin, the wrinkle. Alterations in the ECM are known to cause skin wrinkling, a major characteristic of premature skin aging. Regulation of MMPs is one of the strategies to prevent photodamage to the skin, as their activities contribute to wrinkle formation (Gilchrest, 1990; Hamburger et al., 2003).

14.11.2 Assay Procedure for MMP Inhibition

The assay is performed based on the fluorogenic method using a 96-well fluorescence microplate. When MMP-1 reacts with type-I collagen substrate, it causes collagenolysis, but in the presence of inhibitor, the reaction is slowed down due to the inhibition of MMP-1. MMP-1 activity is measured by hydrolyzing a FRET-tagged substrate to yield a fluorescent product ($l_{ex} = 490/l_{em} = 520$ nm) proportional to the enzymatic activity present (Maity et al., 2011) (Fig. 14.12).

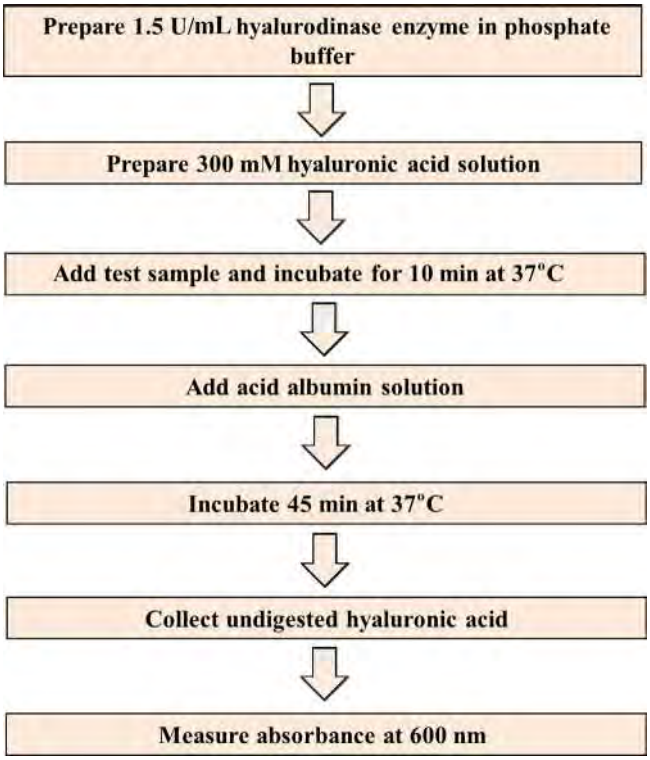

FIG. 14.11 Spectrophotometric assay for hyaluronidase inhibition.

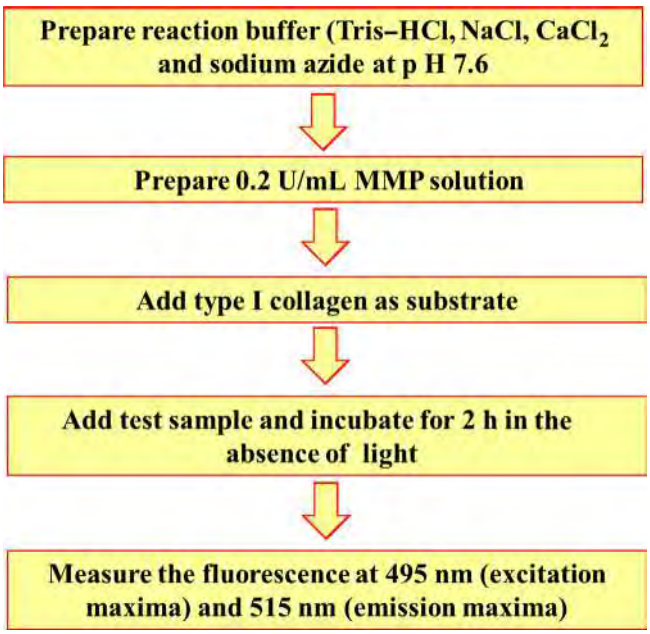

FIG. 14.12 Fluorescence assay for MMP inhibition study.

14.11.3 Natural Products as MMP Inhibitors

MMPs play an important role in several physiological processes, such as the degradation of the ECM, including collagens, elastins, gelatin, and matrix glycoproteins. The use of bioactive molecules from natural sources is very beneficial in combating the harmful effects of MMP-related disorders. Some antioxidants and photoprotective agents include phenolic acids and flavonoids widely present in the human diet that serve as natural MMP inhibitors. A large number of medicinal plants reported to have MMP-inhibitory activity are described in Table 14.9.

TABLE 14.9 Medicinal Plants Having MMP Inhibitory Activity

Name of the Plants	Family	References
Claisiphon novaecaledoniae		Gupta (2016)
Agelas nakamurai	Agelasidae	Gupta (2016)
Clitoria ternatea	Fabaceae	Maity et al. (2012)
Targetes erecta	Asteraceae	Maity et al. (2011)
Terminalia chebula	Combretaceae	Manosroi et al. (2010)
Matricaria recutita	Asteraceae	Kaur and Saraf (2010)
Labisia pumila	Myrsinaceae	Choi et al. (2010)
Calendula officinalis	Asteraceae	Fonseca et al. (2010)
Terminalia chebula	Combretaceae	Manosroi et al. (2010)
Camellia sinensis	Theaceae	Li et al. (2009)
Curculigo orchioides	Hypoxidaceae	Lee et al. (2009)
Camellia sinensis	Theaceae	Li et al. (2009)
Emblica officinalis	Euphorbiaceae	Zaluski and Smolarz (2009)
Curcuma longa	Zingiberaceae	Zaluski and Smolarz (2009)
Curcuma longa	Zingiberaceae	Sumiyoshi and Kimura (2009)
Curcuma xanthorrhiza	Zingiberaceae	Oh et al. (2009)
Berberis aristata	Berberidaceae	Kim et al. (2008)
Camellia japonica	Theaceae	Jung et al. (2007)
Fraxinus chinensis	Oleaceae	Lee et al. (2007)
Melothria heterophylla	Cucurbitaceae	Cho et al. (2006)
Aloe vera	Liliaceae	Barrantes and Guinea (2003)

14.12 INHIBITION STUDY OF TYROSINASE

14.12.1 Importance of Tyrosinase

Tyrosinase, or polyphenol oxidase, is a multifunctional copper-containing enzyme prevalent in plants and animals. It is a key regulatory enzyme that greatly influences the process of melanogenesis within melanocytes. Melanin, a mixture of different biopolymers, determines the color of the skin and hair as well as providing protection from harmful UV radiation. Tyrosinase catalyzes common quinone precursor, which is used further for the synthesis of melanin pigment. Thus, overproduction and accumulation of melanin pigments in the skin leads to the development of dermatological "hyperpigmentation" in clinical conditions, such as solar lentigo, melasma, postinflammatory hyperpigmentation (PIH), and linea nigra. It may also occur due to hormonal imbalance in the body, as in Cushing's disease, Addison's disease, and Nelson's syndrome. Arbutin and kojic acid are known tyrosinase inhibitors commonly used in cosmetic products for skin whitening. Clinically, these depigmenting agents are applied as hyperpigmentation therapy. However, Kojic acid causes dermal sensitization at therapeutic concentration, whereas arbutin has potential cytotoxicity (Burnett et al., 2010; Sarkar et al., 2013; Zhu and Gao, 2008).

14.12.2 Assay Procedure for Tyrosinase Inhibition

The principle of the assay involves conversion of L-tyrosine into L-DOPA in the presence of oxygen and tyrosinase. Then, L-DOPA is converted to L-DOPA-quinone and water in the presence of tyrosinase. The change in absorbance is measured at 280 nm (Biswas et al., 2015) (Fig. 14.13).

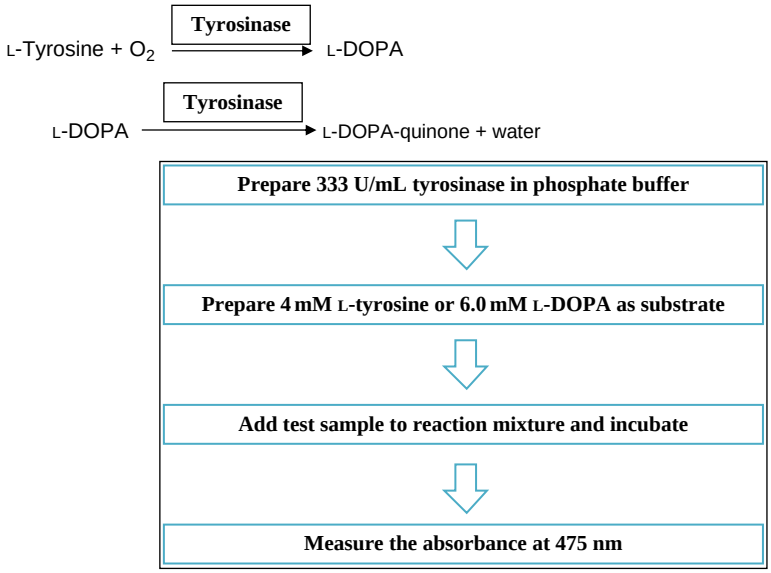

FIG. 14.13 Spectrophotometric study for tyrosinase inhibition assay.

14.12.3 Natural Products as Tyrosinase Inhibitors

Tyrosinase is an oxidoreductase enzyme, having an important role in medicine and cosmetics. The excessive production of melanin causes hyperpigmentation and inhibition of this enzyme often leads to therapeutic benefits against hyperpigmentation and associated disorders. A large number of medicinal plants contain several bioactive components having potential tyrosinase inhibitory activity. Table 14.10 highlights some major plants with tyrosinase inhibition potential.

TABLE 14.10 Medicinal Plants Having Tyrosinase Inhibitory Activity

Plant Name	Family	IC_{50} Value (in μg/mL or % Inhibition)	References
Vetiveria zizanioides	Poaceae	29.13 μg/mL	Liyanaarachchi et al. (2018)
Elaeocarpus serratus	Elaeocarpaceae	20.29 μg/mL	Liyanaarachchi et al. (2018)
Nymphaea nouchali	Nymphaeaceae	30.09 μg/mL	Liyanaarachchi et al. (2018)
Camellia sinensis	Theaceae	11.24 μg/mL	Liyanaarachchi et al. (2018)
Daucus carota	Apiaceae	120.07 ± 0.19 to 410.25 ± 0.41 μg/mL	Jdey et al. (2017)
Frankenia laevis	Frankeniaceae	123.62 ± 1.45 to 730.43 ± 0.75 μg/mL	Jdey et al. (2017)
Berberis aristata	Berberidaceae	50% at 45.45 and 431.11 μg/mL	Biswas et al. (2016)
Rubia cordifolia	Rubiaceae	0.85 ± 0.11 mg/mL	Biswas et al. (2015)
Filipendula ulmaria	Rosaceae	98.30 ± 3.91% at 3 mg/mL	Neagu et al. (2015)
Crataegus pinnatifida	Rosaceae	50%–60% at 500 μg/mL	Huang et al. (2014)
Ficus virens	Moraceae	50% in between 99 and 131 mg/mL	Chen et al. (2014)
Flemingia philippinensis	Fabaceae	50% at 1.28–5.22 μM	Wang et al. (2014)
Hemidesmus indicus	Asclepiadaceae	50% at 0.03 mM	Kundu and Mitra (2014)
Sapium sebiferum	Euphorbiaceae	39.10% ± 3.88% at 2 mg/mL	Fu et al. (2014)
Rhizophora stylosa Griff.	Rhizophoraceae	89.7%	Suh et al. (2014)

Continued

TABLE 14.10 Medicinal Plants Having Tyrosinase Inhibitory Activity—cont'd

Plant Name	Family	IC_{50} Value (in μg/mL or % Inhibition)	References
Bletilla striata	Orchidaceae	50% at 4.3 mg/L	Jiang et al. (2013)
Alhagi camelorum	Fabaceae	63% at 1.14 g/L	Gholamhoseinian and Razmi (2012)
Carthamus tinctorius	Compositae	50% at 1.01 ± 0.03 mg/mL	Chen et al. (2013)
Cudrania tricuspidata		21.54 μM	Zheng et al. (2013)
Sesamum angolense	Pedaliaceae	>7.0 μg/mL (20.3%)	Kamagaju et al. (2013)
Hypericum perforatum	*Hypericaceae*	19.21 ± 1.44% at 250 μg/mL	Altun et al. (2013)
Protea madiensis Oliv.	Proteaceae	>75.0 μg/mL (30.2%)	Kamagaju et al., (2013)
Casta neahenryi	Fagaceae	50% in between 15 and 21 μM	Wu et al. (2012)
Citrus mitis	Rutaceae	0.87 mg/mL	Lou et al. (2012)
Betula pendula	Betulaceae	50% at 119.08 ± 2.04 μg/mL	Germanò et al. (2012)
Eugenia dysenterica	Myrtaceae	90% at 1 mg/mL	Souza et al. (2012)
Eupatorium triplinerve	Asteraceae	50% at 2360 μM	Arung et al. (2012)
Rabdosia serra	Labiatae	21.5 μM	Lin et al. (2011)
Quercus infectoria	Fagaceae	50% at 1.14 g/L	Gholamhoseinian and Razmi (2012)
Pouteria torta	Sapotaceae	100% at 1 mg/mL	Souza et al. (2012)
Lawsonia inermis	Lythraceae	65% at 1.14 g/L	Gholamhoseinian and Razmi (2012)
Gnetum gnemon	Gnetaceae	50% in between 7 and 7.2 μM	Yanagihara et al. (2012)

14.13 INHIBITION STUDY OF DIPEPTIDYL PEPTIDASE-IV

14.13.1 Importance of Dipeptidyl Peptidase-IV

Dipeptidyl peptidase-IV (DPP-IV) (exopeptidase) is serine protease in nature. It is responsible for the cleavage of several peptides, which results in different types of pathophysiological states. The physiologically important peptides cleaved by this enzyme include GLP-1, that is, glucagon-like peptide-1, GIP (glucose induced insulinotropic polypeptide), SDF (stromal cell derived factor), and substance P. GLP-1 is a peptide secreted from the intestinal epithelial cells in response to the glucose challenge, which stimulates insulin secretion from the pancreatic β-cells. DPP-IV enzymes, located at different tissues, including kidneys, the intestinal epithelial brush border, and also freely circulating in the blood, destroy this peptide. Inhibition of this enzyme causes a reduction in the degradation of GLP-1, and thus potentiates the insulinotropic effect of GLP-1. This enzyme has also been proven to be quite useful for the management of Alzheimer's disease, atherosclerosis, and other CVDs.

14.13.2 Assay Methods for DPP-IV Inhibition

14.13.2.1 Fluorogenic Substrate Assay

In this assay, one synthetic tripeptide, Gly-Pro-AMC (contains one glycine and one proline residue) conjugated with a fluorescent compound, AMC (7-amino 4-methyl coumarin), is used. On enzymatic cleavage, AMC splits off and the intensity of the fluorescence is measured using a microplate reader. The excitation and the emission wavelength are set to 380 and 460 nm, respectively (Saleem et al., 2014).

14.13.2.2 Chromogenic Substrate Assay

In this assay, one similar peptide is used in which *p*-NA (*p*-nitro anilide) serves as a chromogenic residue. On enzymatic cleavage, free *p*-NA is generated, which is determined spectrophotometrically at a λ_{max} of 400–410 nm (Kim et al., 2016).

14.13.2.3 DPP Glo Protease Assay

This assay is based on an enzyme-coupled reaction. Here Gly-Pro-Amino Luciferin acts as a substrate, which converts into Luciferin by reacting with DPP-IV. Further, in the presence of luciferase, it gives a fluorescent product, which is detected spectrofluorimetrically at a particular wavelength (Fan et al., 2013).

14.13.3 Natural Products as DPP-IV Inhibitors

Natural extracts play an important role in the treatment of diabetes mellitus for their excellent safety profile. Some phytomolecules that are DPP-IV inhibitors are potential candidates for the treatment of type 2 diabetes mellitus. A list of medicinal plants with potential DPP-IV inhibitory activity are described in Table 14.11.

TABLE 14.11 Medicinal Plants Having DPP-IV Inhibitory Activity

Name of the Plant	Family	IC_{50} Value or % Inhibition	References
Laurencia natalensis	Rhodomelaceae	—	Rengasamy et al. (2017)
Physalis angulata	Solanaceae	13.94 ± 4.08	Riyanti et al. (2016)
Andrographis paniculata	Acanthaceae	37.03 ± 0.65	Riyanti et al. (2016)
Swietenia mahagoni	Meliaceae	38.88 ± 22.25	Riyanti et al. (2016)
Merremia mammosa	Convolvulaceae	17.12 ± 1.95	Riyanti et al. (2016)
Azadirachta indica	Meliaceae	17.78 ± 1.02	Riyanti et al. (2016)
Pterocarpus indicus	Fabaceae	25.0 ± 27.16	Riyanti et al. (2016)
Artocarpus heterophyllus	Moraceae	30.55 ± 4.90	Riyanti et al. (2016)
Ruellia tuberosa	Acanthaceae	30.09 ± 1.30	Riyanti et al. (2016)
Euphorbia cotinifolia	Euphorbiaceae	23.61 ± 3.27	Riyanti et al. (2016)
Lagerstroemia loudonii	Lythraceae	60.22 ± 2.01	Riyanti et al. (2016)
Smallanthus sonchifolius	Asteraceae	52.84 ± 2.01	Riyanti et al. (2016)
Trigonella foenum-graecum	Fabaceae	71.29 ± 0.33	Riyanti et al. (2016)
Ficus religiosa	Moraceae	68.98 ± 1.95	Riyanti et al. (2016)
Palmaris palmate	Palmariaceae	1.65 ± 0.12 mg/mL	Sila et al. (2016)
Urena lobata	Malvaceae	1654.64–6489.88 mg/mL	Purnomo et al. (2015)
Fagonia cretica	Zygophyllaceae	38.1 μg/mL	Saleem et al. (2014)
Hedera nepalensis	Araliaceae	17.2 μg/mL	Saleem et al. (2014)
Momordica charantia	Cucurbitaceae	53.25 ± 0.04% at 0.5 mg/mL	Singh et al. (2014)
Ocimum sanctum	Lamiaceae	66.81 ± 0.05% at 0.5 mg/mL	Singh et al. (2014)
Desmodium gangeticum	Fabaceae	255.5 μg/mL	Bisht et al. (2014)
Pterocarpus marsupium	Fabaceae	273.73 ± 12 μg/mL	Kosaraju et al. (2014)
Eugenia jambolana	Myrtaceae	278.94 ± 16 μg/mL	Kosaraju et al. (2014)
Gymnema sylvestre	Apocynaceae	773.22 ± 19 μg/mL	Kosaraju et al. (2014)
Castanospermum austral	Fabaceae	13.96 μg/mL	Bharti et al. (2012)
Berberis aristata	Berberidaceae	14.46 μg/mL	Chakrabarti et al. (2011)
Mangifera indica extract	Anacardiaceae	182.7 μg/mL	Yogisha and Raveesha (2010)

14.14 INHIBITION STUDY OF α-AMYLASE

14.14.1 Importance of α-Amylase

α-Amylase is an enzyme that hydrolyzes the α-bonds of large, α-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose. It belongs to the GH13 family of glycoside hydrolases. They are endohydrolases in nature and cleave the complex polysaccharides, such as starch, at the internal α-1,4 linkages between the amylose and amylopectin of the starch molecule, whereas the α-1,6 linkage remains unaffected. It is the major form of amylase found in human and other mammals. It is also present in seeds containing starch as a food reserve, and is secreted by many fungi. α-Amylase is a carbohydrate-metabolizing enzyme present in the human gastrointestinal tract, which is released from the pancreas. It is also released in the saliva, referred to as the salivary amylase or ptyalin. The structure of pancreatic α-amylase is 97% homologous to the salivary α-amylase (Van Beers et al., 1995). The inhibition of α-amylase can be an important strategy to lower postprandial blood glucose levels. Inhibitors of these enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a marked decrease in the rate of glucose absorption, thereby blunting the postprandial plasma glucose rise (Bhutkar and Bhise, 2012).

14.14.2 Assay Procedure for α-Amylase Inhibition

14.14.2.1 DNS (3,5-Dinitro Salicylic Acid) Method

This is a chromogenic substrate assay, based on the formation of 3-amino 5-nitro salicylic acid (orange-red colored) on reacting with maltose, liberated due to enzymatic cleavage of starch, and 3,5-dinitro salicylic acid (Kazeem et al., 2013) (Fig. 14.14).

14.14.2.2 The Fuwa Method (Starch–Iodine Method):

In this method, the reaction between starch and iodine to produce a blue-colored inclusion complex is quantitatively estimated using spectrophotometry. The residual amount of starch after enzymatic cleavage is reacted with a solution of 5 mM iodine and 5 mM potassium Iodide in water. The resulting Inclusion complex, with absorption maximum at 580 nm, is read in a microplate reader at that wavelength (Xiao et al., 2006) (Fig. 14.15).

14.14.3 Natural Products as Amylase Inhibitors

There are several medicinal plants reported in traditional medicinal systems to treat diabetes that are considered to be a valuable source of antidiabetic agents. α-Amylase inhibitors lower the levels of postprandial hyperglycemia via control of starch breakdown and are found to be very useful in treating diabetes. The phytoconstituents present in medicinal plants are widely reported for their α-amylase inhibition property. Table 14.12 highlights some medicinal plants with potential α-amylase inhibition properties.

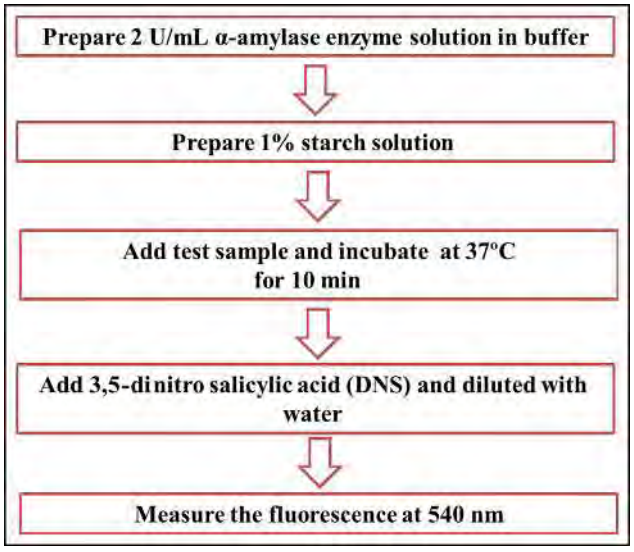

FIG. 14.14 DNS (3,5-dinitro salicylic acid) method for α-amylase inhibition assay.

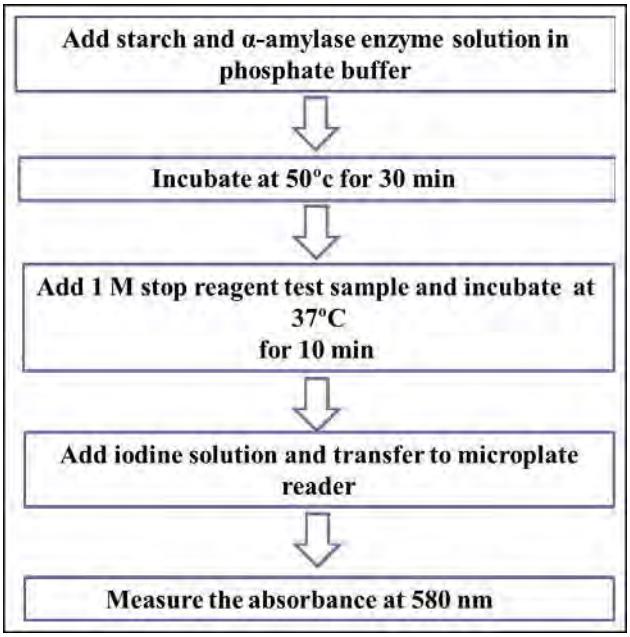

FIG. 14.15 Fuwa method for α-amylase inhibition assay.

TABLE 14.12 Medicinal Plants Having Amylase Inhibitory Activity

Name of the Plant	Family	IC_{50} Value % Inhibition Obtained	References
Momordica dioica	Cucurbitaceae	48 µg/mL	Rao and Mohan (2017)
Nepenthes mirabilis	Nepenthaceae	73.67 ± 10.2 µg/mL	Trinh et al. (2016)
Kandelia candel	Mangroves	7.67 ± 0.9	Trinh et al. (2016)
Ficus racemosa	Moraceae	46.77 ± 23.6 µg/mL	Trinh et al. (2016)
Pterocarpus marsupium	Fabaceae	5.16 µg/mL	Shori (2015)
Asystasia gangetica	Acanthaceae	3.75 µg/mL	Shori (2015)
Eleusine coracana	Poaceae	23.5 µg/mL	Shori (2015)
Urtica dioica	Urticaceae	1.38 ± 0.42 mg/mL	Rahimzadeh et al. (2014)
Juglans regia	Juglandaceae	0.32 ± 0.07 mg/mL	Rahimzadeh et al. (2014)
Tamarindus indica	Fabaceae	At 9 mg/mL 69.72 ± 0.18	Bhutkar and Bhise (2012)
Caesalpinia bonducella	Caesalpiniaceae	At 9 mg/mL 87.26 ± 0.13	Bhutkar and Bhise (2012)
Santalum spicatum	Santalaceae	5.43 µg/mL	Gulati et al. (2012)
Morus alba	Moraceae	1440 µg/mL	Sudha et al. (2011)
Ocimum tenuiflorum	Lamiaceae	8.9 µg/mL	Sudha et al. (2011)
Mangifera indica	Anacardiaceae	37.86 ± 0.32 µg/mL	Dineshkumar et al. (2010)
Murraya koenigii	Rutaceae	59.0 ± 0.51 µg/mL	
Phyllanthus amarus	Phyllanthaceae	36.05–48.92 µg/mL	Tamil et al. (2010)
Ascophyllum nodosum	Fucaceae	44.7 µg/mL	Apostolidis and Lee (2010)
Eleusine coracana	Poaceae	23.5 µg/mL	Shobana et al. (2009)
Andrographis paniculata	Acanthaceae	11.3 ± 0.29 mg/mL	Subramanian et al. (2008)

It can be concluded that the enzyme assay is one of the major in vitro tools for discovering potential therapeutic leads from natural sources. The enzyme inhibitor exerts its action by reducing the enzyme activity, which is linked with the pathophysiology of some diseases. These inhibitor molecules offer different binding characteristics based on their structure and their site of action. To understand the inhibitor characteristics, it is very important to explore the structural knowhow of the enzyme as well as the ligand. The enzyme-based method is able to screen a large number of naturally occurring chemicals in the drug discovery and development process. As plant-based systems continue to play an essential role in healthcare, so the development of rapid, sensitive, in vitro based methods remains a challenge in the initial stage of drug discovery. More focus should be given to the study of the enzyme inhibition mechanism to understand the various modes of interaction and to lead optimization and structure–activity relationships and drug-target residence times of enzyme inhibitors as potential therapeutic agents from medicinal plants/herbal medicine.

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Chapter 15

Evaluation of Herbal Drugs for Antimicrobial and Parasiticidal Effects

Chapter Outline

15.1 HERBS AS ANTIMICROBIALS

Since ancient times, medicinal plants are being used for the treatment of human infections. Interest in drugs from botanicals is growing day by day due to their efficiency, low toxicity, and fewer side effects. The prevalent use of commercial antibiotics for human and synthetic pesticides for crop protection is harmful to human wellbeing, the ecosystem, and the environment. Attention has also been drawn to the antimicrobial properties of plants and their metabolites due to the growing frequency of drug-resistant pathogens of both clinical and agricultural importance. Medicinal plants have the inherent ability to resist pathogenic microorganisms and this has led researchers to investigate their mechanisms of action and how to isolate the active metabolites. This has facilitated exploitation of medicinal plants for the treatment of microbial infections in both plants and human by developing new antimicrobials (Rojas et al., 2006). Numerous encouraging leads have been found with the convergence of empirical uses of different species throughout the globe having antimicrobial potential. The antimicrobial activity of plants can be detected by observing the growth response of various microorganisms. Many methods for detecting the antimicrobial potential of medicinal plants from traditional medicine are available, but because they are not equally sensitive or even based on the same principle, the results obtained will also be influenced by the method selected and the microorganisms used for the test.

It is clear that biological evaluation in general can be carried out much more efficiently on water-soluble, crystalline substances than on mixtures, such as plant extracts. The problems inherent to the antimicrobial screening of plant extracts

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have been surveyed by several authors (Janssen et al., 1987; Rios et al., 1988). In order to evaluate the antimicrobial activity of plant extracts, the following three conditions must be fulfilled.

- The plant extract must be brought into contact with the cell wall of the microorganisms that have been selected for the test.
- The conditions must be adjusted so that the microorganisms are able to grow when no antimicrobial agents are present.
- There must be some means of judging the amount of growth, if any, made by the test organism during the period of time chosen for the test (Skinner, 1955).

The currently available methods for antimicrobial screening fall into three groups, namely, diffusion, dilution, and bioautographic methods. These methods are influenced by several factors, such as the extraction method, volume of inocula, culture medium composition, pH, and incubation temperature. At this point it should be stressed that all available testing methods will only give an idea of the presence or absence of substances with antimicrobial activity in the extract. The potency of the active ingredients can only be determined in pure compounds using a standardized methodology (Mukherjee, 2002).

15.2 METHODS FOR ANTIMICROBIAL EVALUATION

The commonly used methods can be classified by whether or not they require sterile samples. Sterilization by membrane filtration is excluded for aqueous dispersions or emulsions and can result in the loss of antimicrobial activity from aqueous solutions as already mentioned. Sterilization by gamma irradiation is a very effective, inexpensive, but rather time-consuming method. A good alternative is to prepare all samples in an aseptic way (sterilized tubes and flasks, laminar flow, etc.) and to use aqueous ethanol as extraction solvent for the plant material. Because one is never sure of having eliminated all microbes, the methods that require sterile samples (e.g., the liquid [micro broth] dilution method) should not be carried out on plant extracts that have not been sterilized by filtration or irradiation. Among the methods to be considered as valuable for the testing of plant extracts that cannot be sterilized by filtration are hole-plate diffusion and the agar dilution techniques (Mukherjee, 2002). Methods used for the evaluation of antimicrobials are presented in Fig. 15.1.

15.2.1 Diffusion Method

In the diffusion technique, a plant extract to be tested is brought into contact with an inoculated medium (e.g., agar) and, after incubation, the diameter of the zone around the applied extract (inhibition diameter) is measured. In order to lower the detection limit, the inoculated system is kept at a low temperature for several hours before incubation, which favors diffusion over microbial growth and thus increases the inhibition diameter. This method was originally designed to monitor the amounts of antibiotic substances in fermentation cultures and has also been used for obtaining biograms (Janssen et al., 1986) and for the testing of essential oils (Onawunmi et al., 1984). The theory for this technique has been described in detail (Linton, 1983). It is not necessary to sterilize the test samples used in this study.

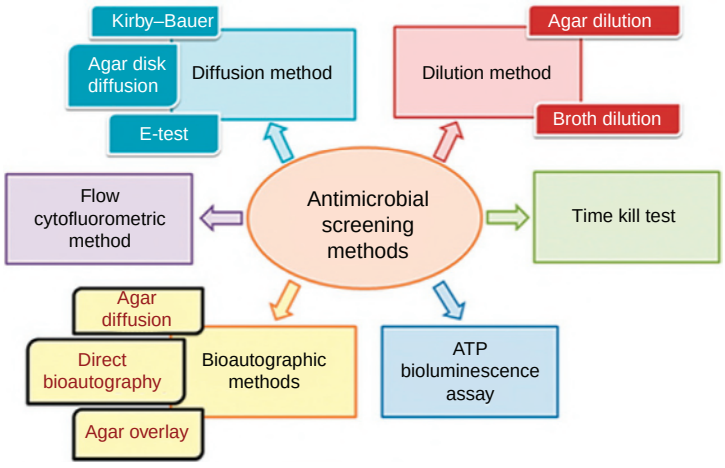


FIG. 15.1 Antimicrobial screening methods.

15.2.1.1 Agar Disk-Diffusion Method

Agar disk-diffusion is a routine method for antimicrobial testing, developed quite some time ago (Heatley, 1944). This is an approved, standard method revealed by the Clinical and Laboratory Standards Institute (CLSI) for the testing of microorganism and yeasts. Though not all fastidious microorganism will be tested accurately by this technique, the standardization has been created to check microorganisms, such as streptococci, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis*, using specific culture media, varied incubation conditions, and interpretative criteria for the zone of inhibition (CLSI, 2006, 2012). During this well-known procedure, agar plates are inoculated with a homogenous inoculant of the test organism. Then, paper disks (about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated. Generally, an antimicrobial agent diffuses into the agar and inhibits the expansion of the test organism, which is measured by determining the diameters of the zone of inhibition (Balouiri et al., 2016).

15.2.1.2 Kirby–Bauer Method

The hole-plate or Kirby–Bauer method is the only suitable diffusion technique for testing aqueous suspensions of plant extracts. In this method, the presence of suspended particulate matter in the sample is tested, which interferes with the diffusion of the antimicrobial substance into the agar. The precipitation of water-insoluble substances in the cylinder or the disk will indeed prevent any diffusion of antimicrobial substances into the agar. Nevertheless, in order to limit precipitation as much as possible in the hole-plate method, preincubation should be carried out at room temperature (25°C) rather than at 4°C. The advantages of the diffusion methods are the small size of the sample used in the screening and the possibility of testing five or six compounds per plate against a single microorganism.

In most studies, inhibition zones are compared with those obtained for antibiotics (Leven et al., 1979). This is useful in establishing the sensitivity of the test organism, but a comparison of the antimicrobial potency of the samples and antibiotics cannot be drawn from this because a large inhibition zone may be caused by a highly active substance present in quite a small amount or by a substance of comparatively low activity but present in a high concentration in the plant extract. On the other hand, the filter paper disk method is a satisfactory and acceptable method for assaying water-soluble antibiotics. The results of a linear diffusion test may indeed be as accurate as those of a $\log_2$ dilution test. The zone diameter of inhibition is indeed inversely related to the minimum inhibitory concentration (MIC), whereas an appropriate MIC can be extrapolated from the zone diameter (Washington, 1988).

15.2.1.3 Antimicrobial Gradient Method (E-Test)

The antimicrobial gradient methodology combines the principle of dilution and diffusion so as to find the MIC value. This method is supported by the possibility of making a concentration gradient of the antimicrobial agent, which is tested in an agar medium. In this methodology, a strip saturated with an increasing concentration gradient of the antimicrobial agent from one end to the other is deposited on the agar surface, which was previously inoculated with the tested microorganism. This methodology is employed for the MIC determination of antibiotics, antifungals, and antimycobacterials. The value of MIC is decided at the intersection of the strip (Baker et al., 1991; Balouiri et al., 2016). This technique can also be performed to investigate the antimicrobial interaction between two drugs for a synergistic interaction study (Hausdorfer et al., 1998; Nachnani et al., 1992).

15.2.2 Dilution Method

In the dilution methods, samples being tested are mixed with a suitable medium that has previously been inoculated with the test organism. After incubation, growth of the microorganism may be determined by direct visual or turbidimetric comparison of the test culture with a control culture that did not receive the sample being tested, or by plating out both test and control cultures. Usually, a series of dilutions of the original sample in the culture medium is made and then inoculated with the test organism. After incubation, the end point of the test is taken as the highest dilution that will just prevent perceptible growth of the test organism (MIC value). These methods are the best for assaying water-soluble or lipophilic pure compounds and to determine their MIC values, and can be carried out in liquid as well as solid media. Also, growth curves of a microorganism can be recorded using this method (Pfaller et al., 2004).

15.2.2.1 Broth Dilution Method

Broth micro- or macrodilution is one of the basic antimicrobial screening methods, used for twofold dilution of the antimicrobial agent. This is estimated in a liquid growth medium, distributed in tubes containing a minimum volume of 2 mL (macrodilution) or in smaller volumes by using a 96-well microtiter plate (microdilution). Then, every tube or well is

inoculated with a microorganism prepared in the same medium after dilution of a standardized microorganism suspension adjusted to 0.5 McFarland scale. After mixing, the inoculated tubes or the 96-well microtitration plate are incubated in appropriate conditions as per the requirements of the test microorganism. The MIC is the lowest concentration of antimicrobial agent that inhibits the growth of the microorganism in tubes or microdilution wells, as detected by the unaided eye (CLSI, 2012). The disadvantages of the macrodilution methodology include the tedious, manual nature of the work, the risk of errors in the preparation of antimicrobial solutions for every test, and the relatively large amount of reagents and space needed, which is not the case for microdilution technology (Balouiri et al., 2016). It is known that the substance size (Gehrt et al., 1995), the type of growth medium (Meletiadis et al., 2001), the incubation time, and the inoculum preparation method can influence MIC values (Rodriguez-Tudela et al., 2003). Therefore, broth dilution has been standardized by CLSI for testing bacteria that grow aerobically (CLSI, 2012), for yeast (CLSI, 2002), and for filamentous fungi (CLSI, 2008).

15.2.2.2 Agar Dilution Method

Only the solid or agar dilution method is suitable for testing nonsterile plant extracts because aerobic organisms do not develop well under the solidified agar. The occasional contaminating culture, which develops on the surface of the agar, is no problem as it can be easily recognized. Moreover, nonpolar extracts, essential oils, suspensions of solids or emulsions, and antimicrobial substances, which do not diffuse through agar media, can be tested directly by incorporating them with the agar media as if they were aqueous solutions. Unlike the diffusion methods, no concentration gradient occurs during the testing procedure. In contrast with the dilution in a liquid medium, however, emulsions of, for example, an essential oil in the medium, may break down during the solid dilution assay (Rios et al., 1988).

Several different test microorganisms may be tested simultaneously on the same dilution (diffusion methods in which several substances or dilutions of one substance may be tested simultaneously against one test microorganism), which makes the agar dilution method very quick and time saving. Considering all of the properties mentioned, the agar dilution method seems to be the most convenient method for routine testing of complex samples, such as plant extracts. The method is also very useful to guide the isolation of antimicrobially active components from plant extracts (Mitscher et al., 1987; Rwanjabo et al., 1988).

This method is very much appreciated for the purpose of assaying pure samples, particularly when a high degree of sensitivity is required, but considered by many researchers to be less suitable than the diffusion methods for qualitative work, for example, the rapid screening of a large number of plant extracts. It is, however, the only method to determine whether an antimicrobial agent is bactericidal or only bacteriostatic to the test organism at various concentrations. The minimum bactericidal concentration can be determined by plating out samples of completely inhibited dilution cultures onto solid or liquid media containing no antibiotic. When the germ does not grow, the sample is bactericidal. All methods described for bacteria are equally suitable for yeasts and for certain other unicellular organisms, but are less so for filamentous mold fungi (Mukherjee, 2002).

15.2.3 Bioautographic Methods

Bioautography, as a method to localize antibacterial activity on a chromatogram, has found widespread application in the search for new antibiotics from microorganisms. Most published procedures are based on the agar diffusion technique, whereby the antimicrobial agent is transferred from the thin layer or paper chromatogram to an inoculated agar plate through a diffusion process. Zones of inhibition are then visualized by appropriate vital stains (Betina, 1973). The problems due to the differential diffusion of compounds from the chromatogram to the agar plate are simplified by direct bioautographic detection on the chromatographic layer (Hamburger and Cordell, 1987). This method, however, requires more complex microbiological equipment and is, in contrast to the contact bioautographic methodology, easily affected by possible contamination from airborne bacteria (Rios et al., 1988).

Although bioautographic methods are suitable for testing highly active antibiotics (MIC values of at least $10\,\mu g\,mL^{-1}$), they did not prove very promising for testing plant extracts, which often contain much less potent antimicrobial agents than the currently available antibiotics.

15.2.3.1 Agar Diffusion

This is also known as the agar contact technique. It is one of the useful techniques available for screening of antimicrobial agents. The testing material containing the antimicrobial agent is transferred by diffusion to an agar plate previously inoculated with the test microorganism. Diffusion is permitted to occur, the recording is then removed, and the agar plate is incubated at 37°C. The result is interpreted by measuring the zone of inhibition (Marston, 2011; Balouiri et al., 2016).

15.2.3.2 Direct Bioautography

In this method, the developed TLC plate is dipped into or sprayed with an inoculum. Then, the bioautogram is incubated at 25°C for 48 h in humid conditions. For determination of the microbial growth, tetrazolium salts are often used (Dewanjee et al., 2015). These salts are converted to corresponding intensely colored formazan crystal by the dehydrogenases of living cells. These salts are sprayed onto the bioautogram, which is reincubated at 25°C for 24 h and at 37°C for 3–4 h (Balouiri et al., 2016). The Mueller Hinton Broth supplemented with agar is suggested to provide a medium with enough fluid to permit the best adherence to the TLC plate and maintain a humidity that is ideal for bacterial growth (Shahat et al., 2008).

15.2.3.3 Agar Overlay Bioassay

This assay method has been developed combining all of the techniques described earlier. This is also known as the immersion bioautography technique. The TLC plate is covered with molten seeded agar medium. The plates are kept at a low temperature for a few hours before incubation so that the tested compounds are diffused properly into the agar medium. Staining with tetrazolium dye is performed under appropriate conditions for individual test organisms (Balouiri et al., 2016). Like direct bioautography, this technique can be applied to any or all microorganisms and provides well-defined inhibition zones and is not sensitive to contamination. It is the simplest technique for the detection of antifungal substances, and conjointly provides consistent results for spore-producing fungi, such as the genus *Aspergillus*, genus *Penicillium*, and *Cladosporium* (Suleiman et al., 2010).

15.2.4 Time-Kill Test

This is an acceptable technique for the determination of the bactericidal or fungicidal effect. It is a powerful tool for gathering information on the dynamic interaction between the antimicrobial agent and the microbial strain. The time-kill test reveals a time-dependent or a concentration-dependent antimicrobial efficacy. For bacteria, this test is well standardized and delineated in the M26-A document of the CLSI (CLSI, 1998). It is performed in a broth culture medium by the utilization of three tubes containing a bacterial suspension (5×10^5 CFU/mL). The first two tubes contain the molecule or the extract tested at final concentrations of 0.25 MIC and 1 MIC, and the third tube is taken as a growth control (Balouiri et al., 2016). The incubation is completed under appropriate conditions at different time intervals (0, 4, 6, 8, 10, 12, and 24 h) (Konaté et al., 2012). The proportion of dead cells is calculated in comparison with the number of living cells (CFU/mL) of every tube by the agar plate count technique. Generally, this technique is used to determine the synergism or antagonism between drugs (two or more) in mixtures (Pfaller et al., 2004).

15.2.5 ATP Bioluminescence Assay

The ATP bioluminescence assay is based on a measurement of the ATP generated by bacteria or fungi. D-Luciferin, in the presence of ATP, undergoes conversion by luciferase to oxyluciferin, which produces light. The amount of emitted light is measured by a luminometer and expressed as a relative light unit (RLU/mol of ATP) (Balouiri et al., 2016). Thus, there is a linear relationship between cell viability and the measured luminescence. The bioluminescence assay has a wide array of applications, such as cytotoxicity tests (Crouch et al., 1993), in situ evaluation of the impact of biofilms, and drug screening on *Leishmania* (Andreu et al., 2012; Balouiri et al., 2016).

15.2.6 Flow Cytofluorometric Technique

The evaluation of the quality of flow cytometry for testing the susceptibility of microorganisms has been utilized in this screening technique. Several scientists have investigated the antibacterial and antifungal activities of many drugs with it. The speedy detection of damaged cells by this approach depends on the use of an appropriate staining method. Propidium iodide (PI), a fluorescent and intercalating agent, is mainly used to stain DNA. Many studies have reported on the effectiveness of flow cytometry as a tool for antimicrobial testing of essential oils against *Listeria monocytogenes*, using combined staining with PI for membrane damage analysis and carboxy-fluorescein diacetate for esterase activity detection. In addition to the lysed cells, three subpopulations (dead, viable, and treated cells) are clearly discriminated by this technique (Balouiri et al., 2016). The treated cells are described as stressed cells and exhibit damage to their cellular parts and further impairment of their growth. Quantification of treated cells has a remarkable application. Indeed, the flow cytofluorometric technique permits the detection of antimicrobial resistance and estimates the impact of the molecule tested on the viability and cell damage of the microorganisms tested (Balouiri et al., 2016).

15.3 ANTIBACTERIAL SCREENING METHOD

The antimicrobial activity of any test drug of natural origin is assayed separately using the agar diffusion method, employing 24 h culture to test bacterial and fungi strains.

For antibacterial screening, bacterial strains usually used are *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus megatorium*, *Pseudomonas cepacia*, *Staphylococcus aureus*, and *Escherichia coli*. The test organisms are seeded into sterile nutrient agar media for bacterial screening. Then, 1 µL of inoculum is mixed uniformly with 20 µL of sterile melted nutrient agar and cooled to 48–50°C in a sterile petri dish. When the agar solidifies, holes of uniform diameter are made using a sterile borer. A quantity of 0.3 µL of each of the test solutions containing different extract solutions at varying concentrations, as well as the standard drug solutions (e.g., tetracycline or other) and DMSO (control-blank), are placed in each hole separately under aseptic conditions. The plates are then maintained at room temperature for 2 h, allowing for diffusion of the solution into the specific medium. All the plates are then incubated at 25°C for 1 week and the zone of inhibition is measured (Mukherjee et al., 1995a, b, 1996, 1997, 1999).

Usually, for the screening of the antibacterial activity of different plant extracts of varying polarity, such as petroleum ether, acetone, chloroform, and methanol, extracts of plant parts are screened for their activity by the method above. After preliminary screening, the effective extracts are further investigated for their activity at different concentrations and the optimum activity of the specific extract is measured. By using this model, several plant species, such as *Nelumbo nucifera* rhizome, leaf extract of *Leucas lavandulaefolia*, *Drymaria cordata*, *Cryptostegia grandiflora*, *Moringa oleifera*, and several species of the genus *Hypericum—H. hookerianum*, *H. patulum*, and *H. mysorense*, have been shown to produce antimicrobial constituents (Mukherjee et al., 1995a, 1997, 1999, 2000, 2001; Saha et al., 1997).

The nature of the extracts of all the above species with reported potent antibacterial potential suggests the use of the plant extracts as a source of active antimicrobial principles against infection caused by susceptible organisms. The occurrence of infrequent variation in concentrations within species and related organisms suggests that the resistance to individual extracts, when it occurs, is due to the intrinsic properties of the species involved rather than acquired characters. For this reason, it would be more effective and much more useful if the plant extract or its active constituents could be exploited in the development of antimicrobial chemotherapeutic agents. This can be considered in the light of the current search for such substances to augment or replace the antibiotics in current clinical use, which, because of the spread of resistance, are less useful than before.

15.4 HERBS WITH ANTIMICROBIAL POTENTIALS

Natural resources have been underexploited in the search for lead antimicrobial metabolites, although there are numerous reports of plants being used as antimicrobial agents in traditional Ayurvedic medicine, Chinese medicine, and even in Western herbal medicine, as plants are capable of acting against bacteria and fungi in their environment because of their strategy of self-protection. To discover the lead compounds responsible for antiinfective activities, there are a large number of research studies going on worldwide on traditional medicinal plants. This research area has drawn more attention from both academic institutes and the pharmaceutical industry in recent years because of the development of resistance to existing antibiotics.

Research on *Hypericum acmosepalum* revealed antibacterial activity against *S. aureus* and *Mycobacterium tuberculosis*. The essential oil constituents present in cinnamon (*Cinnamomum zeylanicum*), and clove (*Syzygium aromaticum*), have been reported to inhibit the growth of molds, yeasts, and bacteria. Both cinnamon oil and clove oil added at 2% in potato dextrose agar were reported to completely inhibit the growth of mycotoxigenic molds, including *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Penicillium* sp. M46, *Penicillium roqueforti*, *Penicillium patulum*, and *Penicillium citrinum*. Both oils have also been documented for the inhibition of many microorganisms, including *Lactobacillus* sp., *Bacillus thermoacidurans*, *Salmonella* sp., *Corynebacterium michiganense*, *Pseudomonas striafaciens*, *Clostridium botulinum*, *Alternaria* sp., *Aspergillus* sp., *Canninghamella* sp., *Fusarium* sp., *Mucor* sp., and *Penicillium* sp. The antibacterial activity of sage, *Salvia officinalis*, has *been reported against S. aureus*, *E. coli*, and *Salmonella typhimurium*. The antibacterial activity of these essential oils in thyme, *Thymus vulgaris*, was reported against *S. aureus*, *E. coli*, and *S. typhimurium*. The essential oils of *Origanum vulgare*, have been reported to exhibit antibacterial activities against *E. coli*, *S. aureus*, and *S. typhimurium*. The Brazilian plant, *Styrax ferrugineus*, has potential activity against *S. aureus*. *Uraria picta* Desv., a suffruticose sparingly branched perennial herb, has been traditionally used as an antidote to the venom of a dangerous Indian snake, *Echis carinata*, having antimicrobial activity against bacteria and fungi. The stem bark of *Flemingia paniculata* revealed significant activities in test organisms against bacteria (both Gram-positive and Gram-negative) and fungi. *Polygonum viscosum* reveals potential inhibition of the growth of drug-resistant *E. coli* and MRSA. *Clausena heptaphylla* (Fam. Rutaceae) was reported to show strong antibacterial activity against *S. aureus*, *E. coli*, and *P. aeruginosa*. Several, medicinal plants having antimicrobial potential are shown in Table 15.1.

TABLE 15.1 Several Medicinal Plants Having Antimicrobial Potential

Plant Name	Compound/Part Used	Activity	Reference
Abrus precatorius L.	St: ethanol (95%) extract	*M. tuberculosis*	Antoun et al. (2001)
Achyranthes aspera L.	Wp: methanol extract	*M. tuberculosis*	Newton et al. (2002)
Acorus calamus L.	Rh: essential oil	*M. tuberculosis*	Chopra et al. (1957)
Actaea spicata L. syn. *Actaea acuminata* Wall. ex Royle	Px: ethanol (95%) extract	*M. tuberculosis*	Grange and Davey (1990)
Adhatoda vasica Nees	Lf: bromhexine and ambroxol (semisynthetic derivatives of alkaloid vasicine)	*M. tuberculosis*	Grange and Snell (1996)
Allium cepa	Allicin	Bacteria, *Candida* sp.	Vohora et al. (1973)
Allium sativum	Allicin, ajoene	General	Naganawa et al. (1996), San-Blas et al. (1993), and San-Blas et al. (1989)
Aloe barbadensis	Latex	*Corynebacterium* sp., *Salmonella* sp.	Martinez et al. (1996)
Aloe vera	Latex	*Streptococcus* sp., *S. aureus*	Martinez et al. (1996)
Anogeissus leiocarpus	Stem bark	Malaria parasite	Shuaibu et al. (2008)
Azadirachta indica	Stem bark	Malaria parasite	Ehiagbonare (2007)
Berberis vulgaris	Berberine	Bacteria, protozoa	McDevitt et al. (1996) and Omulokoli et al. (1997)
Calotropis procera	Latex	*Bacillus subtilis*; *E. coli*; *Proteus vulgaris*; *Salmonella typhimurium*; *Pseudomonas aeruginosa*; *S. aureus*	Ahmad et al. (1998)
Camellia sinensis	Catechin	*Shigella* sp., *Vibrio* sp., *S. mutans*	Vijaya et al. (1995) and Toda et al. (1992)
Capsicum annuum	Capsaicin	Bacteria	Cichewicz and Thorpe (1996) and Jones Jr. and Luchsinger (1986)
Cassia singueana	Root, bark	Malaria parasite	Adzu et al. (2003)
Catharanthus roseus (L.) G.	Ap: dichloromethane and methanol extracts	*M. tuberculosis*	Cantrell et al. (1998)
Curcuma longa	Curcumin	Bacteria, protozoa	Gupta et al. (2015) and Rasmussen et al. (2000)
Elettaria cardamomum	Seeds	*B. subtilis*	Ahmad et al. (1998)
Erythrina indica Lam.	Cajanin	*M. tuberculosis*	Waffo et al. (2000)
Erythrina variegata L. var.	Indicanine C, 5,4-omethylalpinumisoflavone	*M. tuberculosis*	Waffo et al. (2000)
Glycyrrhiza glabra L.	Lico-isoflavone	*M. tuberculosis*	Mitscher and Baker (1998)
Helichrysum aureonitens	Galangin (3,5,7-trihydroxyflavone)	Gram-positive bacteria and fungi	Afolayan and Meyer (1997)
Holarrhena antidysenterica	*Bark*	*B. subtilis*, *E. coli*	Ahmad et al. (1998)
Hydrastis canadensis	Hydrastine	Bacteria, *Giardia duodenale*, Trypanosomes	Freiburghaus et al. (1996)
H. canadensis L.	Alkaloids	*S. aureus*	Ettefagh et al. (2011)

Continued

TABLE 15.1 Several Medicinal Plants Having Antimicrobial Potential—cont'd

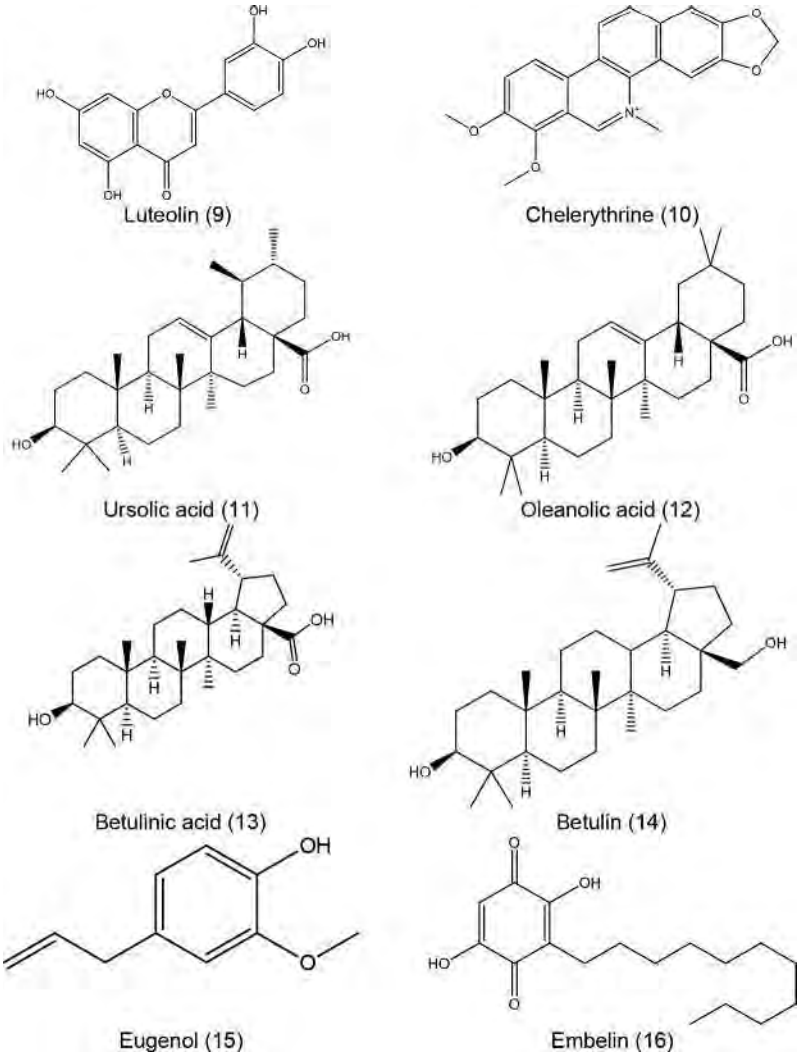
Plant Name	Compound/Part Used	Activity	Reference
H. canadensis	Berberine hydrastine	*G. duodenale*, trypanosomes	Freiburghaus et al. (1996)
Hyrtios sp.	Puupehenone	*Cryptococcus neoformans* and *Candida krusei*	Ahmad et al. (1998)
Hyrtios sp.	Puupehanol and chloropuupehenone	Fungi	Marjorie (1999)
Mahonia aquifolium	Berberine	*Plasmodium* sp., Trypansomes	Omulokoli et al. (1997)
Malus sylvestris	Phloretin	Bacteria	Hunter and Hull (1993)
Matricaria chamomilla	Anthemic acid	*M. tuberculosis*, *S. typhimurium*, *S. aureus*	Hamburger and Hostettmann (1991) and Scheel (1972)
M. chamomilla	Anthemic acid	*M. tuberculosis*	Hamburger and Hostettmann (1991) and Scheel (1972)
Millettia thonningii	Alpinumisoflavone	Schistosoma	Perrett et al. (1995)
Moringa oleifera	Leaves, stem bark, root	Malaria parasite	Shuaibu et al. (2008)
Panax notoginseng	Saponins	*E. coli*, *Sporothrix schenckii*, *Staphylococcus*, *Trichophyton*	Marjorie (1999)
Petalostemum purpureum	Petalostemumol	Bacteria, fungi	Hufford et al. (1993)
Petalostemum sp.	Petalostemumol	Bacteria, fungi	Marjorie (1999)
Piper nigrum	Piperine	Fungi, *Lactobacillus* sp., *Micrococcus* sp., *E. coli*, *Enterococcus faecalis*	Ghoshal et al. (1996)
P. nigrum	Piperine	Fungi	Ghoshal et al. (1996)
Podocarpus nagi	Totarol	*P. acnes*, other Gram-positive bacteria	Kubo et al. (1994)
P. nagi	Nagilactone	Fungi	Marjorie (1999)
Rabdosia trichocarpa	Trichorabdal A	*Helicobacter pylori*	Kadota et al. (1997)
Sarcocephalus orientalis	Indicanine B	*M. tuberculosis*	Waffo et al. (2000)
Terminalia arjuna W	Bark	*B. subtilis*, *E. coli*	Chopra et al. (1992)
Vaccinium spp.	Fructose	*E. coli*	Ofek et al. (1996)
Zingiber officinale Rosc	Rhizome	*B. subtilis*	Ahmad et al. (1998)

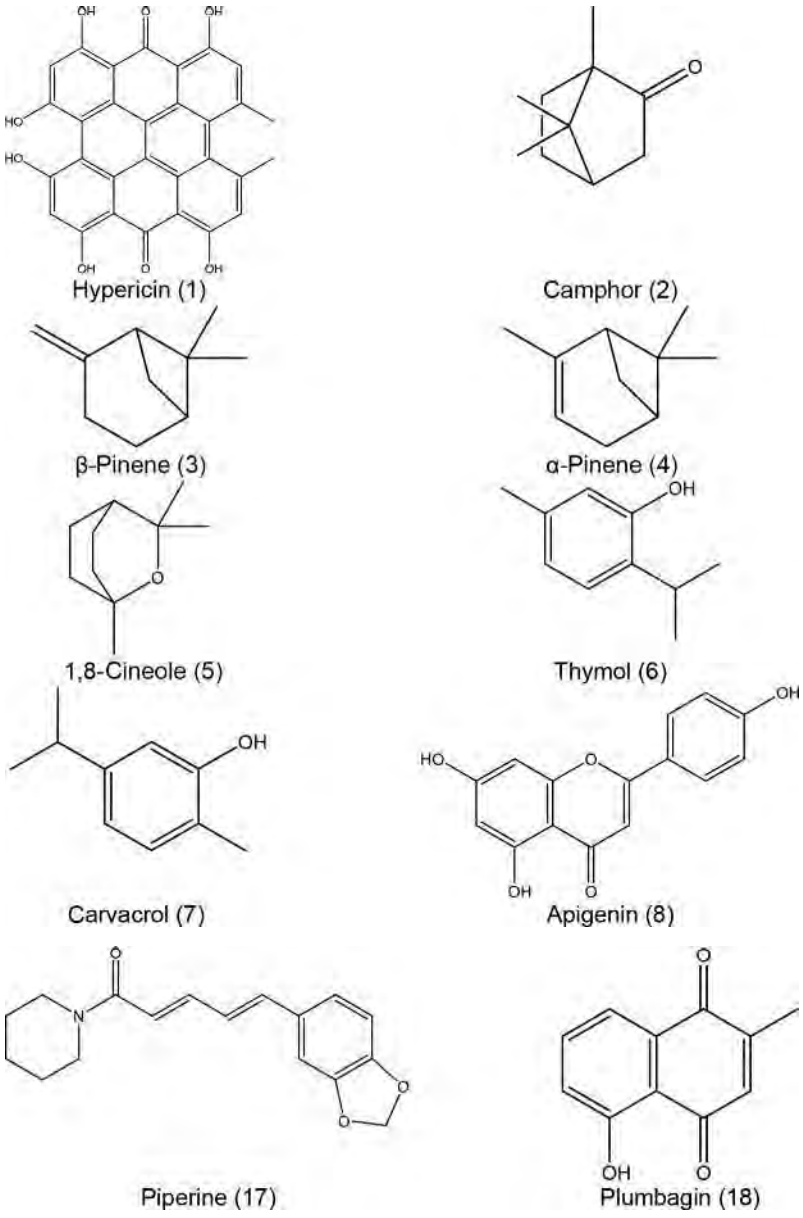
15.5 LEAD PHYTOCONSTITUENTS WITH ANTIMICROBIAL ACTIVITY

There are several reports on the antimicrobial activity of individual plants and their respective antimicrobial agents. From various studies, it is clear that the chemical structures of these agents belong to the most commonly encountered classes of higher plant secondary metabolites. In many cases, investigation with modern methodology has confirmed folkloric accounts of the use of higher plant preparations for the treatment of infections (Mitscher et al., 1987). Up until now, however, all antimicrobial substances from higher plants have been found either to be toxic to animals or not competitive therapeutically with products of microbial origin due to their low potency and narrow spectrum. Therefore, no antimicrobial compound from a higher plant has yet come into significant clinical use. Research, however, continues in the hope of finding plant antimicrobials that are effective for the systemic or topical treatment of human or agricultural infections. Because the screening methodology for the detection of such agents, their isolation from plants, and successive structure activity relation studies are rather easy to perform, there are grounds for cautious optimism for future success (Mitscher and Raghav Rao, 1984).

15.5.1 Plant-Derived Antimicrobials

Secondary metabolites of medicinal plants are a rich source of various potent antimicrobials, as has been reported by several researchers. A major secondary metabolite, hypericin (1), isolated from genus *Hypericum*, possesses antimicrobial efficacy against methicillin-resistant *S. aureus* (Schempp et al., 1999). Other secondary metabolites from *Hypericum* sp. include norlignans and xanthone with potential activity against *S. aureus*. Several lead compounds isolated from medicinal plants that have antimicrobial efficacy, including camphor (2), β-pinene (3), α-pinene (4), 1,8-cineole (5), thymol (6), and carvacrol (7), have been reported against *S. aureus*, *E. coli*, and *S. typhimurium* (Hammer et al., 1999). Phenolic compounds are well known for antimicrobial activities. Apigenin (8) and luteolin (9) showed good antibacterial activity against *S. aureus* strains. Alkaloids are another class of secondary metabolites with potential bioactivity. Several scientific reports suggest that alkaloids from medicinal plants possess antibacterial activities, including Chelerythrine (10), which exhibited promising antibacterial activity against *S. aureus* (Rahman, 2015). Triterpenoids, including ursolic acid (11), oleanolic acid (12), betulinic acid (13), and betulin (14), have antimicrobial efficacy against *E. coli*, *S. aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and some clinical isolates (Fontanay et al., 2008). The essential oil eugenol (15) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane (Devi et al., 2010). Embelin (16) and piperine (17) have potential antimicrobial activity against *Streptococcus mutans* (Dwivedi and Singh, 2014). A naturally occurring naphthoquinone, plumbagin (18) possesses antimicrobial activity against *S. aureus* and *Candida albicans* (Nair et al., 2016).





15.5.2 Antimicrobials From Marine Sources

Several scientific reports have focused on marine organisms and phytoplankton for the discovery of lead bioactive antimicrobials. Naturally occurring diterpenes with unprecedented tetracyclic skeletons, ioniols I and II, tetracyclic brominated diterpenes, were isolated from the organic extract of *Sphaerococcus coronopifolius* and are effective against MDR *S. aureus* and MRSA (Smyrniotopoulos et al., 2008). Ioannou et al. (2011) has reported the isolation of 17 diterpenes from the organic extracts of the brown alga *Dilophus spiralis*. Some of these revealed good antibacterial activity against six strains of *S. aureus*, including multidrug-resistant (MDR) and methicillin-resistant variants. Neomaclafungins A–I (Sato et al., 2012) have been isolated from *Actinoalloteichus* sp. and possess significant antifungal activity against *Trichophyton mentagrophytes*. Leodoglucomides A and B, isolated from a marine-derived bacterium *Bacillus licheniformis* exhibited moderate antimicrobial activity. Peptidolipins B and E exhibit moderate antibacterial activity against MRSA and methicillin-sensitive *S. aureus*. Isolated from *Streptomyces* sp. (Ding et al., 2012). Polycyclic secondary metabolites, citreamicin A, citreamicin, B, citreaglycon A, and dehydrocitreaglycon A, isolated from marine-derived *Streptomyces caelestis*, have antibacterial activity against *Staphylococcus haemolyticus*, *S. aureus*, and *B. subtillis* (Rahman, 2015).

15.6 EVALUATION OF PHYTOPHARMACEUTICALS HAVING ANTIFUNGAL ACTIVITY

Nowadays, evaluation of antifungal agents from natural resources is an emerging trend in research to search for alternative antifungicides. Phytoalexins appear to have a very broad range of fungi for which they are quite toxic in the 10–100 $\mu g\,mL^{-1}$ concentration range. In fact, their nonspecificity could reduce their usefulness to medicine. In spite of their relatively low activity compared with other antibiotics in use today, the relatively small number of useful fungicides for medicine dictates that these potentially useful compounds be investigated for a role in medicine. Appropriate bioassays will indicate which of these compounds will benefit animals and plants that have fungal infection (Mukherjee, 2002).

15.6.1 Screening Methods for Antifungal Agents

Assays using sporelings in liquid media produced the most reliable indication of fungicidal activity. The results of other tests were less reliable and oftentimes complete inhibition of growth did not occur. For most fungi, spores and 1-day-old sporelings were equally sensitive. However, the spores of some fungi were markedly resistant to compounds that inhibited many other fungi. Similarly, 3-day-old sporelings sometimes grew in relatively high levels of the fungicides tested. The growth, which was produced after prolonged incubation, arose from a few cells that survived the potentially fungicidal effects. Mycelial growth in agar media containing phytoalexins was quite variable and was rarely linear throughout the incubation period. The results obtained were thus very difficult to interpret and it was not possible to use mycelial growth to measure the toxicity of all compounds. Skipp and Bailey (1976) divided the growth response of fungi in their bioassays into three types:

(a) Growth started immediately and was essentially linear throughout the assay.
(b) Produced a growth curve with a lag period, but then proceeded at a constant rate.
(c) The rate of growth increased progressively throughout the incubation period.

Type A growth was common for only three fungi tested with all six compounds that were bioassayed. Type C growth was associated with the faster-growing fungi. As a result of these differences, the values obtained for the degree of inhibition of growth rate by the individual compounds varied greatly. Furthermore, the relative sensitivities of the fungi to the phytoalexins were not the same and the relative activity of the phytoalexins was different at the two concentrations.

They conclude that the fungitoxicity of the six compounds they tested was probably best measured by assays in which the effects were tested on the growth of 1-day-old sporelings in liquid media. The walls of young germ tubes are probably very permeable to these compounds. All inocula would have come into immediate contact with the full ambient concentration of the inhibitor and the ratio of volume of inoculum. This test gave the most consistent results; the amount of each phytoalexin required to prevent the growth of each fungus differed by only a small amount, thus, their relative toxicities remained constant. Another advantage of this method is that very small amounts of phytoalexin are required.

Spores of some fungi were much less sensitive that their sporelings. Unlike the cells of 1-day-old sporelings, cells in the germ tubes arising from spores of these insensitive fungi were not killed by the levels of fungicide into which they emerged. Skipp and Bailey (1976) concluded that it is possible that the insensitivity of spores of these species arose because the penetration of phytoalexins into the fungal protoplast was reduced by the low permeability and/or absorptive properties of the spore walls, and that this allowed protoplasts to undergo an adaptive process enabling them, and the resulting germ tubes, to survive. It is clear that spores do not always behave like sporelings and thus results of spore germination assays will not always be relevant to the in vivo situation.

In general, assays using older mycelial inocula in liquid media, which may reflect the situation during lesion limitation, gave results similar to those obtained with 1-day-old sporelings. When prolonged incubation led to increased MIC values, this was probably due to the survival of some cells within the inoculum and subsequent mycelial growth. Assays measuring inhibition of mycelial growth on agar media are widely used. However, these studies confirm that the results of these tests are difficult to interpret. It is inadvisable to calculate values for percentage inhibition from a single measurement of colony diameter made after an arbitrary period.

The lag period in the growth of several fungi was probably caused by the death of most cells within the inoculum. Growth, which eventually occurs, arises from hyphae that survive for some reason. The end of the lag period then coincides with alterations in the sensitivity to the compound by adaptation and/or detoxification. Shepard (1987) states that only recently have the methods for the mass screening of chemicals to control fungi been developed. Variation between tests is a problem and relative potency was introduced as a useful concept in dealing with this variability.

Media used

Solid agar and liquid broth culture media "C" and "D" as specified in the Pharmacopoeia of India (1985) may be used for sensitivity, turbidity, and spore germination, and to determine the zone of inhibition.

Cultures

Pure cultures of the organisms are to be procured from reputed laboratories/organizations and to be cultured in the laboratory where the test is being performed. The fungal strains usually used for this study are *Aspergillus niger*, *A. flavus*, *C. albicans*, *Candida tropicalis*, *Cryptococcus neoformans*, and *Trichophyton lignorum*.

15.6.2 Sensitivity Test

This is performed by the tube dilution technique. A series of test tubes ($16 \times 125\,mm^2$), containing 9 mL of sterile culture medium "C" and 1 mL of various concentration of test drugs (10 tubes for each concentration) are taken. All tubes are inoculated with microorganisms to be tested and then incubated at 20–25°C for 48 h. The turbidity produced is observed thereafter by determining the absorbance at 530 nm.

15.6.3 Turbidity Method

This is performed as per the method described in Pharmacopeia (Indian Pharmacopoeia, 2014). One milliliter of each concentration of the test drug to be screened is used. To each tube, 9 mL of nutrient medium "C," previously seeded with the appropriate test organism, is added. One control containing the inoculated culture medium and another blank, which is identical with the control but treated immediately with 0.5 mL of dilute formaldehyde solution, is used. All the tubes are incubated at the specified temperature for 48 h. The growth of the test organisms is measured by determining the absorbance at 530 nm in a spectrophotometer.

15.6.4 Spore Germination Method

Spore suspensions of 7-days-old culture are prepared in the test compounds and the standard drug for comparison, griseofulvin (in specific concentrations). A control is prepared to be identical with this, but without using the test compounds. A drop of spore suspension is placed on a sterilized slide and incubated in a humid chamber for 12 h, then the number of spores germinated is scored to calculate the percentage of spore germination.

15.6.5 Disk-Diffusion Method

The filter paper disk agar diffusion method is used (Mukherjee et al., 1995a, b). First, 20 mL of the sterilized medium "D" is put into each petri dish. Then, 2 mL of a 24-h-old broth culture of subcultured organisms is distributed evenly over the surface of the plates. Sterilized Whatman filter no. 1 disks (6 mm diameter), thoroughly moistened with different concentrations of test and standard drugs, are placed on the surface of the plate. Disks moistened with sterile water are used as a control.

The test organisms are seeded into sterile SDA media for fungal screening. In a sterile Petri dish, 1 µL of inoculum is mixed uniformly with 20 µL of sterile melted nutrient agar and cooled to 48–50°C. When the agar solidifies, holes of uniform diameter are made using a sterile borer. Test solutions of 0.3 µL containing solutions of different extracts at varying concentrations as well as the standard drug solutions (e.g., griseofulvin or other) and DMSO (control-blank) are placed in each hole separately under aseptic conditions. The plates are then maintained at room temperature for 2 h and the solution is allowed to diffuse into the specific medium. All the plates are then incubated at 25°C for 1 week and the zone of inhibition is measured (Mukherjee et al., 1995a, b). By using these models, several plant species, such as *N. nucifera* rhizome, leaf extract of *Cassia tora*, and several species of the genus *Hypericum—H. hookerianum*, *H. patulum*, and *H. mysorense*, have been shown to produce antifungal potentials (Mukherjee et al., 1995b, 1996, 2001).

15.6.6 High-Throughput Method for Antifungal Activity

In this method, a high-throughput antifungal activity screening is performed by using a cheese-mimicking matrix distributed in 24-well plates. This method allowed rapid screening of a large variety of antifungal agent candidates: bacterial fermented ingredients, bacterial isolates, and preservatives. This screening method achieved a screening rate of 1600 assays per week and can be implemented to evaluate antifungal activity of microorganisms, fermentation products, or purified compounds compatible with dairy technology (Garnier et al., 2018).

15.7 EVALUATION OF THE ANTIMYCOBACTERIAL ACTIVITY OF HERBS

Several plant extracts have been reported for their antimycobacterial activity. This activity is usually screened using mycobacteria cultured in various types of broth and agar-based media. *M. tuberculosis* is slow growing and its screening requires a few weeks and stringent safety measures are needed because of its virulence. Several investigators have used nonpathogenic species of mycobacteria, such as *M. avium*, *M. intracellulare*, and *M. kansaii*, which. Like *M. tuberculosis*, are slow growing. They have also used alternative species, such as *M. chelonei*, *M. fortuitum*, and *M. smegmatis*, which are quicker growing, thus permitting tests to be completed within a few days. Most commonly, the test methods employed are the disk diffusion and the broth dilution methods. In the disk diffusion method, paper disks impregnated with the tested extract are placed on a semisolid (agar based) medium that has been inoculated with mycobacteria. After incubation, the diameter of zones of inhibition around the disks is measured (Mukherjee, 2002).

The main disadvantages with this method are that nonpolar compounds may not diffuse properly into the agar media, so that active compounds may be missed, and that it is not possible to obtain reliable quantitative results for comparative purposes. In the broth dilution method, the MIC is determined using a series of tubes containing serial dilutions of the tested sample (Satim and Washington, 1991).

There is a need for high-throughput, rapid screening methods that can be automated; these have been reviewed by Gordon et al. (1996). The first rapid methods to be developed involved measuring the evolution of $^{14}CO_2$ from *M. tuberculosis* cultured in a medium containing ^{14}C-palmitic acid and this formed the basis for the BACTERC system (Becton-Dickinson, Oxford, UK). This is used for the susceptibility testing of clinical isolates and can provide results in an average of 5 days compared with 3–4 weeks for conventional methods. However, the BACTERC system is not suitable for high-throughput screening due to the technical difficulties involved in measuring $^{14}CO_2$. Chung et al. (1995) developed an assay based on measuring the uptake of radiolabeled uracil into *Mycobacterium aurum*, a fast growing and nonpathogenic species that appears to be a good model for *M. tuberculosis* as it has a similar profile of sensitivity to anti-TB drugs. The latter method may be used for high-throughput screening and does not require containment facilities, but the separation from unincorporated uracil is labor intensive. The major disadvantage of the above method is the need for radiolabeled substrates, but this has been overcome with the development of assays in which mycobacterial viability is determined using either bacterial or firefly luciferase. The bacterial enzyme uses reduced flavin (produced by viable mycobacteria) to oxidize an added aldehyde substrate (decanal), which is accompanied by the production of light at 490 nm. Firefly luciferase is dependent upon ATP generated by the mycobacteria to decarboxylate luciferin, resulting in the production of light at 562 nm. Light production may be measured easily using a luminometer in a high-throughput system. Several species of mycobacteria, including *M. tuberculosis*, have been genetically modified by inserting the genes for the production of bacterial luciferase; only viable bacilli emit light when decanal is added and there is no requirement for growth so that susceptibility testing may be carried out rapidly. Similarly, the gene for firefly luciferase has been incorporated into a number of mycobacteria species, including *M. aurum* (Chung et al., 1995).

Colorimetric assay methods are appropriate for use in microtiter plates and the results are obtained simply by employing a photometer. Gomez-Flores et al. (1995) found the AN assay for testing against *M. avium* complicated; it depends on the flexibility of viable microorganisms to convert dimethylthiazol diphenyltetrazolium (MTT) to formazan crystals. Another similar methodology utilizes the oxidation–reduction dye Alamar blue, which changes color from blue to pink in the presence of viable *M. tuberculosis* (Yajko and Madej, 1995). These assays have the benefit of being straightforward and not requiring radioactive substrates; however, many days are needed for the growth of the microorganism. M. TB is primarily a living infective agent residing within the acidic vacuoles of scavenger cells. This setting might have an effect on the action of anti-TB medication, such as antibiotic drugs (activity reduced) and pyrazinamide (activity increased), and it should be valuable to judge the flexibility of tested compounds to inhibit M. TB in cultured human macrophages (Crowle and May, 1990).

Crude extracts of several plant species, particularly those having ethnomedical uses, have been screened for in vitro antimycobacterial activity; however, a comparatively small number of active compounds have been isolated. In several cases, the research was interrupted because the extracts exhibited very little or no activity at the best concentration of the test sample. Newton et al. (2000) reviewed antimycobacterials that were tested against several species of mycobacteria and that were explored further to evaluate the character of the constituents responsible for the activity. Compared with microorganisms, plant species have up to now been unsatisfying as a supply of potent medication agents.

Several plant extracts and compounds do have potential antimycobacterial properties, including *Borrichia frutescens*, *Ferula communis*, genus Heracleum most, *Karwinskia humboldtiana*, *Leucas volkensii*, herbaceous plants, *Oplopanax horridus*, sage multicaulis, and *Strobilanthes cusia*. In some cases, compounds have been isolated that have antimycobacterial activities comparable to anti-TB medication, for instance, (*E*)- and (*Z*)-phytol and phytanol, which were isolated from *L. volkensii* (Rajab et al., 1998). It is assumed that natural products may provide useful leads toward new drugs for TB

treatment. Bromhexine is a semisynthetic derivative of vasicine, which is found in *Adhatoda vasica*, and Bromhexine and ambroxol (metabolite of Bromhexine) are widely used as mucolytics, such compounds having a pH-dependent inhibitory potential against in vitro M. TB, which may be useful in the treatment of tuberculosis (Grange and Snell, 1996).

15.8 EVALUATION OF ANTIMALARIAL ACTIVITY

Human malaria is one of the most important diseases in the world, with a corresponding mortality rate of more than 1 million deaths per year (WHO, 1994). The declining efficacy of classical medication in relation to the rapid extension of *Plasmodium falciparum* chloroquine-resistant strains (Plowe et al., 1995) has led to a need for new efficient antimalarial drugs. In Africa, where malaria is endemic, access to chemical treatment is reduced in rural areas, and cultural practices still remain important (WHO, 1994). Consequently, 75% of the population has recourse to traditional medicine for treating the afflictions of early childhood. Thus, one of the principal approaches of chemotherapeutic research against malaria parasites consists of investigating the antiparasitic activity of plants currently employed in traditional medicine (Gasquet and Delmas, 1993).

The techniques of using *P. falciparum* and *Plasmodium vivax* alive have been the basis of in vitro screening procedures for herbal drugs for over half a century. A major breakthrough in screening for antimalarial activity came in 1948 through the discovery of the rodent malaria parasite *Plasmodium berghei* at Kreyberg in the then Belgian Congo. This parasite, which readily infects laboratory mice and rats, has proved to be valuable for the estimation of activity in chemotherapeutic research programs in which more than 300,000 compounds have been screened (Peters, 1987). The next major contribution to screening procedures was the in vitro cultivation of the intraerythrocytic stage of *P. falciparum*, the cause of human tertiary malignant malaria. WHO Scientific Group on the Chemotherapy of Malaria and Resistance to Antimalarials and World Health Organization (1973) have fixed guidelines for antimalarial screening of drugs in four stages as follows:

(i) Primary screening establishes whether compounds have activity against malaria parasites.
(ii) Secondary screening sets out to further qualify and quantify antiparasitic activity and to determine the safety and comparative activities of analogs.
(iii) Tertiary screening deals with the study of nonhuman and human parasites in primates other than man.
(iv) The fourth stage is clinical testing. In assessing the activity of plant extracts for the presence of compounds with antimalarial activity, the techniques of primary and secondary screening, as outlined by Peters (1987), can be utilized. For initial screening, either in vivo or in vitro techniques, as described, may be employed.

15.8.1 Selection of Blood Schizontocides for In Vivo Models

Two major antimalarial screening tests have been used during the past 50 years. The avian parasite *Plasmodium gallinaceum* was used as a primary test prior to and during World War II, but this test has now been superseded by one using the rodent parasite *P. berghei*. The mouse model with *P. berghei* needs careful standardization in order to avoid variable results and factors, such as parasite strain, host, and drug administration, must be carefully controlled. The N strain (N = normal) of *P. berghei* develops rapidly in many strains of mice but is slow to develop in other strains. *P. berghei* (N strain) has been maintained over many years by syringe passage and no longer produces gametocytes. The continuous exposure of the N strain to chloroquine has resulted in the development of the NS strain, which has a low level of chloroquine; the pharmacokinetics and/or metabolism of drugs may well vary with mouse age. Male mice of approximately 20-g weight tend to be optimal for antimalarial testing. The environment and the diet of the mice must be carefully controlled. There are two different types of test with *P. berghei* that may be used for the screening of plant extracts, the Peters' test and the Rane's test (Mukherjee, 2002).

15.8.2 Peters' Test

The test is performed by the principal of the 4-day suppressive test of blood schizontocidal action. Male mice (e.g., Swiss albino) weighing 20 ± 2 g are maintained at 22°C (±2°C) in batches of five and fed on a standard diet. Blood from a donor mouse with rising parasitemia (about 20% infected erythrocytes) is diluted in a tissue culture medium (TC 199) so that each 0.2 mL contains 10^7 infected red cells. Each mouse receives 0.2 mL intravenously via the tail on day zero. The plant extract is either suspended or dissolved by trituration or sonication after the addition of a 0.2% solution of Tween 80 or 0.5% carboxymethyl-cellulose or dimethylsulfoxide. Initially, the dose range should be between 1 and 100 mg kg^{-1} of aqueous concentration and administered regularly from the first day of infection for the next 4 days, either by subcutaneous or oral routes. After that, blood is drawn from the tail and stained with proper staining agents (Giemsa) and the results are recorded

in terms of parasitized RBCs as a percentage of the total ED_{50} values. Standard deviations are calculated by the use of appropriate computer programs. For large-scale screening, a single arbitrary dose (e.g., 100 mg kg^{-1}) may be given instead of a series of doses (Mukherjee, 2002).

15.8.3 The Rane Test

This method is based on the effect of the inoculum of *P. berghei*, which can kill mice within 6 days after a single dose administration of test compound. An inoculum (10^6 infected donor cells) is given intraperitoneally on day 1 and plant extract solutions or sonicated suspensions in arachis oil are given subcutaneously at an initial dose range of 640, 320, 160, and 80 mg kg^{-1} on day 4. Activity is judged to be survival to more than twice that of the controls. The minimum effective dose (MED) is obtained and compared with the maximum tolerated dose that produces no more than one in five toxic deaths. Lower dose levels may be used in order to obtain the MED. Although the test is somewhat crude in comparison with the 4-day test, it has proved valuable in identifying new drug leads because it gives a measure of efficacy against toxicity. It is not always possible to compare results obtained in the Rane test with those obtained from the Peters' test. Neither of these tests is suitable for the identification of long-acting compounds and if this information is required, tests utilizing *Plasmodium yoelli* or *Plasmodium vinckei* in mice may be used. Alternative tests with *P. yoelli* are also available for tissue schizontocidal and gametocytocidal activities (Mukherjee, 2002).

15.8.4 In Vitro Models for the Selection of Blood Schizontocides

In vitro screening tests can be carried out rapidly and are economical by way of the amount of test drug and of animals required. The major disadvantage of in vitro tests is that no data are obtained on drug kinetics, metabolism, or toxicity. However, in vitro tests do lend themselves to high-volume screening, although surprisingly little use has been made of them to date in primary screening (Peters, 1987). The use of *P. berghei* in vitro has not been developed because of the success of the in vivo tests. For screening, extracts of the cause of human malaria can be used in traditional medicine; in vitro tests offer advantages in that *P. falciparum*, the cause of human malaria can be employed. In vitro tests are useful for guiding fractionation of plant extracts by chromatographic separation techniques.

The method as modified by Fairlamb et al. (1985) has been used for the assessment of the antimalarial activity of plant extracts. The test employs 96 flat-bottomed wells in microtiter plates. The plant extracts are dried and dissolved or micronized in ethanol and six different concentrations are made by 10-fold dilution in the RPMI 1640 medium of the test samples. The concentration of ethanol for testing is kept below 0.1%. Inoculum (50 µL) in the culture medium containing the plant extract at 10-fold dilution with a concentration ranging from 1000 to 0.01 µg/mL is added to each well. Human blood (50 µL, 0 +ve, 5% hematocrit) containing 1% parasitemia is added to each well. Dilutions to produce 1% parasitemia are made with uninfected washed red blood cells. All tests are performed in duplicate. Two series of controls are set up, one with parasitized blood without addition of plant extract and the other with uninfected red blood cells. After incubation in a 3% O_2, 4%, CO_2, and 93% N_2 gas phase for 18 h at 37°C, 50 µL of G-[^{3}H] hypoxanthine (40 Ci mL^{-1}) is added to each well and incubation is continued at 37°C for a further 18–24 h. The Titertek 530 Cell Harvester (Flow Laboratories) is used to harvest the red blood cells. Normal saline is used to wash the wells and glass fiber membrane is used for filtration. Dried membranes are placed in scintillation fluid and incorporation of [^{3}H] hypoxanthine are measured by a scintillation counter. The percentage inhibition of incorporation is calculated from the following equation:

$$\%\text{inhibition of incorporation} = 100 \times \frac{\left(\text{dpm infected blood with test substance}\right) - \left(\text{dpm uninfected blood}\right)}{\left(\text{dpm infected blood}\right) - \left(\text{dpm uninfected blood}\right)}$$

IC_{50} values are determined. The data obtained by this method have been shown to correlate with the in vivo data obtained with *P. berghei*. The test may be modified by using candle jars to supply the required reduced oxygen atmosphere and staining with counting techniques may be used instead of incorporation of [^{3}H] hypoxanthine. In order to preserve isolates of *Plasmodium* species for in vitro tests, deep freezing (cryopreservation) techniques are used. Cryopreservation is economical in the use of parasite stocks, which would otherwise have to be passaged either in vivo or in vitro. In vitro tests for the selection of tissue schizontocides are also available utilizing *P. gallinaceum*, *P. fallax*, *P. berghei*, and *P. yoelli* (Peters, 1987). When the level of drug action is determined in vitro, the host's immune response plays a part. The 4-day test may, in some instances, prove to be too crude and the test may require modification. In most plasmodial infections, parasitemia levels may require modification. In most plasmodial infections, parasitemia levels will increase logarithmically up to a level of 2% parasitemia, at which point host factors intervene. Further information for secondary and tertiary

screening includes studies on different routes of administration, long-acting drugs, mode of action, biochemical studies in mode of action, drug potentiation and antagonism, dose-activity regression analysis, morphological changes in parasites, action against preerythrocytic and sporogenic stages, different laboratory models, drug-resistant lines, toxicity evaluation, and action on nonhuman primates. The estimation of the antimalarial potential in herbal drugs can also be made using the methods described below (Mukherjee, 2002).

15.8.5 Toxicity to Macrophages

Toxicity is evaluated on peritoneal macrophages (BALB/c mouse) incubated in a cell medium for an incubation period of 24–28 h at 37°C in 5% CO_2, with a varying concentration of different extracts. The percentage of viable macrophages is determined microscopically and the extracts appearing toxic are detected.

15.8.6 In Vitro Antimalarial Assays

The antimalarial activity of plant extracts is assessed against two strains of *P. falciparum* maintained in continuous culture according to the methodology described by Trager and Jensen (1976) and Trager and Polonosky (1994); the chloroquine-sensitive strain 3D7 and the chloroquine-resistant strain W2. Parasitemia is evaluated after 48 h by a flow cytometric technique derived from the protocol published by Reinders et al. (1995). Cells are run on a Facsort flow cytometer. The antimalarial activity of the extracts is measured by the IC_{50}, representing the concentration of drug that induced a 50% decrease in parasitemia compared with the control culture and chloroquine is tested on the two strains for standard antimalarial activity. May Grundwald Giemsa-stained thin blood smears are used for comparing microscopically the results obtained by flow cytometry.

15.9 ANTIGIARDIAL EFFECT OF HERBS AND ITS EVALUATION

Giardiasis is a common gastrointestinal infection caused by a protozoal parasite, *Giardia lamblia*. The incidence of infection ranges from 20% to 60% in the world.

Walia et al. (1986), has reported that the endemicity of giardial infection is high (87%) because of the inadequate hygiene conditions in several regions of India. In infants and children, high ubiquity is also noticed (Black et al., 1977). One notable feature of giardiasis is the variability of the symptoms. A large population of patients does not show any clinical symptoms (Farthing, 1989). Modern chemotherapy is effective in acute infection but creates drug resistance and several side effects, including disturbance of microflora of the bowel, nausea, abdominal cramps, furry tongue, and metallic and unpleasant taste (Jokipil and Jokipil, 1979). So, scientists are interested in exploring herbal drugs, especially in the form of preparations (Rasayanas), which are used to rejuvenate strength by boosting the immune system of the host. Pippali Rasayana, prepared from *Piper longum* and *Butea monosperma*, showed a significant antigiardial activity proven by experimental and clinical studies.

15.9.1 In Vitro Activity

An exponentially grown (24-h old) culture of *G. lamblia* having approximately 2000 trophozoites (0.1 mL) is poured in a cavity slide. A test sample (0.1 mL in TYI-S-33 medium) with the desired concentration is added in the cavity slide. Then, the slide is mounted with a coverslip and the edges are sealed with paraffin wax. The total system is incubated at 37°C for 24 h. The results are calculated in terms of live and dead trophozoites to determine the antigiardial efficacy of the tested compound (Tripathi et al., 1999).

15.9.2 In Vivo Screening

In this method, noninfected Swiss mice (18–20 g) are used. Pure cultures of *G. lamblia are subcultured in* TYI-S-33 medium and incubated for 48 h in appropriate culture conditions. After incubation, the culture is centrifuged and the supernatant is discarded. The remaining pellet is suspended with fresh media. Inoculum (0.25 mL) having live trophozoites ($0.5–1.0 \times 10^6$ cells/mL) is injected into mice (intrajejunally). After a proper incubation period (48 h), the infected animals are divided into two groups having six animals in each group. The first group is treated with the test compound (different doses) by oral administration for the next 5 days, whereas the second group remains untreated and serves as a control. The infected animals are then sacrificed and the jejunum is removed and thoroughly washed with phosphate buffer saline (pH 7.2).

The washed aliquot is observed under a microscope for live trophozoites. The percentage of recovery is measured in the comparison with the first group (E = infection + treatment by compound) and the second group (C = only infection) and calculated by the following formula (Tripathi et al., 1999):

$$\%\text{Recovery} = \frac{C - E}{C} \times 100$$

15.9.2.1 Specific Immune Response

A. Hemagglutinating antibody (HA) titer

Serum is separated from the blood and an HA titer is determined using a microtiter plate. Aliquots are prepared in PBS (pH 7.2) and 50 µL is added to the microtiter plate. 1% SRBC suspension (25 µL) is also added to each well and mixed properly. The plates are incubated at room temperature from 1 to 2 h and agglutination is measured. The result is calculated in terms of highest dilution of test serum giving 100% agglutination and presented as the HA titer.

B. Plaque-forming cell (PFC) assay

Spleens are separated, washed, and re-suspended in an RPMI-1640 medium having a cell concentration of 10^6 cells/mL. The bottom layer is prepared by using 1.2% agarose with NaCl (0.15 M) in glass petri plates. A 20% SRBC suspension along with 0.6% agarose in RPMI media and 10^5 cells/mL are spread over the bottom layer and the system is incubated at 37°C for 1–2 h. Lastly, freshly diluted guinea-pig serum is added and incubated further at room temperature for 45 min. The plaques formed are counted and the values are represented as 10^5 spleen cells (Gerne and Nordin, 1963).

15.9.2.2 Nonspecific Immune Response

(a) Macrophage migration index

The peritoneal exudate cells (PECs) are collected, cleaned, and suspended in RPMI-1640 medium, supplemented with 10% fetal calf serum. This suspension is packed into capillary tubes of uniform diameter (microhematocrit). One side of the capillary tube is sealed and the cells are settled with the application of low-speed centrifugation. Then, the portion of the capillary with the cells is cut at the cell-liquid interphase, transferred to a migration chamber suspended with RMPI-1640 medium having fetal calf serum (10%), and incubated overnight at 37°C. The PECs are migrated (A1) to the migration chamber; this area of migration is marked on Whatman No. 1 filter paper and untreated cells are also similarly marked by using camera lucida. The ratio of migration (area) of cells is presented as a macrophage migration index (Saxena et al., 1991).

(b) Phagocytic activity

The phagocytic activity was determined according to the method of Leijh et al. (1977) with some modification. PECs were collected from treated and untreated (control) animals, cleaned with PBS, and suspended in RPMI-1640 medium supplemented with fetal calf serum (10%) having a final cell concentration of 10^6 cells/mL. In a 16-well plate (cluster plate), aliquot (1 mL) is added and incubated at 37°C for 1 h with 5% CO_2.

The cell monolayer is cleaned and the nonadherent cells are incubated for 1.5 h with [^{14}C]-leucine-labeled *E. coli*. The results of each well are recorded by a liquid scintillation counter.

15.10 AMOEBICIDAL ACTIVITY OF HERBS AND ITS EVALUATION

In 1969, amoebiasis was defined by the United Nations as "a condition during which a patient is harboring the organism *Entamoeba histolytica* within the bowel." This condition may occur even if the patient exhibits no clinical symptoms of the malady (Wilcocks and Manson-Bahr, 1972). *E. histolytica* may exist either as mobile trophozoites or as cysts, with the former generally being between 10 and 40 µm in diameter, although morbific ones are larger. Cysts are sometimes 10–15 µm in diameter with one to four nuclei and are found within the lower part of the intestine. The protozoa sometimes live and multiply within the lumen of the large intestine, living in interdependence with the microorganism flora as commensal organisms and also the host, who may exhibit no symptoms of disease, can pass infective cysts in the feces. If the cysts are transferred to a different host, they endure the acid conditions of the abdomen and unleash trophozoites within the bowel. The precise factors that make *E. histolytica* morbific are not completely understood (Keene et al., 1986). The trophozoites could invade different organs as well as the liver, brain, and lungs, forming native necroses called abscesses. Gentle cases of amebiasis cause very little disturbance to the patient; however, a severe infection will cause amoebiasis, which can be fatal.

A number of natural compounds, as well as several alkaloids, have amoebicidal activity as does tubulosine (Steck, 1972), cryptopleurine (Martin et al., 1982), and berberine (Druey, 1960). Many quinolizidine alkaloids isolated from species of the genus Sophora that are used in Japanese folk medicine have been shown to possess amoebicidal activity; they include matrine (Neal, 1983), its N-oxide, and cytisine (Janot, 1953). Dita bark and *Aristolochia macrophylla* are utilized

in Asian countries and also the Philippines to treat infectious disease and it is believed that indole alkaloids, such as alstonine, are responsible for their activity (Steck, 1972). The internal secretion of the organic compound conessine has marked amoebicidal activity and it is the key alkaloid of *Holarrhena antidysenterica* that was introduced into Europe as a cure for amoebiasis in the 19th century (Druey, 1960). Conessine showed marked aspect effects that rendered it unsuitable for clinical use (Steck, 1972). Crude extracts from species of the genus *Tabernaemontana* have been shown recently to possess in vitro amoebicidal activity (van Beek, 1984).

In any program of assessment for potential amoebicidal activity, a major downside has been the development of a speedy screening technique. The expansion of amoebae on their own (axenic culture) was initially achieved in a diphasic medium (Diamond, 1961). Later, a transparent liquid medium was developed and an axenic culture of *E. histolytica* in such a medium has provided a straightforward technique for the development of in vitro screening of potential amoebicidal agents (Diamond, 1968). Enhancements in check procedures have resulted from the employment of biological research techniques for the assembly of many uniform strains of *E. histolytica* and simplification of the medium (Diamond, 1986).

In order to work out whether or not the in vitro check can be used for crude extracts of plants, a series of three organic compound fractions from *Cinchona ledgeriana* (Nilgiris variety) leaves were tested and fabricated for the amoebicidal result (Keene et al., 1986). In this study, the IC_{50} values that ranged from 0.21 to 3.0 $\mu g\,mL^{-1}$ were indicative of the presence of compounds with greater activity than the pure cinchona alkaloids.

When screening material for chemotherapeutical activity, it is advantageous to get an early indication of specificity of action in distinction to nonspecific cytotoxicity. Some activity against DNA- and protein-synthesis is often obtained from the inhibition of uptake of [^{3}H]-thymidine into guinea-pig ear keratinocytes (GPK cells) in vitro.

Emetine and a pair of 3-dehydroemetines (as hydrochlorides) have ratio <1, confirms the highly cytotoxic nature of these compounds. On the other hand metronidazole has a ratio of >455, which is considered as it acts under anaerobic conditions depending on the nitro group reduction to produce an intermediate product that binds to DNA (Goldman, 1980). The toxicity to amoebicidal concentration of 5-chloro-8-hydroxyquinoline, helminthic, and antimalarial drugs are 36, 9, and >7, severally indicating some specificity for amoebicidal activity (Page, 2008). Quinidinone, with the keto substituent at C-9 rather than the secondary radical in the antimalarial drug, showed exaggerated amoebicidal and cytotoxic activities compared with the antimalarial drug. The foremost attention-grabbing compound rumored is aricine that has as amoebicidal IC50 worth of 3.6 $\mu g\,mL^{-1}$ and a toxicity to amoebicidal concentration of >27.

15.10.1 In Vivo Models for Amoebiasis

Several animal models are employed for the study of amebiasis. The rat is employed for enteric infections and the hamster is used for hepatic infections. *E. histolytica* is mostly used to evaluate chemotherapeutic analysis. Amoebae are subjected to direct injection into cecum or liver. For hepatic amoebiasis, a small sponge is soaked with amoeba and incorporated into the liver.

In the in vivo screening models, the animals are treated seven times by oral administration of the check drug, the primary dose being given sometime before challenge infection and also the second 2 h after infection. The remaining five doses are given on the next 5 consecutive days after infection. Metronidazole and emetine are used as standard drugs. Hepatic infections in golden hamsters are introduced by the injection of trophozoites of axenic *E. histolytica* into the liver lobes. The inoculum per hamster is approximately 1×10^5 cells/mL.

The control group contains infected and untreated animals (6 nos.) On day 6, an autopsy of the hamsters is performed and the liver tissue smear is observed under a microscope to detect the mobile trophozoites. Animals having no liver necrosis are considered as a cure.

In the same investigation (Bhutani et al., 1987), a polyaxenic strain of. *E. histolytica* was used to infect weaning rats by inoculation of trophozoites (1×10^5 cells/mL) in the cecal lumen. On day 7 of infection, the animals are sacrificed to observe the amoeba and intestinal lesions. Animals free from intestinal lesions and amoeba are accepted as cure.

Hepatic lesions are easier to study because livers may be excised, weighed, and given arbitrary scores, depending upon the extent of liver damage. With experimental intestinal amoebiasis, cecal wall lesions are assessed macroscopically and the presence or absence of amoebae judged by microscopical examination of lumen contents. The antiamoebic properties of drugs may be assessed by a graded score of lesions and by the presence or absence of amoebae (Neal, 1983). For successful in vivo testing, a virulent strain of *E. histolytica* is required, the strain of rat must be at its most susceptible age, and inoculation normally occurs as soon as possible after weaning. Cholesterol has been shown to increase the severity of cecal infections and care must be exercised over the animal diet. Liver infections produced by combined amoebae and bacteria tend to be severe and high drug doses may be required. When axenic cultures are used, liver lesions are less severe and such models may be more valuable for therapeutic studies (Mukherjee, 2002; Balouiri et al., 2016).

15.10.2 In Vitro Models for Amebiasis

In vitro models for amoebicidal activity have been developed because of the limitations of the in vivo models. The expansion of amoebae on their own (axenic culture) was initially achieved in a diphasic medium and was a serious advance in the development of an in vitro check procedure. Later, a clear, single-phase liquid medium was developed and axenic cultures of *E. histolytica* in such a medium have provided a straightforward methodology for the development of in vitro screening tests (Diamond, 1961, 1968, 1986). In vitro test method was performed to observe the growth of amoebae in a solid medium (Gillin et al., 1982) to determine viability. Later, this procedure has been used for testing of the plant extracts (Phillipson et al., 1987).

15.10.2.1 Materials and Medium for In Vitro Amoebicidal Test

The medium and solutions are sterilized by membrane filtration through 0.22 µm filters and an Amicon pressure vessel is used. Dimethyl sulfoxide, glassware is autoclaved for sterilization.

Growth media is sometimes prepared from basic TPS 1 medium (Diamond's medium) with inactivated horse serum (Difco), a mixture of vitamins, penicillin, and streptomycin mixture and kept at +4 degree. 1 M sodium hydroxide is used for the adjustment of pH 6.9. Basic TPS 1 medium (Diamond's medium) consists of Trypticase (BBI), Panmede (Ox liver digest), glucose, l-cysteine hydrochloride, vitamin C, sodium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and distilled water. Water-soluble vitamins (B, biotin, folic acid) and lipid-soluble vitamins (A, D, E, K) are used to prepare the vitamin mixture. Sterilization is performed by membrane filtration and kept at +4°C. Ampicillin and streptomycin solution is prepared in the ratio of 2:1 by dissolving in distilled water and stored at −20°C for further use. Glucose and phosphate buffer are prepared in distilled water and sterilized by membrane filtration and kept at 4°C (Keene et al., 1986).

15.10.2.2 Maintenance of Cultures

E. histolytica (NIH 200 strain) culture is maintained in Falcon flasks (Nune) and subcultured twice a week. The cultures are observed under an inverted microscope to examine growth as well to detect the presence of any contaminants. Subculturing is done when the amoebae cells are in the exponential phase and the cells are attached to the walls of the falcon. The falcons are chilled (30 min) to detach the cells from the wall and the cell suspension is subjected to centrifuge (1000 rpm) for 05 min. The pellets portion is seeded into media (1 mL) and the cells are counted by hemocytometer. To adjust the cell count (05 amoeba cell/ hemocytometer square), media are used. An appropriate amount of suspension (0.3 mL) is added to each falcon and suspended with media. The seeded falcon flasks are incubated at 37°C (Keene et al., 1986).

15.10.2.3 Amoeba Cell Count

An amoeba cell suspension (20 µL) is added with equal volume of 1% trypan blue. Dead cells of amoebae are stained with blue color and living cells remain colorless. The living cells of amoebae are counted in the four corner squares of the hemocytometer slide. The amoeba cells counted are expressed by dividing by four to get the actual cell number $\times 10^6$ cells/mL suspension (Keene et al., 1986).

15.10.2.4 Cryopreservation of Amoebae

During subculturing of Amoeba cells, a mixture of sterile glucose solution, phosphate buffer solution, and dimethyl sulfoxide are added to the cell suspension, which is further poured into sterilized ampules. After keeping the ampules at room temperature (15 min), the ampules are kept in liquid nitrogen and stored for future use. Once needed for use, an ampule is thawed by placing in a water bath (37°C) and the contents are placed in a small falcon supplemented with the media and incubated at 37°C. The medium is to be changed at a 24-h interval and cells are subcultured when needed (Keene et al., 1986).

15.10.2.5 Amoebicidal Test Protocol

Sufficient test compounds are prepared by serial dilution within the range of 0.01–100 µg/mL by dissolving them in the proper amount of media and dimethyl sulfoxide. The solution is sterilized by membrane filtration. An amoebal suspension (0.1 mL) is added to a sterilized flat sided tube (12 in number), which is further incubated at 37°C for 72 h. Each test method is made in triplicate and untreated cells serve as a control. After incubation, the tubes are chilled and the suspension is centrifuged to obtain a pellet. The pellet is then suspended with the media and an equal volume of 1% trypan blue. The live and dead cells of amoeba are counted in a hemocytometer. A calculation of the percent inhibition of each concentration is performed and the values are expressed in terms of probit values, which are plotted against the log concentrations of the tested compounds. Cumulative index (CI) values are determined (Keene et al., 1986).

15.11 ANTIMICROBIAL RESISTANCE

Antimicrobial resistance has become a major challenge for multiple antimicrobial agents in pathogenic bacteria. Bacteria have been able to evolve efficaciously to become resistant to antibiotics through continuous use. In addition to this problem, antibiotics sometimes produce adverse effects on the host that include hypersensitivity, as well as immunosuppressant and allergic reactions. As a result, antibiotics become ineffective and this is responsible for healthcare-associated infections that persist in the host, which increases the risk of morbidity and mortality (Andersson and Hughes, 2010). Many different types of antibiotic resistance are found to characterize the different patterns of resistance, including MDR, extensively drug-resistant (XDR), and pandrug-resistant (PDR) bacteria.

- Multidrug-resistant (MDR)

  MDR means "resistant to more than one antimicrobial agent." MDR is based on in vitro antimicrobial susceptibility test results, when the test resistant to multiple antimicrobial agents or antibiotics. Another method used to characterize bacteria as MDR, is when they are "resistant to one key antimicrobial agent" (Hidron et al., 2008). MDR bacterial isolates may threaten public health due to the resistance to one key antimicrobial agent, but they often demonstrate cross or coresistance to multiple classes of antimicrobials, which makes them MDR.
- Extensively drug-resistant (XDR)

  XDR has been used as an acronym for several different terms, such as "extreme drug resistance," "extensive drug resistance," "extremely drug resistant," and "extensively drug resistant." Bacteria are classified as XDR due not only to their resistance to multiple antimicrobial agents, but also to their ominous likelihood of being resistant to all, or almost all, approved antimicrobials (Siegel et al., 2007).
- Pandrug-resistant (PDR)

  PDR means "resistant to all antimicrobial agents." A bacterium is considered to be PDR when it is found to be resistant to all approved and useful antimicrobials (Siegel et al., 2007).

15.11.1 Mechanisms for Antimicrobial Resistance

Several mechanisms are responsible for the development of the antibiotic resistance mentioned below. The details are highlighted in Fig. 15.2

(i) mutations of the antibiotic target,
(ii) changes in cell permeability and multiple efflux pumps, and
(iii) horizontal transfer of resistance genes.

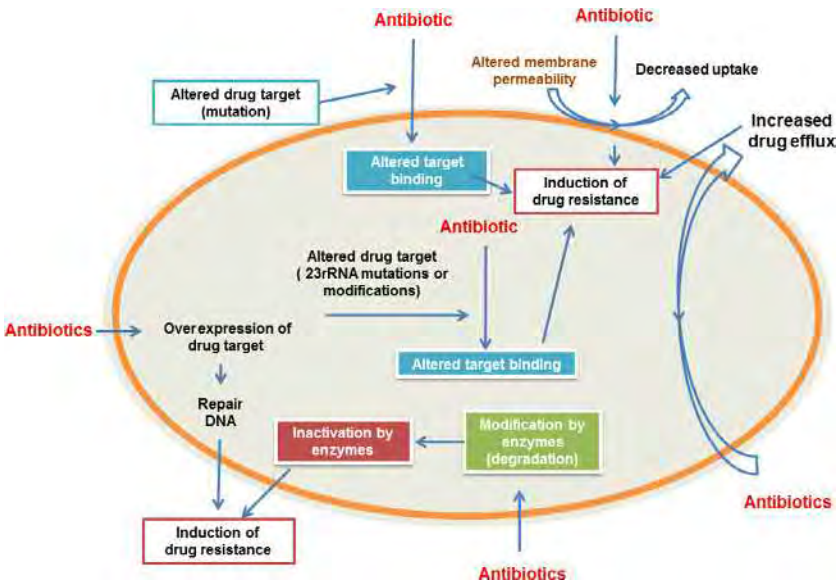


FIG. 15.2 Mechanisms of antimicrobial resistance.

15.11.2 Test Methods Used for Antimicrobial Resistance

Several antimicrobial susceptibility testing methods are used to determine the antimicrobial resistance of a bacterium. Screening tests for MRSA can be assessed on the basis of sensitivity, specificity (and positive predictive value and negative predictive value), turn-around time (time between taking the specimen and reporting the result), and ease of performance. In all methods, the use of control strains is required to ensure that the method is performing correctly, and both BSAC and NCCLS standardized methods include recommendations for susceptible and resistant control strains. Dilution methods including agar dilution and broth dilution are used for MDR, especially MRSA. As per NCCLS (2003), other methods are used for the determination of MRSA, including E-test, break-point method, agar screening method, tube coagulase test, Latex agglutination, automated methods, quenching fluorescence method, and molecular methods (Archer and Pennell, 1990; Bignardi et al., 1996).

15.11.3 Herbs Having Anti-MRSA Potential

From ancient times, various medicinal plants have been used in the treatment of several communicable and noncommunicable diseases. Several scientific reports suggest that *Acorus calamus*, *Lawsonia inermis*, *Hemidesmus indicus*, *H. antidysenterica*, *Punica granatum*, *Plumbago zeylanica*, *Camellia sinensis*, *Delonix regia*, *Terminalia chebula*, *Emblica officinalis*, and *Terminalia bellirica* have in vitro antimicrobial action against MRSA (Aqil et al., 2006; Phongpaichit et al., 2005). St John's Wort (*Hypericum perforatum*), a medicinal plant being used widely as an antidepressant in herbal medicine, has been reported to produce a major antibacterial metabolite with a MIC value of 0.1 mg/mL against methicillin-resistant *S. aureus* (MRSA) and penicillin-resistant variants (Rahman, 2015). Other medicinal plants and their plant parts have significant inhibitory potential against MRSA, including leaves of *C. sinensis*, *Azadirachta indica*, *H. antidysenterica* (Kurachi) bark, *P. granatum* (Pomegranate) rind, *H. indicus* (Anantamul) stem, and *P. zeylanica* (Chitra) root (Mehrotra et al., 2010; Aqil et al., 2005, 2006; Hena and Sudha, 2011;). Several scientific documents suggest that *Acanthus ilicifolius* L. leaf; *Aegle marmelos* (L.) fruit; *Aframomum melegueta*, *Ageratum conyzoides* L. leaf, *Alnus nepalensis*, *Arctium minus* aerial parts, *Argyreia speciosa* leaf; *Blumea balsamifera* (L.), *Boswellia sacra*, *Brandisia hancei* Hook, *B. monosperma* leaf, *Callicarpa farinosa* Roxb. Bark, *Callistemon lanceolatus* leaf, *C. sinensis* (L.), *Cirsium palustre*, *Curculigo orchioides*, *Dalbergia paniculata* Roxb., and *Desmodium caudatum* (Thunb.) also possess MRSA inhibitory potential (Zuo et al., 2012; Kenny et al., 2014; Dubey and Padhy, 2013; Lai et al., 2010; Hasson et al., 2011; Sahu and Padhy, 2013; Chung et al., 2014). Several plants and their isolated compounds that have been evaluated for antimicrobial activity are explained in Table 15.1.

Several phytoconstituents isolated from medicinal plants also show in vitro activity against MRSA. Phytoconstituents with reported anti-MRSA activity include β-asarone from *A. calamus* rhizome, Prenylated flavonoids from *D. caudatum* root, and eupomatenoid-5 from *P. regnellii* leaves. Kaempferol-3-*O*-(2″,3″,4″-tri-*O*-galloyl)-α-L-rhamnopyranoside, quercetin-3-*O*-(3″,4″-di-*O*-galloyl)-α-L-rhamnopyranoside, and quercetin-3-*O*-(2″,3″,4″-tri-*O*-galloyl)-α-L-rhamnopyranoside are three novel galloylated flavonol rhamnosidesides from *C. tergemina* leaves that have anti-MRSA activity. Seven prenylated flavonoids and one prenylated chromanochroman isolated from *D. caudatum* also show in vitro anti-MRSA activity (Chan et al., 2014; Sasaki et al., 2012). Berberine isolated from *Hydrastis canadensis* L. leaf shows inhibitory potential against the MRSA strain (Cech et al., 2012). Some techniques have been subjected to standardization by the NCCLS, CLSI, and EUCAST, marking major remarkable steps on this field. However, some modifications of the standardized protocols for antimicrobial testing are often performed in the case of botanicals. For this study, the use of solvents may also affect the growth of the tested microorganisms and for this some minor methodological modifications to standardized protocols can be a solution to ensure accurate experimental protocols to further explore antimicrobials from natural resources.

Infections caused by microorganisms have become an imperative clinical threat, with significant associated morbidity and mortality, which is mainly due to the development of antimicrobial resistance. The public health threat of MDR, including MRSA, is increasing in developing, as well as in developed, countries. Antimicrobial susceptibility testing has been comprehensively used to discover novel antimicrobials from natural resources. Scientific evidence suggests that several medicinal plants and their active phytoconstituents have demonstrable anti-MRSA activity and have a prospect of being considered as a potential treatment option for MRSA infections. Due to the presence of various secondary metabolites, the medicinal plants used in traditional medicine have a great potential for development of leads in antimicrobial resistance. These aspects may be explored further for the evaluation of the antimicrobial potential of the extract, enriched fractions, and the isolated compounds from different medicinal plants that have been used in therapy for a long time.

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Chapter 16

Antiviral Evaluation of Herbal Drugs

Chapter Outline

16.1 ANTIVIRAL POTENTIALS OF HERBS

Although vaccines have been very successful in controlling many viral diseases, some diseases are likely to be controlled only by antiviral chemotherapy. The concept of antiviral drugs has only been accepted slowly, partly because of the toxicity of many of the earlier antiviral agents. In contrast to the development of antibiotics, attempts to develop antiviral drugs have indeed met a variety of problems. Being strictly dependent on cellular metabolic processes, viruses possess only limited intrinsic enzyme systems and building blocks that may serve as specific targets for a drug. Moreover, contrary to a bacteriostatic compound, an effective antiviral drug should not only display considerable specificity in its antiviral action, but should also irreversibly block viral synthesis in order to stop cell suicide due to the viral infection and restore normal cell synthesis (Vanden Berghe et al., 1986). In addition to this inhibition, the antiviral agent must have a broad spectrum of activity, favorable pharmacodynamic properties, and not be immunosuppressive. In the ideal situation, the antiviral drug checks the infection while the immune system prepares to destroy the last virus particles (Munro et al., 1987). This point is critical for those immune-compromised by illness (AIDS, cancer) or drug therapy (transplants, cancer). A frequent cause of death in these instances is from viral infections, so that adjuvant antiviral chemotherapy is vital in these circumstances (Shannon and Schabel, 1980).

Many viral infectious diseases still cause high mortality. Although antiviral chemotherapy has shown outstanding progress, antiviral agents are still required. The emergence of drug-resistant viruses during treatment raises a potential problem for effective therapy. Furthermore, new viral pathogens may be discovered. Biologically active substances of plant origin have long been known as viral inhibitors. These antiviral compounds may be extracted from sources, such as higher plants, which have, for various reasons, been explored considerably less than the traditional ones.

The first clinically useful antiviral drug was methylisatin–thiosemicarbazone (Methisazone), which was active against pox viruses, especially smallpox virus. Methisazone (25 mg/kg) was found to inhibit Variola virus in mice and was later

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used successfully to treat cases of eczema vaccinatum and to treat and prevent smallpox. Kaufman et al. (1962), an ophthalmologist, successfully treated a herpes eye infection with an antineoplastic drug, Idoxuridine. At the same time, a group of chemists at the Du Pont chemical company in the United States investigated the antiviral activity of a molecule called Amantadine. It was active against influenza virus type A. In rapid succession, further nucleoside analogs, cytosine arabinoside, trifluorothymidine, and adenine arabinoside, were found to inhibit herpes virus. In the 1970s, the antiherpes activity of a new compound, 9-(2-hydroxyethoxymethyl) guanine (Acyclovir), was detected by J. Bauer at the Wellcome Research Labs. Within a decade, the same group was to discover azidothymidine (Zidovudine), the first effective inhibitor of the newly emerged HIV. The alkaloid from the Australian horse chestnut (Castanospermine australe), isolated in the 1980s, was also found to be active against HIV.

A research program to detect and isolate antiviral compounds from higher plants is best carried out by a multidisciplinary team, consisting of at least a pharmacognosist and a virologist. The antiviral screening system should meet all requirements of any good assay, including validity, lack of ambiguity, accuracy, reproducibility, simplicity, and reasonable cost. Moreover, because we are dealing with plant extracts, the antiviral screen should be highly selective, specific, and sensitive; it is advisable to discriminate a true antiviral activity from a viricidal one at this stage. Because most of the aforementioned requirements are better met by in vitro testing, we not only prefer in vitro screening of the plant extracts, but also the use of the same bioassay to guide the isolation of the antivirally active compounds from the plant extracts. The antiviral activity of the pure compounds then has to be confirmed in a later stage by in vivo assays (Leven et al., 1982; Vanden Berghe et al., 1986).

One of the possible strategies for finding new antiinfective drugs may involve the search for compounds with chemotherapeutic activities supplementary to, and structures widely different from, those in current use. These compounds could be extracted from sources that have, for various reasons, been explored considerably less than the traditional microorganisms, including, among others, higher plants (Mitscher and Rao, 1984). Considering the enormous number and the amazing structural diversity of the currently available antimicrobially and antivirally active plant constituents, one might hope that promising systemic and/or locally acting antiinfective agents might be discovered in the plant kingdom (Vanden Berghe et al., 1986; Vlientinck et al., 1988).

The increase of drug-resistant viruses in treatment raises an important issue for effective treatment. Moreover, new viral pathogens might be found. Along these lines, there is a strong requirement for promptly accessible antiviral medications at moderate costs with minimal side effects. Henceforth, traditional medicines ought to be investigated as novel antiviral agents, as many of these ancient medicaments, containing different plant metabolites, have potent antiviral activities (Chattopadhyay and Khan, 2008).

The design of new antiviral drugs potentially focuses on the structural dynamics and replication cycles of the various infections causing viruses. A suitable example is the invention of acyclovir, which hinders certain proteins of herpes infections responsible for the triggering of disease. Ethnomedicines are a vast repository of structural diversities and extensive bioactivities that can serve as a huge source of potential antiviral drugs. A significant number of medicinal plants from Ayurveda and the traditional Chinese system of medicine serve as potential remedies to decrease the severity of illness caused by viruses (Chattopadhyay et al., 2009; Khan et al., 2005; Jadhav et al., 2012).

Research on the antiviral potentials of plants was first started in 1952, and 12 out of 288 plants were found to be effective against influenza (Chattopadhyay and Naik, 2007). Numerous screening studies have been conducted in the last few years to determine the antiviral efficacy of medicinal plants using in vitro and in vivo assays. A few out of a 100 British Colombian medicinal plants showed antiviral efficacy against respiratory syncytial virus (RSV), corona virus, influenza virus, and herpes simplex virus (HSV) (McCutcheon et al., 1995), while the marine algae *Spirulina* showed antimutagenic, immunomodulatory, and antiviral activities (Chamorro et al., 1996). Interestingly, Cyanovirin N, an 11-kDa protein of blue green algae, inactivates HIV-1 by binding with its glycoprotein120 (Clercq De, 2000), while sulfated polysaccharides of seaweeds and algae showed anti-HIV and anti-HSV activities (Schaeffer and Krylov, 2000).

The plant kingdom is highly diverse and ranges from unicellular microscopic plants to long lived, huge trees. To screen each and every plant or their individual parts for the identification of antiviral components is a huge task. Several examples of plants having antiviral properties and newly identified active compounds from them are reported in various journals. One of the examples that can be cited here is cyanovirin N (CV-N), an 11-kDa protein isolated from the cyanobacterium *Nostoc ellipsosporum*, which exhibits the property of inhibiting HIV-1 infection and also possesses broad-spectrum activity. The phytochemical, Podophyllotoxin, isolated from the aqueous extract of *Podophyllum peltatum* L., inhibited HSV type 1 (HSV-1) (Bedows and Hatfield, 1982). The acetone extract of another plant, *Phyllanthus urinaria*, also suppressed HSV-2 and HSV-1 (Yang et al., 2007). Bessong et al. (2006) reported a comparison of various plants and their individual parts (stem, leaves, roots, and so forth.) in repressing viral reverse transcriptase (RT) and integrase, the two basic enzymes in HIV disease. After comparing all the extracts and fractions of the various plants, it was found that the *n*-butanol fraction of *Bridelia micrantha* (Hochst) exhibited the highest anti-RT activity. It has also been reported that the aqueous extract from the

roots of *Carissa edulis* (Forssk.) Vahl, a plant grown in Kenya, displayed noteworthy activity against HSV for both wild type and resistant strains (Tolo et al., 2006). Polyphenol-rich extract from the medicinal plant *Geranium sanguineum* L. has been reported to show a strong antiinfluenza virus activity, as well as antioxidant and radical scavenging capacities (Sokmen et al., 2005)

Hepatitis A, B, C, D, and E viruses are the leading causes for the prevalence of viral hepatitis and liver inflammation. Despite the fact that presentation to any of these infections prompts intense disease, in any case, types B, C, and D are unique in causing chronic infection. Plants belonging to the genus *Phyllanthus* of the Euphorbiaceae family were extensively used as a traditional remedy against these infections. Clinical investigations were additionally intended to look at the inhibitory effects of different species of *Phyllanthus*, that is, *P. amarus* (L.), *P. niruri* (L.), and *P. urinaria* (L.) (Wang et al., 1995). The screening of 56 different Chinese medicinal herbs led to the identification of two potent plant extracts against Duck hepatitis B virus, namely, *Ardisia chinensis* and *Pithecellobium clypearia* (Leung et al., 2006). Similarly, this also led to the identification of *Oenanthe javanica* (Blume) DC flavones (OjF). They acted as a strong inhibitor of HBsAg and HBeAg secretion (involved in viral pathogenesis) in 2.2.15 cells and also reduced DHBV-DNA levels in the HBV-infected duck model (Wang et al., 2005). Because of the strong prevalence of HCV infection in poor countries, screening for the identification of anti-HCV potentials from medicinal plants are still ongoing. According to Hussein et al. (2000) various plant extracts, such as methanol extracts *Acacia nilotica* L. Willd ex Delile, *Boswellia carterii*, *Embelia schimperi*, *Quercus infectoria*, *Trachyspermum ammi* L., and aqueous extracts of *Piper cubeba* L., *Q. infectoria*, and *Syzygium aromaticum* L., were found to possess significant inhibitory activity against HSV.

Combination therapy for treating diseases is an age-old practice of traditional medicine in which several plants are mixed together to develop an effective formulation for a particular disease. Such combination therapies have also been tried for the inhibition of viral hepatitis. As an example, a Chinese herbal medicine, prepared by liquid fermentation broth of *Ganoderma lucidum* supplemented with an aqueous extract of *Radix Sophorae flavescentis*, was potent against hepatitis B virus activity in vitro and in vivo. Viral infections are a matter of great concern for this planet. Plants having broad-spectrum activity against many viruses could be evaluated for isolation and identification of molecules for treating viral infections. As an example, glycyrrhizin, a bioactive component of licorice (*Glycyrrhiza uralensis* Fisch), and lycorine, isolated from *Lycoris radiata* L., showed strong anti-SARS-CoV activity, and was initially used for treating other indications (Li et al., 2005a, b).

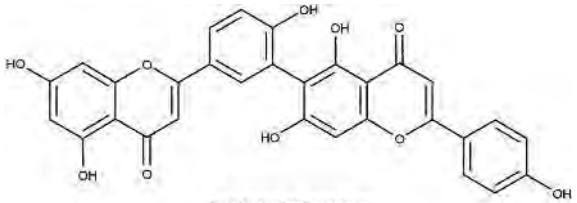
A variety of herbal preparations have shown potentials for inhibiting viruses that cause serious infections among humans, such as measles viruses (Olila et al., 2002), human rotaviruses (HRV) (Husson et al., 1994; Takahashi et al., 2001), respiratory syncytial virus (RSV), human rhinoviruses (Glatthaar-Saalmuller et al., 2001), the coxsackie group of viruses (Evstropov et al., 2004; Su et al., 2006), neurotropic Sindbis virus (NSV) (Paredes et al., 2001), and various strains of polio virus (Andrighetti-Frohner et al., 2005; Melo et al., 2008). One such illustration is the atomic investigation of the heated water concentrates of *Stevia rebaudiana* L., which blocked a section of different irresistible serotypes of HRV into permissive cells by an anionic polysaccharide having a molecular weight of 9800 with uronic acid as a noteworthy sugar constituent (Takahashi et al., 2001). Thus, an alkaloid concentrate of *Haemanthus albiflos* globules repressed RNA amalgamation of HRV spread in MA-104 cells (Husson et al., 1994).

16.2 PHYTOCONSTITUENTS HAVING ANTIVIRAL POTENTIALS

In contrast to the many publications on antibacterial and antifungal screening of plant extracts that have appeared in the last decades, far fewer antiviral screening studies of plant extracts have been reported. This is due chiefly to the complexity of the different techniques involved in such research, which consequently requires the know-how and dedication of a multidisciplinary team. Nevertheless, many antiviral agents have been isolated from natural sources and partly or completely characterized. From these studies, several substances have emerged as true antivirals having a good chemotherapeutic index based on the viability of infected cells and on in vivo tests. Thus, different 3-methoxy flavones and synthetic derivatives have shown to be promising leads for the development of antirhinovirus drugs (Van Hoof et al., 1984; Vlientinck et al., 1988), whereas the spanonin, glycyrrhizic acid, was found to be highly active in vitro against herpes simplex (Pompei et al., 1979), varizella-zoster (Baba and Shijeta, 1987), and human immunodeficiency viruses (Ito et al., 1987). Whether these compounds have any clinical potential, that is, in the therapy of the corresponding viral diseases, remains to be determined. There, in vivo bioavailability and other pharmacokinetic parameters are subjects of future study.

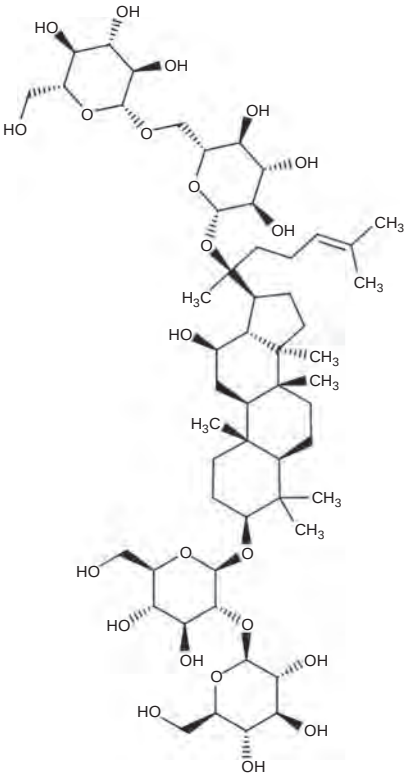
Because of the problems of drug resistance stated earlier and of side effects, the pharmaceutical industry is looking forward toward natural products (mainly medicinal plant extracts) as a source of possible antiviral agents. Approximately 2500 medicinal plant species have been recorded globally to treat a myriad of inflictions and diseases. Polyphenols, alkaloids, flavonoids, saponins, quinones, terpenes, proanthocyanidins, lignins, tannins, polysaccharides, steroids, thiosulfonates, and coumarins are prominent bioactive phytochemicals that have been observed to combat viral infections, as they are harmless to the systems of the human body. Some selected anthraquinones and anthraquinone derivatives are noted for their

antiviral and viricidal activities against viruses representing several taxonomic groups. One of these compounds, hypericin, has activity against vesicular stomatitis virus, HSV types 1 and 2, parainfluenza virus, and vaccinia virus (Andersen et al., 1991). The mechanism of inhibition of HIV-1 infection by purified extracts of *Prunella vulgaris* in the lymphoid cell line MT-4, in the monocytoid cell line U-937, and in peripheral blood mononuclear cells is that it antagonizes HIV-1 infection by preventing HIV-1 viral attachment to the CD-4 receptor (Yao et al., 1992). The mannose-specific lectins from the orchid species *Cymbidium hybrid*, *Epipactis helleborine*, and *Listera ovata* were highly inhibitory to HIV-1 and HIV-2 in MT-4 and showed a marked anti-HCMV, antirespiratory syncytial virus and antiinfluenza A virus activity in HEL, HeLa, and MDCK cells, respectively (Balzarini et al., 1992). Five Australian *Phyllanthus* species have been found to have an antiviral effect on the duck hepatitis B virus endogenous DNA polymerase (Shead et al., 1992). Hudson and Towers (1995) noted the influence of reaction parameters on antiviral assays of phytochemicals.

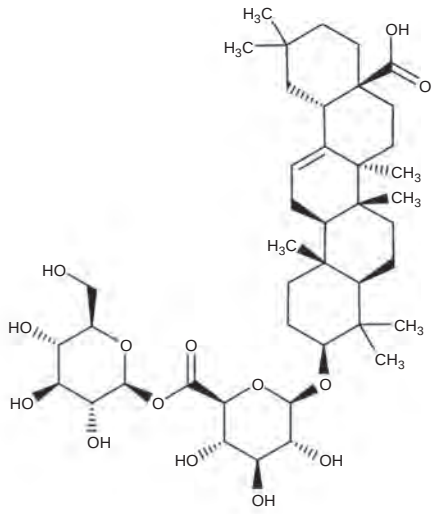


Robustaflavone

Dammarane saponins, ginsenoside Rb1 (GRb1), and Chikusetsusaponin (Chi-III) have been found to possess antiviral activity against HSV-I using an in vitro plaque elimination assay. Chi-III prevented plaque formation at half the concentration of GRb1 (Fukushima et al., 1995).



Ginsenoside Rb1



Chikusetsusaponin

Hudson and his coworkers have assayed 31 species of medicinal plants used in the treatment of diseases of viral origin in the Yunnan province of China for the inhibition of Sindbis virus and Murine Cytomegalovirus in mammalian cell cultures in which 16 species displayed antiviral activity (Hudson and Towers, 1995). The inhibitory effect of ferulic acid and isoferulic acid on murine interleukin-8 production in response to influenza virus infections in vitro has been reported (Hirabayashi et al., 1995) and the effect of isoferulic acid was found to be greater than that of ferulic acid. Hayashi et al. (1995)

found a direct inhibitory activity of the steam distillate prepared from fresh plants of *Houttuynia cordata* (Saururaceae) against HSV-1, influenza virus, and HIV-1, without showing cytotoxicity, but not against Poliovirus and Coxsackie virus. Montanha et al. (1995a) evaluated the action of a series of 19 Aporphine alkaloids against HSV-1 in cell cultures. On the basis of viral titer reduction, six alkaloids were found to be active. Ellagitannins isolated from *Phyllanthus myrtifolius* and *P. urinaria* (Euphorbiaceae) showed activity against Epstein–Barr virus DNA polymerase. Flavonoidal constituents, such as biflavonoids and robustaflavones, exhibited strong inhibitory effects against influenza A and B viruses. The antiviral potential of the flavonoids of *Chamaesyce thymifolia* has been reported; they showed high cytotoxicity on HEp-2 cells and moderate inhibitory activity against HSV-1 and bovine viral diarrhea virus (Amaral et al., 1999). Sotanaphun et al. (1999) isolated Sclerocarpic acid from the stem bark of *Glyptopetalum sclerocarpum*, which exhibited antiviral activity against Herpes simplex virus types 1 and 2. Rhamnan sulfate, a natural sulfated polysaccharide isolated from *Monostroma latissimum*, showed potent inhibitory effects on the virus replication of HSV-1, HCMV, and HIV-1 in vitro (Lee et al., 1999). Matsuse et al. (1999) tested aqueous and methanolic extracts of 39 Panamanian medicinal plants for anti-HIV effects. Seven of these were found to be moderate inhibitors of HIV-protease enzyme. Serkedjieva and Ivancheva (1999) investigated the inhibitory effect of five extracts from the Bulgarian medicinal plant *G. sanguineum* on Herpes simplex virus types 1 and 2. Yoosook et al. (1999) evaluated the anti-HSV-2 activities of *Barleria lupulina* and *Clinacanthus nutans*. The results suggested a therapeutic potential against HSV-2 for *B. lupulina*, but not for *C. nutans*. The antiviral activities of various water and methanol soluble substances isolated from *G. lucidum* against HSV types 1 and 2, influenza A virus, and vesicular stomatitis virus were studied using cytopathic effect inhibition assay and plaque reduction assay (Eo et al., 1999). Kudi and Myint (1999) investigated the antiviral activity of medicinal plant extracts against Poliovirus, Astrovirus, HSV, and Parvovirus. Most of the extracts showed activity against more than one virus at a dose rate of between 100 and 400 µg/100 µL (Eo et al., 1999). Bourne et al. (1999) assessed 19 plant products in vitro by plaque reduction assay to determine their activity against a commonly transmitted pathogen, Herpes Simplex virus type 2. Docherty et al. (1999) found that Resveratrol, a phytoalexin, inhibited HSV-1 and HSV-2 replication in a dose-dependent reversible manner.

Anani et al. (2000) prepared methanol extracts from 19 medicinal plants of Togo and analyzed them for antiviral and antibiotic activities. Ten of the 19 showed significant antiviral activity against one or another of the test viruses (Herpes Simplex, Sindbis, and Poliovirus). Hudson et al. (2000a, b) evaluated ethanolic extracts of 11 plants, endemic to Madagascar, in order to determine the potential of Malagasy plants as sources of antiviral activities. Nine of the extracts had significant activity against HSV, whereas only four were active against the Sindbis virus. A bioactive flavonoid, "Baicalein," isolated from the Chinese medicinal plant *Scutellaria baicalensis* Georgi showed antiviral properties using the high-speed countercurrent chromatography (HSCCC) technique (Li and Chen, 2005).

Many other substances, including flavonoids, phenolics, tannins, triterpenes, and alkaloids, interfere with host cell replication at their antivirally active concentrations or only exhibit extracellular viricidal activities. Some of these viricides, including flavonoids and tannins present in foodstuffs, might be important, because they can inhibit virus replication of picorna-, rota-, and arena viruses in the gastrointestinal tract of humans and animals.

Artemisia capillaris was found to possess an active 6,7-dimethylesculetine having potent cytotoxic potential. In the fruits of *Schisandra chinensis* (schizandraceae) used in oriental medicine, 22 lignans were identified, some of which, such as Wuweizisu C and Gomisine N, are very active. The same method threw light on the mechanism of the antihepatotoxic action of such well-known compounds as glycyrrhizin and its intestinal metabolites, which are protective against the first stages preceding hepatic lesions. Other tests of this type are used to track down active substances. From *Taxus baccata*, Potier's group isolated new analogs of taxol, a terpenic compound displaying very good antileukemic and antitumor activities. Taxol promotes the polymerization of tubulin into microtubules and inhibits their depolymerization. The choice among various fractions obtained by extraction was guided by the antitubulin activity in an in vitro test. Many substances that are present in only trace quantities and are difficult to purify have been studied chemically; for example, the demonstration of xylose-bearing derivatives is new in this series of compounds. Regarding structure activity relationships, in vitro cytotoxicity tests have shown that the activity is mainly related to the presence of a complex ester function in the compound. A list of plant extracts and their phytoconstituents that have antiviral potentials are listed in Table 16.1.

In recent years, a lot of development has taken place regarding the identification of antiviral molecules from plant sources and the molecular mechanistic approach. Compounds, such as Spiroketalenol ether derivatives isolated from rhizome extract of *Tanacetum vulgare*, have been reported to block virus entry and also arrest the synthesis of HSV-1 gC and HSV-2 gG glycoproteins (Fernandes et al., 2012). Samarangenin B from the roots of *Limonium sinense* exhibited inhibition of HSV-1α gene expression (Kuo et al., 2002), whereas Oxyresveratrol from *Artocarpus lakoocha* plant was found to inhibit the early and late phases of viral replication of HSV-1 and HSV-2, respectively (Chuanasa et al., 2008). Also, Pterocarnin A compound isolated from *Pterocarya stenoptera* inhibited HSV-2 from binding and penetrating to the host cells (Cos et al., 2003). The structures of some of the potential phytoconstituents having significant antiviral activity are depicted in Fig. 16.1.

TABLE 16.1 Several Viruses Inhibited by Plants and Their Active Constituent(s)

Plant Species	Family	Active Constituent	Activity	Reference
HIV virus				
Buchenavia capitata	Combretaceae	*O*-demethyl-buchenavianine	Produces partial protection against the cytopathic effect of HIV in cultured human lymphoblastoid cells	Vlietinck et al. (1997)
Ancistrocladus korupensis D. Thomas and Gereau	Ancistrocladaceae	Michellamines D and Michellamines F	Exhibited in vitro HIV-inhibitory activity	Halloch et al. (1997)
Schumanniophyton magnificum	Rubiaceae	Schumannificine	Activity against HIV and anti-anti-HSV	Vlietinck et al. (1997)
Berberis vulgaris	Berberidaceae	Berberine	Antiviral effects against HIV-1	Manske and Brossi (1990)
Glycyrrhiza glabra	Leguminosae	Licopyranocoumarin	Inhibit giant cell formation in HIV-infected cell cultures without any observable cytotoxicity	Vlietinck et al. (1997)
Syzygium claviflorum	Myrtaceae	Betulinic acid	Potent against HIV	Fujioka et al. (1994) and Cichewicz and Kouzi (2004)
Curcuma longa	Zingiberaceae	Curcumin	Active against HIV	Mathew and Hsu (2018)
Rheum palmatum	Polygonaceae	Sennoside A	Active against HIV-1	Esposito et al. (2016)
Securigera securidaca	Fabaceae	Kaempferol	Active against HIV-1	Behbahani et al. (2014)
Olea europaea	Oleaceae	Maslinic acid	Anti-HIV	Qian et al. (2011)
Artemisia annua	Asteraceae	Artemisinin	Anti-HIV activity	Lubbe et al. (2012)
HSV virus				
Actinodaphne hookeri	Lauraceae	Actinophnine	Active against HSV-1	Montanha et al. (1995a)
Peganum harmala	Zygophyllaceae	Harmaline and harmine	Have antiviral effect against the DNA-containing herpes virus type 1 (HSV-1)	Rashan (1990)
Pedilanthus tithymaloides	Euphorbiaceae	Luteolin	Active against HSV-2	Ojha et al. (2015)
Mallotus peltatus	Euphorbiaceae	Ursolic acid	Active against HSV-1 and HSV-2	Bag et al. (2012)
Achyranthes aspera	Amaranthaceae	Oleanolic acid	Active against HSV	Mukherjee et al. (2013)
Terminalia chebula	Combretaceae	Chebulagic acid and punicalagin	Active against HSV-1	Lin et al. (2011)
Artocarpus lakoocha	Moraceae	Oxyresveratrol	Inhibit viral replication	Chuanasa et al. (2008)
Influenza virus				
Syzygium aromaticum	Myrtaceae	Eugenol	Inhibit the activation of extracellular signal-regulated kinase, p38-mitogen-activated protein kinase, IkB kinase (IKK)/ NF-kB signal pathways	Dai et al. (2013)

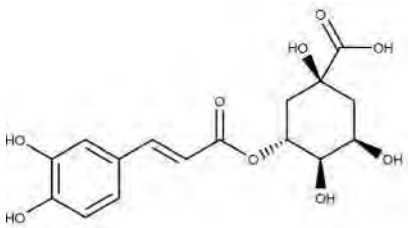
TABLE 16.1 Several Viruses Inhibited by Plants and Their Active Constituent(s)—cont'd

Plant Species	Family	Active Constituent	Activity	Reference
Houttuynia cordata	Saururaceae	Quercetin 3 rhamnoside	Inhibit replication in the initial stage of antiinfluenza A virus infection by indirect interaction with virus particles	Choi et al. (2009)
Caesalpinia sappan	Fabaceae	3-Deoxysappanchalcone	Antiinfluenza, apoptosis, and antiinflammation	Yang et al. (2012)
Angelica keiskei	Apiaceae	Xanthokeistal A	Neuraminidase inhibitor	Park et al. (2011)
Rubus coreanus	Rosaceae	Gallic acid	Influenza virus	Lee et al. (2016)
Lonicera japonica Thunb	Caprifoliaceae	Chlorogenic acid	Influenza A (H1N1/H3N2) virus	Ding et al. (2017)
Melaleuca alternifolia	Myrtaceae	Terpinen-4-ol, terpinolene	Anti-A/PR/8 virus (H1N1)	Garozzo et al. (2011)
Hepatitis B virus (HBV)				
Liriope platyphylla	Asparagaceae	LPRP-Et-97543	Inhibit viral gene expression and replication. Inhibit viral promoter activity	Huanga et al. (2014)
Piper longum Linn.	Piperaceae	Piperine	Inhibit the secretion of HBV surface antigen	Jiang et al. (2013)
Swertia macrosperma	Gentianaceae	Swermacrolactones and luteolin	Inhibit secretion of HBV surface antigen	Wang et al. (2013)
Phyllanthus niruri L.	Phyllanthaceae	Nirtetralin A	Anti-HBV activities	Wei et al. (2012)
Phyllanthus urinaria	Phyllanthaceae	Ellagic acid	Effective against hepatitis B	Kang et al. (2006)
Hepatitis C virus (HCV)				
Bupleurum kaoi	Apiaceae	Saikosaponin b2	Inhibiting early HCV entry, including neutralization of virus particles, preventing viral attachment	Lin et al. (2015)
Ruta angustifolia	Rutaceae	Chalepin and pseudane IX	Inhibited HCV at the postentry step and decreased the levels of HCV RNA replication and viral protein synthesis	Wahyuni et al. (2014)
Syncephalastrum racemosum	Syncephalastraceae	Ursolic acid	Anti-HCV activity	Fu et al. (2013)
Embelia ribes	Primulaceae	Quercetin	Active against HCV	Bachmetov et al. (2012)
Vaccinium virgatum Aiton	Ericaceae	Proanthocyanidin	Inhibit HCV replication	Takeshita et al. (2009)
Respiratory syncytial virus (RSV)				
Schefflera heptaphylla	Araliaceae	Dicaffeoylquinic acids	Inhibition of virus–cell fusion in the early stage and the inhibition of cell–cell fusion at the end of the RSV replication cycle	Li et al. (2005a, b)

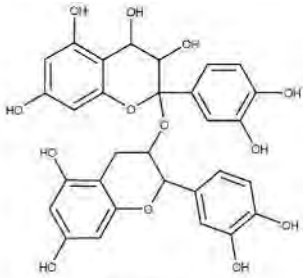
Continued

TABLE 16.1 Several Viruses Inhibited by Plants and Their Active Constituent(s)—cont'd

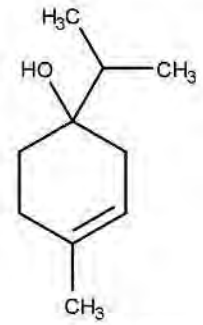
Plant Species	Family	Active Constituent	Activity	Reference
Citrus reticulata	Rutaceae	Tangeretin and nobiletin	Affected the intracellular replication of RSV. Tangeretin down regulated the expression of RSV phosphoprotein (P protein)	Xu et al. (2014)
Rosmarinus officinalis	Lamiaceae	Carnosic acid	Inhibit replication of RSV	Shin et al. (2013)
Cimicifuga foetida L.	Ranunculaceae	Cimicifugin	Inhibit viral attachment and internalization	Wang et al. (2012)
Vesicular stomatitis virus (VSV)				
Melia azedarach L.	Meliaceae	1-Cinnamoyl-3,11-dihydroxymeliacarpin (CDM)	CDM blocks VSV entry and the intracellular transport of VSV-G protein and confined it only to the Golgi apparatus	Barquero et al. (2006)
G. glabra	Fabaceae	Glycyrrhizin	Inhibit phosphorylation enzymes and latency of VSV	Fiore et al. (2008)
Calendula arvensis	Asteraceae	Oleanolic acid	Inhibit VSV multiplication	De Tommasi et al. (1991)
Dengue virus (DEN)				
Magnolia grandiflora	Magnoliaceae	Honokiol	Inhibits dengue virus type 2 infection	Fang et al. (2015)
Scutellaria baicalensis	Lamiaceae	Baicalein	Viricidal against DEN-2	Zandi et al. (2012)
Epstein–Barr virus (EBV)				
Glycyrrhiza radix	Leguminosae	Glycyrrhizic acid	Interferes with an early step of EBV replication cycle	Lin (2003)
Saururus chinensis	Saururaceae	Manassantin B	Inhibitory effects toward EBV lytic replication	Cui et al. (2014)
Polio virus (PV)				
Baccharis gaudichaudiana	Compositae	Apigenin	Anti-PV type 2	Visintini et al. (2013)
Dianella longifolia	Xanthorrhoeacea	Chrysophanic acid	Inhibit PV 2 and PV 3 replication	Semple et al. (2001)
Pterocaulon sphacelatum	Asteraceae	Chrysosplenol C	Inhibit PV	Semple et al. (1999)
SARS-corona virus (SARS-CoV)				
G. glabra	Fabaceae	Glycyrrhizin	Anti-SARS-CoV	Fiore et al. (2008)
Lycoris radiata	Amaryllidaceae	Lycorine	Anti-SARS-CoV	Li et al. (2005a, b)
Ebola virus				
Aglaia foveolata	Meliaceae	Silvestrol	Active against Ebola virus	Biedenkopf et al. (2016)
C. longa	Zingiberaceae	Curcumin	Active against Ebola virus	Mathew and Hsu (2018)



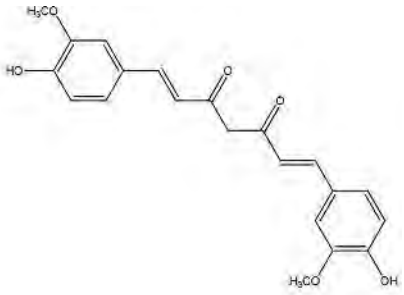
Chlorogenic acid



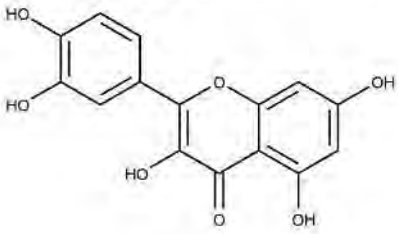
Curcumin



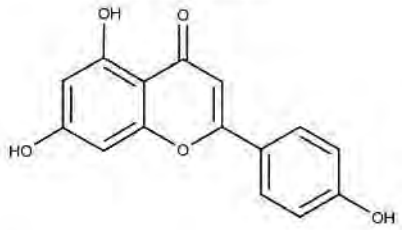
Terpinen-4-ol



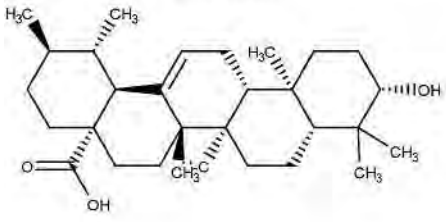
Procyanidin



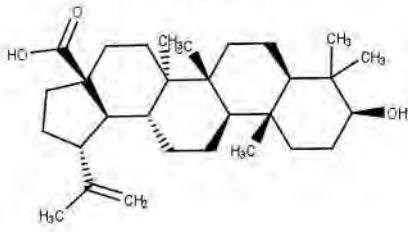
Quercetin



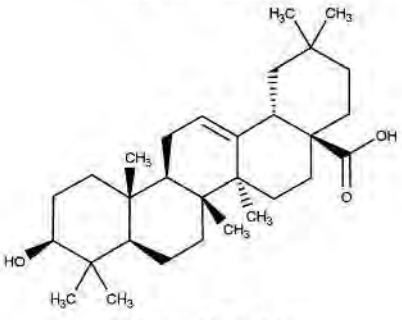
Apigenin



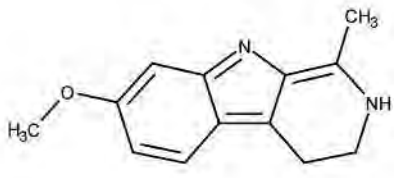
Ursolic acid



Betulinic acid



Oleanolic acid



Harmaline

FIG. 16.1 Several important antiviral compounds from plant sources.

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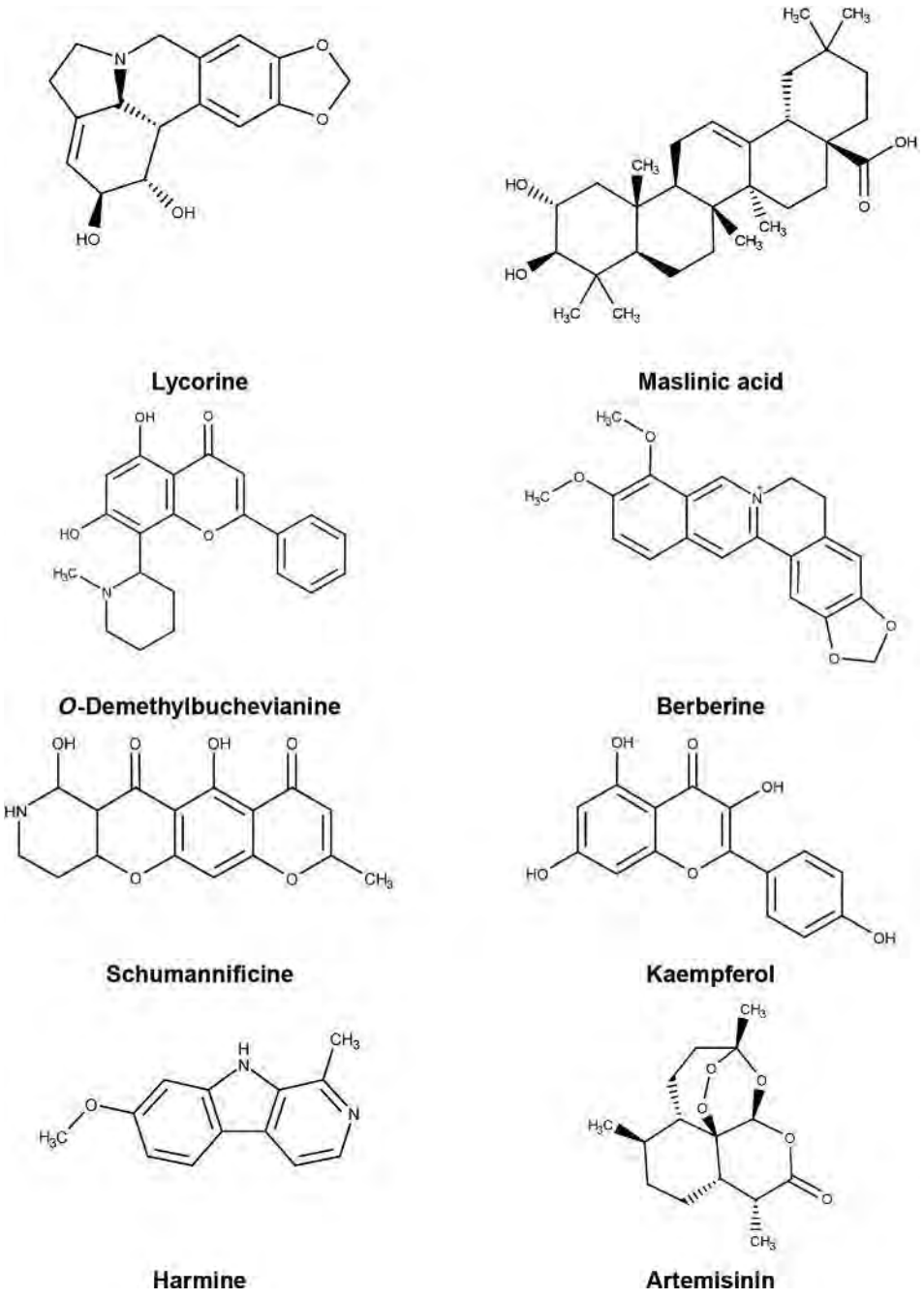
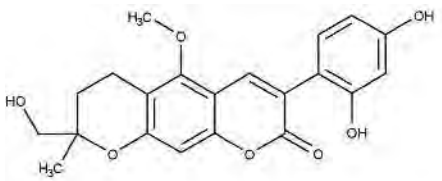
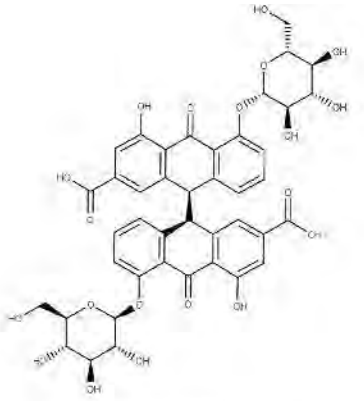


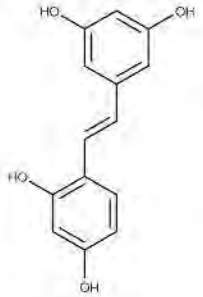
FIG. 16.1 Cont'd



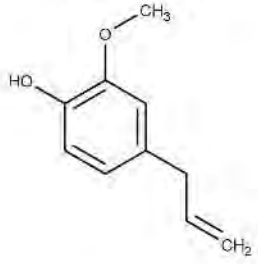
Licopyranocoumarin



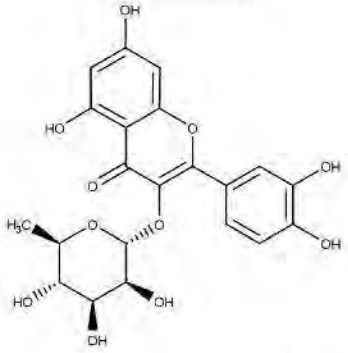
Sennoside A



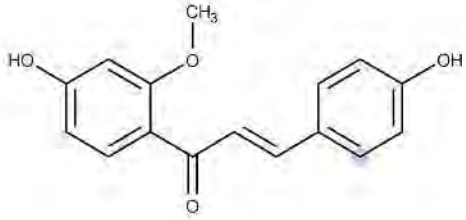
Oxyresveratrol



Eugenol



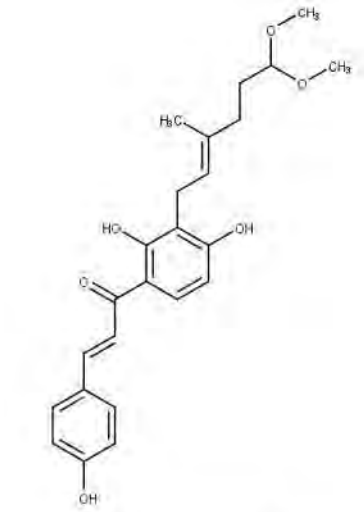
Quercetin 3 rhamnoside



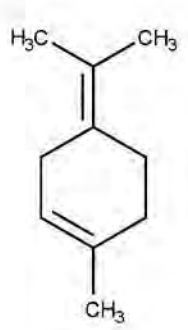
3-Deoxysappanchalcone

FIG. 16.1 Cont'd

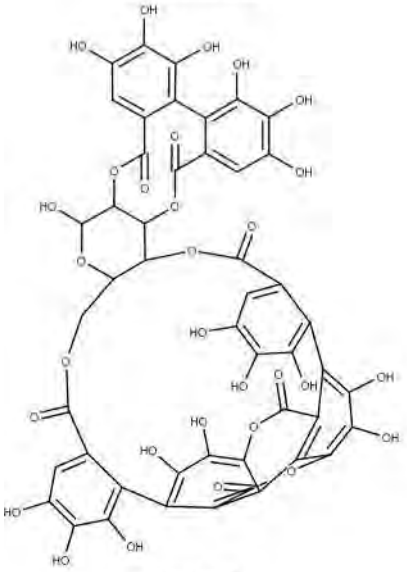
(Continued)



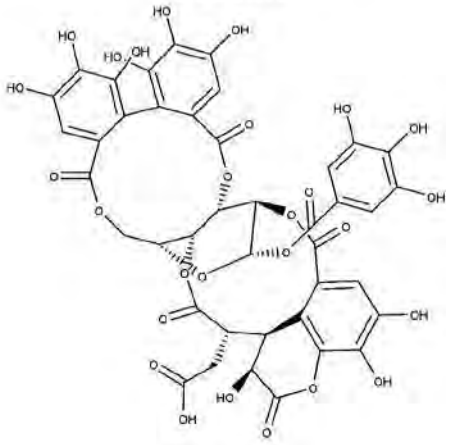
Xanthokeistal A



Terpinolene

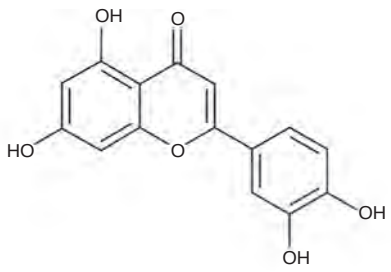


Luteolin

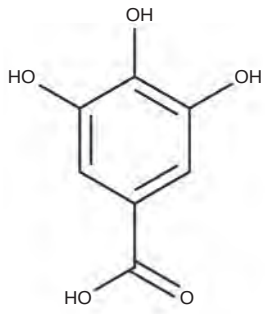


Chebulagic acid

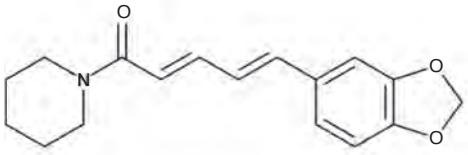
FIG. 16.1 Cont'd



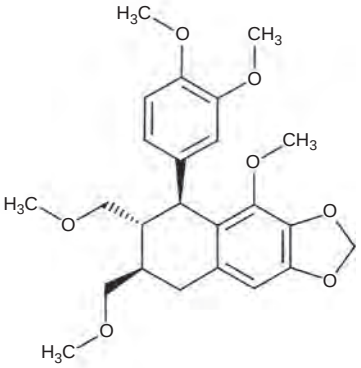
Punicalagin



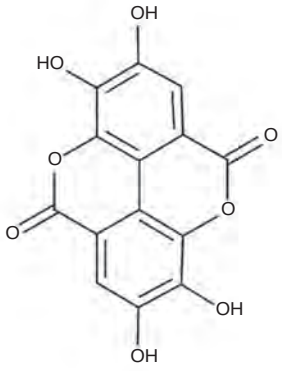
Gallic acid



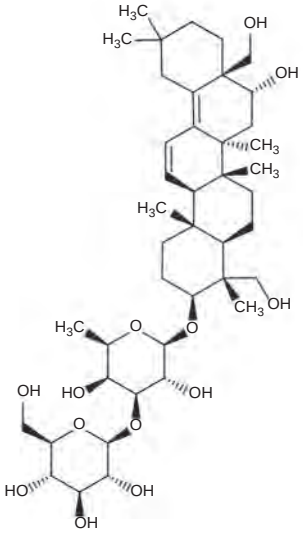
Piperine



Nirtetralin



Ellagic acid



Saikosaponin B2

FIG. 16.1 Cont'd

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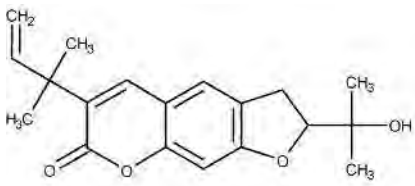
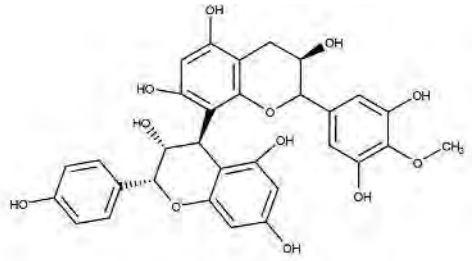
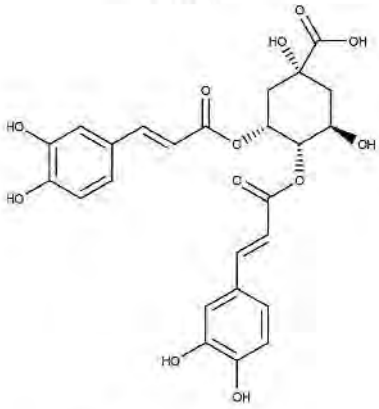


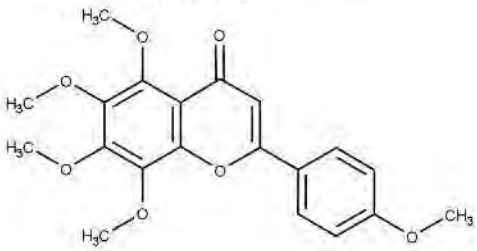
Chalepin



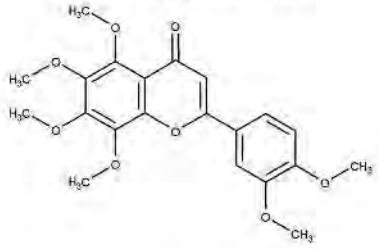
Proanthocyanidin



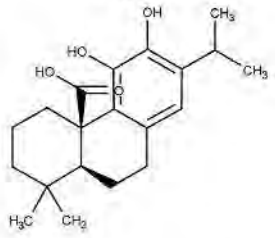
Dicaffeoylquinic acid



Tangeretin

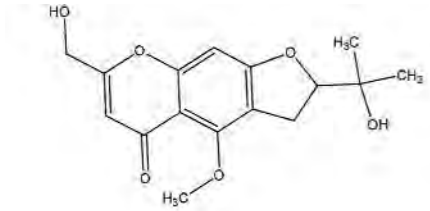


Nobiletin

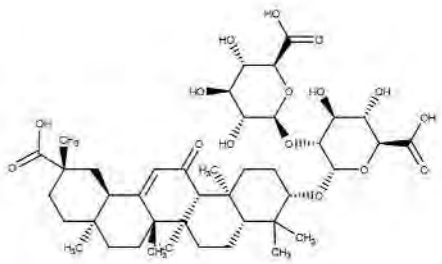


Carnosic acid

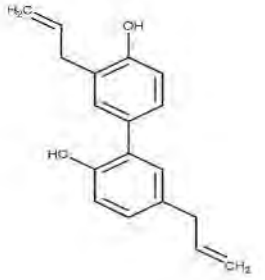
FIG. 16.1 Cont'd



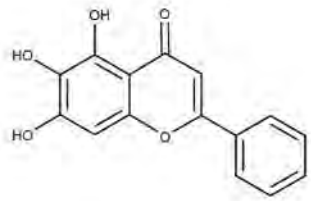
Cimicifugin



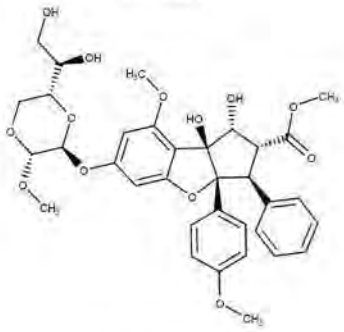
Glycyrrhizin



Honokiol



Baicalein



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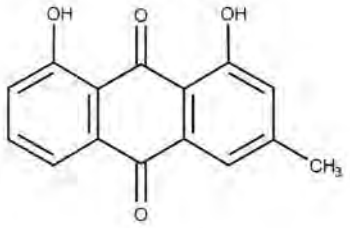
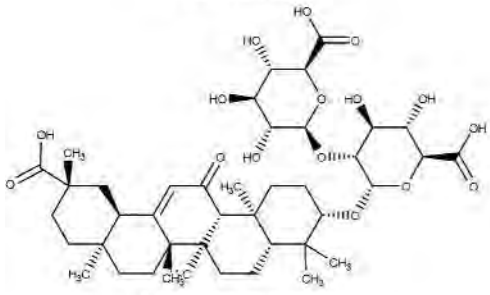
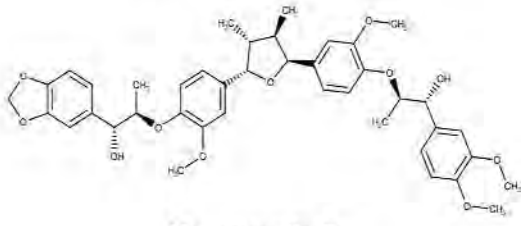


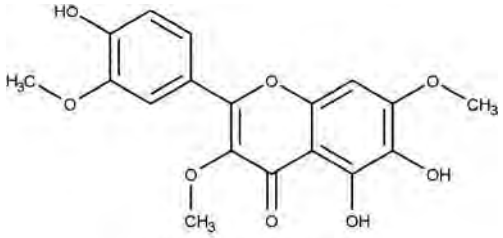
Chrysophanic acid



Glycyrrhizic acid



Manassantin B



Chrysosplenol C

FIG. 16.1 Cont'd

16.3 IN VITRO PERSPECTIVES FOR ANTIVIRAL EVALUATION

Many of the antiviral drugs now known have been discovered by random search in the laboratory. Most labs use a biological test system in which new molecules are added to tissue culture cells at a range of concentrations (e.g., 100–1000 µg/mL); the drug-treated cells (and untreated cells as control) are then infected with a known multiplicity of infective virus particles. Thousands of compounds can inhibit viral replication in a cell culture. In general, the more complex the regulatory mechanisms of a virus, the easier it is to find molecules that inhibit it. A broad estimate of the ratios of the activity of antiviral compounds in a cell culture, animal models, and humans is 1000:10:1.

During the evaluation of antiviral agents, many test conditions, such as the cell culture system, virus strain, virus challenge dose, virus input multiplicity of infection, and time of harvesting, can affect or even alter the test results. To test the inhibitory activity of a new antiviral agent, it is first necessary to select the host cell system(s) in which the virus replication can be measured. Viruses vary considerably in their ability to replicate in cultured cells. Many viruses can cause CPE while some of them can form plaques. Others can produce some specialized functions, such as hemagglutination, hemadsorption, or cell transformation. Virus replication in cell cultures can also be detected by the presence of viral products, namely, viral DNA, RNA, or polypeptides.

The *antiviral tests selected may be based on:*

(a) Inhibition of the virus-induced cytopathic effect (CPE) in which the 50% effective dose (ED_{50}) of the antiviral agent is expressed as the concentration that inhibits CPE in half of the quadruplicate cultures.

(b) Plaque reduction assay in which the dose of the drug required to reduce the plaque number by 50%, that is, ED_{50} is calculated.

(c) Virus yield reduction assay in which the drug concentration required to reduce 90% (1 $\log_{10}$ reduction), or 99% (2 $\log_{10}$ reduction) of the virus yield as compared with the virus control (infected cultures without drug) are determined from the dose–response curves and are expressed as ED_{90} or ED_{99} of the antiviral agent.

(d) Assay systems based on the measurement of specialized functions and viral products; a number of viruses do not produce plaques nor do they cause CPE readily, but they may be quantified by certain specialized functions based on their unique properties, for example, hemagglutination and hemadsorption tests used to study the antiviral activity against myxoviruses and ELISA, used to determine the extent of virus replication and, thus, obtain a measure of the inhibitory effect of various antiviral agents on virus replication, etc. (Hu and Hsiung, 1989).

16.3.1 MTT and Neutral Red (NR) Assays

Colorimetric assays quantify cell viability through enzyme-mediated biochemical reactions owing to ingress of certain dyes inside living cells. Mosmann, Borenfreund, and Puerner first advocated the application of tetrazolium (MTT) and Neutral Red (NR) assay, respectively, to quantitate cell viability and the cytotoxicity to cells in vitro. Parida et al. (1999) compared the efficacy of two colorimetric assays (MTT and Neutral Red) to determine viral infectivity in microculture virus titration employing Polio virus type-3 and Dengue virus type-4. MTT assay, also known as tetrazolium assay, has been exploited extensively to reveal the protective efficacy of therapeutic agents and plant extracts against cancer, HIV-1, HSV, Polio virus type 3, DEN-4 virus, and to the determination of neutralizing antibody levels to HIV and Respiratory syncytial virus. MTT assay using microculture virus titration (MCVT) was applied for the determination of infectivity titers of Influenza viruses and was found to be compatible with the well-established procedure of egg infectivity assay.

Unlike MTT, neutral red dye uptake assay has not been substantially exploited in virologic research. NR dye assay was earlier performed for the study of the antiviral efficacy of basil leaves extract against Polio virus type 3. MTT, a tetrazolium salt, is a yellow-colored dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] which gets cleared by mitochondrial succinate dehydrogenase enzyme into a blue-colored formazan in active cells. This product does not form crystals when it interacts with isopropyl alcohol and thus can be accurately measured. There is no need to harvest the viable cells, wherein the cell viability can be directly measured by a spectrophotometer (Parida et al., 1999). The assay procedure has been discussed in Section 16.6.7.

16.3.2 WST-1 Cell Proliferating Reagent Assay

A colorimetric assay based on the cleavage of the tetrazolium salt WST-1 has been developed for human cytomegalovirus (HCMV) antiviral susceptibility testing and adapted to a microtiter plate format. Bedard et al. (1999) developed a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 for human cytomegalovirus antiviral susceptibility testing.

16.4 FACTORS INFLUENCING ANTIVIRAL EVALUATION

(i) *Host cell system*

The response of different cell cultures to a given antiviral agent, including the drug metabolism and toxicity, among other factors, may vary greatly. To perform antiviral testing against a particular virus infection, it is necessary to obtain a suitable host cell system for that virus infection. The same antiviral agent may behave very differently in different cell culture systems, although the same virus strain is used.

(ii) *Virus strain and passage history*

The variability of sensitivity to a given antiviral agent has been noted among different strains of HSV and CMV. Drug-resistant strains have emerged to some antiviral agents, especially in the herpes virus group. The passage history of a virus strain may also affect its sensitivity to some antiviral agents.

(iii) *Virus input multiplicity of infection (MOI)*

MOI can influence substantially the evaluation of antiviral activity by the plaque reduction method or the virus yield reduction assay. High MOI will decrease the sensitivity of the virus to an antiviral agent.

(iv) *Virus yield harvesting time*

This may also contribute to the disparity among antiviral evaluation results, even when the virus MOI is kept constant (Hu and Hsiung, 1989).

(v) *The influence of reaction parameters on antiviral assays of phytochemicals*

The activities of several known antiviral phytochemicals are profoundly affected by the presence of serum components. For example, the terthiophene, α-terthienyl (α-T) was inhibited in a concentration-dependent manner by serum. In the case of the carboxylic acid derivative of α-T, the compound appeared to have no antiviral activity at all in the presence of serum, yet in its absence this compound was as effective as α-T. In contrast, the complex anthraquinone hypericin required a small amount of serum for maximal antiviral activity. The reactions were also strongly affected by the order of incubation of the components: virus, compound, serum, and light (Hudson and Towers, 1995).

16.5 HOST CELLS, ORGANISMS, AND CULTURE MEDIA

The viruses to be selected for initial evaluation of plant extracts are obviously of major importance. They must be chosen to represent the different groups of viruses according to their morphology and various multiplication mechanisms and a range of virus diseases for which chemical control would be useful. Besides the need for control, also the prevalence of the viral diseases and the resulting projection of the market potential, which are determined by the antigenic abundance of the causative viruses and the problems this represents for vaccine control, are important selection criteria (Grunert, 1979).

In vitro methods are therefore more appropriate, because they allow simultaneous screening of a battery of viruses. In vivo screening of extracts against a broad array of viruses, in contrast, is not only very expensive but also extremely laborious. In vitro antiviral bioassays utilize thinly confluent monolayers of tissue culture cells with sufficient susceptibility to the infecting viruses that a visibly cytopathogenic effect (CPE) occurs, for example, rounding up. Shrinking or detaching of cells from the monolayer can be produced and readily observed microscopically. A monolayer of cells consists of animal or human cells, such as chick embryo, rabbit, or green monkey kidney cells (Vero cells), or human skin fibroblasts and carcinoma cells (HeLa cells), grown in a culture medium. Such continuous cell lines used in virology are mostly "transformed" cells that can be maintained for an indefinite number of generations.

The host cells require an appropriate tissue culture medium in which they can survive for at least 1 week without having to renew the medium. Renewal of the medium causes changes in intra- and extracellular products and alters the virus concentration. The medium must have a stable pH during the whole incubation time and may contain only a small amount of serum, because blood products tend to absorb many compounds. Mostly, a defined synthetic medium, supplemented by some type of serum (such as fetal bovine, calf, or horse), a buffer on sometimes bacterial and fungal inhibitory antibiotics, is used. According to experience, Vero cells, which allow the growth of many human viruses with visible CPE, grown in the medium described by Hronovosky et al. (1975), and slightly modified by lowering the pyruvate concentration, are most suitable for antiviral screening of plant extracts (Van den Berghe et al., 1978).

Many combinations of test viruses are possible, but a battery of six viruses seems to be quite acceptable. Virus types and strains may vary in sensitivity, but have to be selected as a function of their ability to multiply in the same tissue culture when cell culture models are used as screening systems. In this way, an objective comparison of antiviral activities is possible, whereas toxicity tests are minimized. Moreover, virus multiplication must cause a visible CPE within a reasonable period of time, preferably within a week after infection.

16.6 METHODS FOR ANTIVIRAL EVALUATION OF HERBAL DRUGS

An in vitro screening bioassay involves the detection of inhibition of the visible CPE on monolayer tissue culture cells after inoculation of the cells with a pretitered virus suspension and incubation in a maintenance medium containing the plant extract or test component. Virus suspension is characterized by their "virus titers," which are expressed as the number of infectious units per volume. An infectious unit is defined as the smallest amount of virus capable of producing a reaction after virus inoculation and can be carried out by two generally applied methods, namely, the plaque test (PT) and the 50% endpoint titration technique (EPTT).

In the plaque test, monolayers of cells grown in plastic or glass Petri dishes are inoculated with dilutions of a viral suspension. After adsorption of infectious virus particles to the host cell, the monolayers are overlaid with an agarose-containing medium so that the newly formed virus particles are localized by the solid agar over layer. These newly formed infectious particles spread from the initially infected cell to the adjacent cells and develop well-circumscribed foci of cellular degeneration. These areas of dead cells are called "plaques" and are visualized by staining the cell monolayer with a vital dye, such as Neutral Red. The plaques may also be detected microscopically by determining the multinucleated cells (e.g., measles) or by immunofluorescence. The concentrations of viral suspensions measured by counting the plaques are expressed as plaque-forming units per mL (PFU mL^{-1}). In the endpoint titration technique, the concentration of infectivity is measured by determining the highest dilution of the suspension, which produces CPE in 50% of the cell cultures inoculated. This dilution is called the 50% tissue culture dose endpoint (TCD_{50}). Dilutions are therefore made in a maintenance medium and a certain volume of each of them (0.05–0.1 mL) is added to four or more tube cultures. The proportion of positive cultures is registered for each dilution and the precise dilution at which 50% of the inoculated tube cultures are infected is calculated. At this dilution, the inoculum contains, on average, one TCD_{50} or one tissue culture (infectious) dose for 50% of the tissue culture tubes.

The influence of a plant extract on virus multiplication can be determined as a viricidal or an antiviral activity. The viricidal activity is measured by titration of the residual infectious virus after incubation of the plant extract with a virus suspension of at least 10^6 TCD_{50} mL^{-1}. On the other hand, the antiviral activity is determined by comparing the virus titers of infected cells, which have been cultured with a maintenance medium containing plant extracts or test substances and a maintenance medium without test material (Colegate and Molyneux, 1993).

16.6.1 Preparation of Samples for Antiviral Testing

In contrast with antibacterial screening, no solvents, other than physiological buffer solutions, should ideally be used in the in vitro antiviral screening of plant extracts because the samples have to be added to tissue culture cells. It has been observed that many nonpolar plant extracts, prepared and evaporated, are reasonably soluble in dimethyl sulfoxide (DMSO), especially if little or no water is present in the sample and the dissolving sample is heated on a water bath. Viricidal and antiviral determinations may then be carried out on test solutions containing not more than 10% and 1% DMSO, respectively. Therefore, dissolved samples of nonpolar plant extracts in DMSO are added dropwise to the maintenance medium in a ratio of 1:10 or 1:100 under stirring. As already mentioned, the maintenance medium may contain antibiotics, such as penicillin G (20 µg mL^{-1}), neomycin (1 µg mL^{-1}), and amphotericin B (1 µg mL^{-1}), in order to avoid sterilization of the test solutions. Any contamination by bacteria or fungi would indeed ruin the in vitro antiviral bioassay (Colegate and Molyneux, 1993).

There are various methods for validation of antiviral activity. The major techniques have been highlighted in the preceding sections and in Fig. 16.2.

16.6.2 Extracellular Viricidal Evaluation Procedure

Most currently used antiseptics and disinfectants kill pathogenic bacteria and fungi at 25°C within 5 min when present in a concentration of about 0.5% (3-log titer reduction). Because it has been noticed that most of these preparations fail to kill all pathogenic viruses under these circumstances, a method has been developed for testing the in vitro viricidal effect of plant extracts, as will be described in the following section.

Thoroughly mix the preincubated (25°C) plant extracts, dissolved in a physiological buffer, or their twofold dilutions (e.g., 1/2 to 1/16), with the same volume of a preincubated (25°C) virus suspension of, for example, 10^6 PFU mL^{-1} or TCD_{50} mL^{-1} in physiological buffer. Incubate the mixture at 25°C for 5 min. Stop the incubation by the addition of a 10-fold volume of ice-cold maintenance medium and filter the mixture immediately through a 0.22 µm filter to eliminate all possible precipitate. Then, filter the ice-cold filtrate through a 0.01 µm filter to concentrate residual virus on the filter and separate the virus from possibly cytotoxic plant components, which pass the filter. Remove the residual virus from the filter

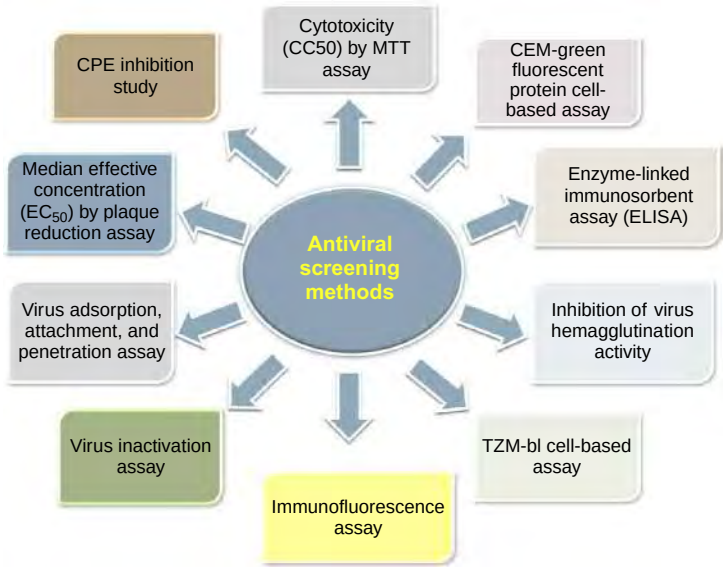


FIG. 16.2 Antiviral screening methods.

with maintenance medium, supplement with 5% serum (1 mL), sonicate in an ice-bath for 30 s, and titrate in 10-fold dilutions at 37°C by plaque formation or in microtiter plates according to the EPTT. Carry out a virus control in a physiological buffer containing no plant extract simultaneously.

An essential step of this methodology is the separation of all cytotoxic plant components from the residual virus, which has to be measured at 37°C. Cytotoxic substances have a greater influence on the activity of an extracellular virus at 37°C than at 25°C. This step, however, can be omitted when the plant extracts to be tested are not toxic to the host cells under the conditions of the evaluation procedure (Colegate and Molyneux, 1993).

16.6.3 End Point Titration Technique (EPTT)

The EPTT technique is performed on preemptied confluent monolayers of Vero or other cells, grown in the holes of microtiter plates, which are infected with serial 10-fold dilutions of a virus suspension (100 µL). Starting with monolayers containing 10^4 cells per hole and a virus suspension of, for example, 10^7 $TCID_{50}$ mL^{-1} or PFU mL^{-1}, infect the first monolayers of cells with a multiplicity of infection (MOI). The antiviral activity is expressed as the virus titer reduction at the maximal nontoxic dose (MNTD) of the test substance, that is, the highest concentration (µg mL^{-1}) that does not affect the monolayers under the antiviral test condition. Viral titer reduction factors, that is, the ratio of the viral titer reduction in the absence (virus control) and presence of the MNTD of the test sample of 1×10^3 to 1×10^4 indicate a pronounced antiviral activity and are suitable as selection criteria for further investigation of plant extracts (Colegate and Molyneux, 1993). The EPTT is more suitable for testing complex samples, such as plant extracts, for many reasons.

(i) First, the concentration of many compounds in the extract remains constant, and consequently the proportion of toxic versus active compounds does not change.

(ii) Second, the exact duration of the antiviral action can be determined by using the EPTT, because the action starts from the moment the extract is added to the cells.

(iii) Third, the EPTT using serial diluted extracts deals with a dynamic process, because the cells are subsequently infected with different MOI.

(iv) This system allows the correlation of all possible MOI values in the same microtiter plate with decreasing amounts of plant extracts, so that the noncytotoxic concentration of plant extracts can be determined. At the same time, a correlation between extract toxicity and antiviral activity according to the corresponding MOI can be determined in the same microtiter plate.

(v) It can be stated as a general rule that the detected antiviral activity should be stable in at least two subsequent dilutions of nontoxic concentration of the extract; otherwise the activity is directly correlated with the toxicity of the extract or is only viricidal. Moreover, a true antiviral product has to protect the cells, which have been infected with low virus dilutions (starting from 0.1 PFU per cell onward).

(vi) Finally, it should be stressed that all possible steps of virus manipulation are to be completed before the plant extracts are added. This means that an antiviral product $TCD_{50}\ mL^{-1}$), under nontoxic conditions, must act on virus replication steps after uncoating. When the cells are completely protected only in the lower MOI ($0.1\ TCD_{50}\ mL^{-1}$), replication processes before uncoating may be involved.

16.6.4 Effect of Test Compounds on Virus CPE Inhibition

An important aspect of the inhibition of viral cytopathic effect (CPE) is the determination of $TCID_{50}$ (50% tissue culture infective dose). After harvesting, dilute the virus stock 10-fold. Add 0.1 mL of each dilution in 10 wells each of a 96-well microtiter plate. Add 0.1 mL of cell suspension of 10,000 cells/well, incubate at 37°C with 5% CO_2 atmosphere, and observe for vial CPE on alternate days. Take a final reading on the fifth day and calculate $TCID_{50}$ as per the method of Reed and Muench (1938), from which $TCID_{50}$ can be further calculated from the log value.

$$\mathrm{TCID}_{50} = \left(\frac{(\%\text{CPE at dilution next above } 50\%) - 50}{(\%\text{CPE at dilution next above } 50\%) - (\%\text{CPE at dilution next below } 50\%)} \right)$$

An antiviral drug will inhibit the CPE of a virus. Therefore, for detecting an antiviral agent, the amount of inhibition of CPE of a virus can be observed microscopically (Kenny et al., 1985). For this purpose, trypsinise the monolayer and allow to seed in 96-well microtiter plates. After a 24-h incubation, wash the monolayer in each well and add different test drug dilutions and incubate. After 24 h, wash the cell culture and inoculate with 0.1 mL of 10 $TCID_{50}$, 50 $TCID_{50}$, and 100 $TCID_{50}$ of the virus suspensions in different wells. Incubate them for 1 h at 37°C in an incubator for the adsorption of the virus onto the cells. After incubation, remove excess virus suspension by washing with RPMI. Add 0.1 mL of selected concentration of the test compound and keep both virus and cell control wells with 0.1 mL of RPMI containing 2% sheep serum. Observe the plates under a microscope every 24 h until the virus control shows 100% CPE and tabulate the results (Hu and Hsiung, 1989).

16.6.5 Virus Yield Assay

Trypsinize the cell monolayer, allow to seed in a 96-well microtiter plate and incubate for 24 h at 37°C with 5% CO_2 atmosphere. Remove the medium, wash the cell monolayer with RPMI without serum, and add 0.1 mL of different virus suspensions in different wells containing the cell layer and incubate for 1 h for virus adsorption; wash off the excess virus suspension after adsorption. To each well, add the selected concentration of the test drug diluted in RPMI containing 2% sheep serum. To the virus control and cell control, RPMI is added (2% serum) and incubated for 24 h. Freeze the plates at −70°C and thaw at room temperature a couple of times to liberate the associated virus. Determine the virus titer by the end point dilution method and express as $TCID_{50}$ (Cinatl et al., 1997).

16.6.6 MTT Assay

Trypsinize the monolayer culture and allow to seed in a 96-well microtiter plate. After a 24-h incubation, wash the monolayer in each well and add different test drug dilutions and incubate. After 24 h, wash the cell culture and inoculate with 0.1 mL of 10 $TCID_{50}$, 50 $TCID_{50}$, and 100 $TCID_{50}$ of the virus suspensions in different wells. Incubate for 1 h at 37°C in a CO_2 incubator for adsorption of the virus onto the cell. After incubation, remove excess virus suspension by washing with RPMI without serum. Add 0.1 mL of the selected concentrations of the test compound and keep both the virus and cell control wells with 0.1 mL of RPMI containing 2% sheep serum. Incubate the plates at 37°C for 72 h. After 72 h, discard the old media from the cell cultures and add 50 µL of 2 mg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to each well and incubate for 3 h. After 3 h of incubation, discard the MTT and add 100 µL of isopropyl alcohol to each culture and set aside for 10 min. Record the absorbance using an Elisa plate reader at 540 nm. In this experiment, the effect of the test drug on mitochondrial synthesis due to viral infection is studied.

16.6.7 Plaque Reduction Assay

The "plaque" is a confined region of contaminated cells formed by multiplying virus particles. The plaques are revealed either as regions of dead/decimated cells recognized by cell stains or as zones of polluted cells by immunostaining. The blended cell monolayer is infected with a log10 dilution of viral plaque-forming unit (PFU) in the absence or presence of the test drug and permitted to adsorb (1 h at 37°C in 5% CO_2); afterward, the cells should be washed twice with prewarmed

minimum essential medium (MEM). The overlaid drug dilutions are arranged in the overlay medium on the contaminated culture, without the test drug. Then, 45 mL of carboxy methyl cellulose is added to 9 mL of the medium; the plates are incubated for 3–5 days, then settled with 10% formalin or 4% formaldehyde for 30 min. The cells are stained with methylene blue (1 mL/well) or 1% gem violet (w/v), washed, and dried to check the plaques (dark areas) by low-power amplification of a binocular microscope.

The antiviral impact should be measured as the percentage inhibition of plaque formation:

$$\left[\left(\text{mean number of plaques in control}\right)-\left(\text{mean number of plaques in sample}\right)\right]\times 100 \,/\, \text{Mean number of plaque in control.}$$

The concentration of the test drug required to exert 50% of virus inhibition (IC_{50} or EC_{50}), as compared with the infection control, is evaluated from the graphical plot as dose–response curves by regression analysis (Chattopadhyay et al., 2015).

16.6.8 Inhibition of Virus Hemagglutination Activity (HA)

Viruses, for example, influenza, can agglutinate RBCs due to surface HA proteins, which can be analyzed visually by blending viral dilutions with RBC. This can be utilized to inspect the inhibitory impact of any medication on HA. The 10-overlay serially diluted (1–1000X) test drug is used alongside the diluted viral stocks (1:4 to 1:128). This dilution (50 mL/well) is added to drug-containing wells. It should be preincubated for 45 min and mixed with RBC (1/20 in PBS) sample solution. Here, up to a specific dilution, the viral particles possibly lose their capacity to agglutinate RBCs, which shows a linkage of the drug with the viral HA.

16.6.9 Immunofluorescence Assay

Known quantities of virus (MOI 5–10) are used for infecting both untreated or drug-treated cells and allowed to adsorb for 45–60 min. The unabsorbed virus particles are washed and fresh media is added to incubate for 24–36 h. Then, cells are washed with PBS, fixed with paraformaldehyde (3%–4%), and permeabilized with acetone or triton X-100. The cells are again washed and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min. Then, the cells are incubated with mouse or rabbit antibody against a specific viral protein for 1–4 h at 37°C. After that, the cells are subjected to repeated washing and incubated with a fluorescent-tagged secondary antibody for 1 h and washed again. After washing, the cells are visualized under a fluorescent microscope and compared with the fluorescence of untreated and drug-treated cells. On the other hand, for quantitation, the cells are trypsinized after treatment and fixed with 4% paraformaldehyde. The cells are then washed, permeabilized, and labeled with a fluorescent-tagged antibody, followed by propidium iodide (PI: 50 mg/mL in PBS). The cells are then counted in a fluorescent-activated cell counter to quantitate the fluorescence percentage.

16.6.10 Enzyme-Linked Immunosorbent Assay

Known quantities of virus (MOI 5–10) are used for infecting both untreated and drug-treated cells, adsorbed for 1 h, washed, and incubated (2–4 days) for evaluation of the inhibition of the virus-induced cytopathic effect (CPE). The virus stock is centrifuged after freeze thawing and diluted for ELISA. Each well of a plate coated with a virus-specific antibody is mixed with 100 mL of controls or test drug and incubated at room temperature (1 h) with Horseradish peroxidase conjugate, alkaline phosphatase, or b-D-galactosidase-labeled virus-specific antibody. The wells are washed five times and the substrate (100 mL) is added and incubated in the dark for 10 min. The reaction is stopped by adding 5% H_2SO_4 solution and the absorbance is read at 450 nm. The 96-well plates are seeded with a quadruplicate cell monolayer having a log 10 dilution of the test drug followed by infection with the virus. After 16–20 h of incubation at 37°C, the monolayers are fixed with 0.05% glutaraldehyde and examined for virus-specific protein(s) on the cell surface. The ELISA should be performed with a monoclonal antibody to the specific protein of the corresponding virus and protein and the OD (optical density) is measured at 450 nm. The concentration is calculated by 50% reduction in OD values (EC_{50}) from extrapolating graphical plots followed by the determination of SI (ratio of CC_{50}:EC_{50}) in which the results are expressed as a percentage of virus-infected cells (virus control) (Chattopadhyay et al., 2015).

16.6.11 Virus Inactivation Assay

The test drug and the virus (10^4 PFU/mL) are mixed to incubate for 1 h at 37°C. Then, immediately dilute the virus-drug mixture to 100-fold (final inoculums 50 PFU/well) with media containing 2% FBS to get a subtherapeutic concentration of

the test drug. Following that, mix the monolayer, with the virus inoculums seeded using a 12-well plate. Alternately, add the virus-test drug mix diluted to 100-fold, with no incubation period, with the respective cells for infection. Allow to adsorb for 1 h at 37°C, discard the diluted inoculums, and wash the cells with PBS. Pour an overlay medium (with 2% FBS), and incubate at 37°C for 72 h, followed by plaque reduction assay. Count the viral plaque numbers obtained from infections set in the presence of the test drug and compare it with the control.

16.6.12 Attachment Assay

Viral attachment to the cell surface is carried out at 4°C, as it permits binding, but stops viral entry, by ELISA. Briefly, incubate susceptible cells (2×10^4 cells/well) in 96-well plates and allow growth overnight. The cell monolayers are cooled at 4°C. The cells are infected with the virus (MOI 5) using heparin in presence of the test drug for 3 h at 4°C as a control. After washing the wells with ice-cold PBS, fix with prechilled 4% paraformaldehyde in PBS for 1 h on ice, blocked with 5% BSA at 4°C. The samples are incubated at 37°C for 1 h with a primary antibody in PBST PBS with 0.05% Tween 20 along with 5% BSA. The wells are washed twice with PBST, 5% BSA, and again only with PBST twice, at 5-min intervals on a shaker. This is mixed with secondary antibody in PBST with 5% BSA and incubated at 37°C for 1 h. The reaction is developed with a 3,3′,5,5′-tetramethyl-benzidine two component microwell peroxidase substrate for 20 min; the reaction is stopped with 1 M phosphoric acid. The absorbance is measured at 450 nm, and the values are expressed as the fold change of absorbance relative to the mock infection control (Lin et al., 2011).

16.6.13 Penetration Assay

The cell monolayers are cooled and grown in 12-well plates at 4°C for 1 h. Subsequently, infect the prechilled cells with HSV-1 (100 PFU/well) and incubate for 3 h at 4°C to allow for viral adsorption. Incubate the infected cell monolayers in the presence of test drug or heparin (100 mg/mL) for an additional 20 min at 37°C to facilitate HSV-1 penetration. The extracellular virus is inactivated by citrate buffer (pH 3.0) for 1 min, and the cells are washed with PBS followed by overlay with DMEM containing 2% FBS. After 48 h of incubation at 37°C, the viral plaques are stained and counted (Lin et al., 2011).

16.6.14 Virus Adsorption Assay

Add the plated cells (0.8×10^5 cells/well for a 12-well plate) grown overnight at 30% confluence (300 mL) with virus dilution and DEAE dextran at a final concentration of 20 mg/mL. After adsorption (2 h at 37°C in CO_2 incubator), place the plates in a rocker to prevent the cells from drying and add fresh medium (1–2 mL) containing the test drug to each well and incubate for 40–48 h in 5% CO_2 at 37°C for subconfluent growth. After removing the media, add fixing solution (1–2 mL) to each well and incubate for 5 min at room temperature (β-galactosidase activity decreases dramatically if the fixing solution is left for >10 min). Then, discard the fixing solution, wash the cells twice with PBS, stain, and incubate at 37°C for 50 min. Finally, stain the plates to count the number of blue syncytia and express the titration values as the number of stained cells multiplied by the viral dilution (Chattopadhyay et al., 2015).

16.7 VIRUS-SPECIFIC ANTIVIRAL ASSAYS

16.7.1 Human Immunodeficiency Virus (HIV)

(a) TZM-bl cell-based assay

This assay is applicable for HIV-1, simian immunodeficiency virus (SIV), and simian-HIV and is carried out in TZM-bl cells as it reveal the reduction in Tat-induced luciferase (Luc) reporter gene expression after a single round of virus infection. Place the TZM-bl cells (4×10^4/well) in a 24-well plate and incubate overnight. In a separate vial, mix the HIV-1NL4.3 (MOI 0.05) virion with the test drug or vehicle for 1 h at 37°C, then add to TZM-bl cells and incubate for 4 h. Wash the cells (with cold PBS), and add fresh media with the test drug to culture for 48 h, using untreated HIV-1-infected cells (negative) and azidothymidine (AZT)-treated cells (positive) as controls. Wash the cells twice with PBS, lyse with 1X lysis buffer, and add the supernatant with the substrate, which should then be analyzed for luciferase activity in an optiplate using a fluorimeter. The results are expressed as percentage inhibition:

$$\text{luminescence in the experimental group}\left(\text{test drug or AZT}\right) / \text{luminescence of infected cells without the drug} \times 100$$

and the percent inhibition is calculated by subtracting the above value from 100 (Wan et al., 2012).

(b) CEM-green fluorescent protein (GPT) cell-based assay

CEM-GFP is a stable T-cell line-containing a plasmid encoding GFP and is suitable for HIV-1NL4.3 (MOI 0.05) culture. For postinfection, incubate the cells (2.0×10^5/well) with the test drug up to 8 days, using AZT and solvents (used to prepare the test drug) as control(s). Lyse the virus-infected cells with 1X Promega cell lysis buffer (150 mL), and transfer to a culture plate to read the absorbance at 485 nm (excitation) and 520 nm (emission) by a fluorimeter. The results can be expressed as percentage inhibition:

$$\text{GFP fluorescence in the experimental group / fluorescence in infected cells without the test drug} \times 100$$

with the he percent inhibition calculated by subtracting the above value from 100 (Chattopadhyay et al., 2015).

16.7.2 Hepatitis B Virus

(a) Hep AD38 assay

Place Hep AD38 cell suspension (6×10^5 viable cells/mL of Hep AD38 seeding medium) in a 96-well microtiter plate, and incubate at 37°C for 3 days. Discard the medium and wash the cell monolayers with warm (37°C) DPBS. To the proper wells, add 100 µL of HepAD38 assay medium that contains either test or control compounds at the desired concentrations. Also include wells with Hep AD38 assay medium alone as "virus only" controls. Incubate at 37°C for 3 days. On day 3, wash the cells once with warm DPBS and add fresh medium containing the appropriate compound to the wells. After 24 h, transfer the supernatants to v-bottomed 96-well plates and remove cellular debris by centrifugation (15 min, 2500 rpm at 4°C). Transfer 90 µL of the clarified supernatants to new v-bottomed plates and store at −70°C for quantification of HBV DNA.

Thaw the supernatants that were collected and add 90 µL of 2X denaturation solution to each well and mix. Incubate at 37°C for 20 min. Cut the nylon membrane to size and prepare it for blotting by wetting it first with distilled water and then 20X hybridization buffer, SSC (Saline sodium-citrate). Dot-blot the denatured supernatants on to the nylon membrane as directed by the manufacturer. Wash the blot with 200 µL of neutralization solution followed by 200 µL of 20X SSC. Remove the blot, rinse it briefly in 2X SSC, and then crosslink the DNA to the nylon filter by UV irradiation.

Prehybridize the blot at 42°C for 1 h in 20 µL of hybridization solution. Prepare a 32P-labeled probe by random priming using a portion of the HBV genome as a template. Purify the probe through a Clontech Chroma Spin column. Denature the probe by boiling for 5 min and add it immediately to the hybridization solution. Hybridize the nylon filter overnight at 42°C. Wash the nylon filters twice with 50 mL of 2X SSC, 0.1% SDS (sodium dodecyl sulfate) at room temperature for 20 min and twice with 50 mL of 0.2X SSC, 0.1% SDS at 65°C for 20 min. Expose the nylon filters to a molecular imager screen for 4 h and scan on a phosphor imager to obtain the data. To determine the percent inhibition of HBV replication, subtract the background value (counts of radiation detected from the nylon filter itself) from all control and experimental values. Divide the average values of the experimental wells (cells treated with test compounds) by the average value for the "virus only" control (cells not treated with compound or tetracycline during the experiment) and multiply this number by 100 (King and Ladner, 2000). The above mentioned in vitro studies are listed in Table 16.2.

16.8 CYTOTOXICITY STUDIES

Drug development programs include preclinical screening of immense quantities of chemicals for specific and nonspecific cytotoxicity against numerous sorts of cells, which is imperative to show the potential therapeutic target and safety evaluation. The screening of plant extracts or pure compounds for investigating their antiviral properties can be more significant with cytotoxicity measures (Meyer et al., 1982).

It is essential for an investigational item to establish the antiviral activity at concentrations that can be accomplished in vivo without inducing toxicity to cells. Moreover, in a cell culture display, antiviral activity of an investigational item can be the aftereffect of host cell death after exposure to the item. Cytotoxicity tests utilize a series of increasing concentrations of the antiviral product to determine what concentration results in the death of 50% of the host cells. This value is referred to as the median cellular cytotoxicity concentration (CC50 or CTC50 or CCIC50). The relative effectiveness of the investigational product in inhibiting viral replication compared with inducing cell death is defined as the therapeutic or selectivity index (CC50 value/EC_{50} value). It is desirable to have a high therapeutic index giving maximum antiviral activity with minimal cell toxicity. According to US FDA guidelines, it is recommended to determine CC50 values in both stationary and dividing cells from multiple relevant human cell types and tissues to establish the potential for cell cycle, species, or tissue-specific toxicities. Studies determining cytotoxicity and therapeutic indexes should be conducted before the initiation of phase 1 clinical studies. There are a number of advantages for in vitro testing using cell cultures, which include

TABLE 16.2 In Vitro Evaluation for Antiviral Potentials: Determination of the Viral Infectivity in Cultured Cells During Virus Multiplication

Virus Assay	Specificity and Application
End point titration technique (EPTT)	• Determination of virus titer reduction in the presence of twofold dilutions of test compound(s)
Virus-induced cytopathic effect inhibition (CPE)	• For viruses that induce CPE but do not readily form plaques • Determination of virus-induced CPE in monolayers, cultured in liquid medium, infected with a limited dose of virus, and treated with a nontoxic dose of the test substance(s)
Virus yield reduction assay	• Determination of the virus yield infected with a given amount of virus and treated with a nontoxic dose of the test substances(s) • Virus titration is carried out after virus multiplication by the plaque test or the 50% tissue culture dose end point test (TCD_{50})
MTT assay	• Determination of inhibition of virus infection treated with a nontoxic dose of the test substances(s) by MTT reagent • Inhibition is determined by calculating optical density (OD) in an ELISA plate reader
Plaque reduction assay	• Only for viruses that form plaques • Titration of residual virus infectivity after extracellular action of test substance(s) • Cytotoxicity should be eliminated, for example, by dilution, filtration, etc., before the titration
Assays on specialized functions and viral products	• Determination of virus-specific parameters, e.g., hemagglutination and hemadsorption tests (myxovirus), inhibition of cell transformation (Epstein–Barr virus, EBV), immunological tests detecting antiviral antigens in cell cultures (EBV, HIV, HSV, and CMV), TZM-bl cell-based assay (HIV), CEM-green fluorescent protein cell-based assay (HIV), Hep AD38 assay (HBV), immunofluorescence assay, enzyme-linked immunosorbent assay (ELISA) • Reduction or inhibition of virus-specific polypeptides synthesis in infected cell cultures, e.g., viral nucleic acids, determination of the uptake of radioactive isotope labeled precursors or viral genome copy numbers
Other assays for validation of antiviral activity	• Virus inactivation assay, virus adsorption assay, virus attachment, and penetration assay

analysis of species specificity, feasibility of using only small amounts of test substances, and facility to do mechanistic studies (Guidance for Industry, 2006).

After confirming the cytotoxic concentration, the drug concentrations are selected for antiviral studies based on the percentage viability of cells and are used to study the antiviral activity by CPE inhibition assay, virus yield assay, followed by MTT assay.

16.8.1 Cytotoxic Study by Trypan Blue Dye Exclusion Technique

Any compound that is cytotoxic to cells inhibits the cell proliferation and kills the cells. Trypan blue is a dye that is capable of penetrating dead cells; therefore, the dead cells take up the blue stain whereas the viable cells do not. This method gives an exact number of dead and viable cells (Strober, 2001).

16.8.2 Determination of Cell Metabolic Function by Protein Estimation

Protein content is widely used for estimating total cellular material and can be used in growth experiments. The colorimetric method of estimating protein is more sensitive. The cell pellets are treated with 11% cold trichloroacetic acid to remove amino acid pools and dissolved in alkaline cupric sulfate and folin ciocalteau phenolic reagent. Folin's reagent and cupric sulfate together react with amino acid to give a blue color and this color intensity is proportional to the protein concentration, which can be measured colorimetrically (Maya et al., 1995).

To proceed with the same technique, the cells from the wells were trypsinized using 100 µL trypsin and transferred into Eppendorf tubes and centrifuged at 5000 rpm for 10 min to obtain pellets. The cell pellets are dissolved in NaOH and diluted

to 0.1 N. The test drug is to be added to 200 µL of protein sample, mixed, and left for 10 min. To this, 100 µL of test reagent is added with constant mixing and left for 40 min in incubator. The absorbance was read at 655 nm using an Elisa reader (Bio-Rad). The color development was correlated with the cell number as follows:

$$\%\text{Growth inhibition} = 100 - \frac{\text{OD of the sample}}{\text{OD of control}} \times 100$$

The cytotoxic concentration found by dye exclusion techniques gives superficial data. The selected concentrations from a trypan blue dye exclusion study are used further for estimating proteins.

16.8.3 Determination of Mitochondrial Synthesis by MTT Assay

MTT (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide) in live cells enters the cells and enzyme succinate dehydrogenase present in mitochondria reduces it to formazan blue product. The color intensity is directly proportional to the number of live cells.

To perform this process, the plates were seeded with HEp-2 cells at 10,000 cells/well. They are incubated for 24 h. After 24 h, the medium is discarded and drug concentrations were added and incubated for 72 h. Then, 50 µL of 2 mg/mL of MTT is to be added and incubated for 3 h and then 100 µL of Isopropyl alcohol is added and absorbance is read at 540 nm in an ELISA plate reader (Bio-Rad). The results are tabulated and percentage growth inhibition is calculated using the following formula:

$$\%\text{Growth inhibition} = 100 - \frac{\text{Mean OD of the individual test group}}{\text{Mean OD of control group}} \times 100$$

The concentrations of the test drug used in the previous experiments can be further confirmed by studying the mitochondrial synthesis by MTT assay. The formazan blue color formation is directly proportional to the number of viable cells and therefore the absorbance is to be read at 540 nm.

16.8.4 Brine Shrimp Bioassay

In this test, brine shrimp (*Artemia salina*) eggs are hatched in artificial sea water (38 g/L of sea salt). The Brine Shrimp test (BST) bioassay experiment is performed according to the procedure described by Meyer et al. (1982). Generally, samples of the test drugs for the experiment are prepared in methanol solution, which acts as control vehicle. After 48 h of incubation, 10 brine shrimps are transferred to each sample vial using a Pasteur pipette and artificial sea water is added to make 5 mL. Sample vials are previously prepared by dissolving specific concentrations of test drugs with different dilutions. The solvent is then evaporated overnight. Survivors are counted after 24 h and the LC_{50} values, with 95% confidence intervals are determined using probit analysis, as described by Finney (1971). Control vials are prepared using methanol only. Three replicates are prepared for each concentration of the test drugs.

Control disks are prepared using only methanol. Replicates are prepared for each dose level. To begin the bioassay, brine shrimp eggs are hatched in a shallow rectangular dish ($22 \times 32\,cm^2$) under the same conditions described in the literature except that natural instead of artificial seawater is used. Ten shrimps are selected and transferred into each sample vial by means of a 23-cm disposable Pasteur pipette and the final volume in each vial is adjusted to 5 mL using natural seawater. A drop of dry yeast suspension (3 mg in 5 mL seawater) is added as food to each vial. The vials are maintained under illumination. Survivors are counted with the aid of a stereomicroscope, after 6, 24, and 48 h, and the deaths at each dose level and control are determined. No deaths are usually observed to occur in the control after 48 h.

The brine shrimp test (BST) represents a rapid, inexpensive, and simple bioassay for testing plant extract lethality, which, in most cases, correlates reasonably well with cytotoxic properties (McLaughlin, 1991). Following the procedure of Meyer et al. (1982), the lethality of the test drugs/plant extracts to brine shrimp is determined.

16.8.4.1 Lethal Concentration Determination

The lethal concentrations of test drugs/plant extract resulting in 50% mortality of the brine shrimp (LC_{50}) and 95% confidence intervals are determined from the 24 and 48-h counts and the dose–response data are transformed into a straight line by means of a trend line fit linear regression analysis; the LC_{50} is derived from the best-fit line obtained. Caffeine ($LC_{50} = 306$ µg/mL) (Meyer et al., 1982) is used as a positive control and methanol (500 µL) as a solvent and a negative control in the bioassay experiments.

The current scenario of viral diseases is lethal and there is an upsurge in new viral diseases and resistance to existing viral infections worldwide. The currently accessible antivirals, however effective, are exorbitant and past the reach of a majority of individuals. Along these lines, the advancement of safe, effective, and low-cost antiviral medications, for example, RT inhibitors, is among the top priorities, as many viral infections are not yet treatable and have high death rates. For the past few years, substantial work has been carried out regarding the effectiveness of medicinal plants on HIV infection (Premanathan et al., 1999; Calabrese et al., 2000; Asres et al., 2001) and an increasing popularity of over-the-counter plant products containing orthodox drugs has been observed. The main focus is to lower the adverse effects associated with viral infections and an inclination toward synergistic interactions of multiple molecules present in plant extracts. Be that as it may, because mostly pharmacological mechanisms of the combinations are not studied, antagonistic impacts or remedial disappointments have been seen (Chan et al., 2000). A prerequisite that should be considered significant for medicinal plants is to identify and standardize the method of extract preparation, the suitable season for collecting plant material, and the details of its administration (Chattopadhyay et al., 2006). As a lot of plant extracts and subsequent formulations have shown significant outcomes, it seems to be rational to endorse the idea of the study of medicinal plants as a quest to find potential antivirals.

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Chapter 17

Plant Metabolomics and Quality Evaluation of Herbal Drugs

Chapter Outline

17.1 METABOLOMICS: A NEW "OMICS" PLATFORM TECHNOLOGY

Metabolic activity is considered to be the most essential characteristic of life. The central tenets of molecular biology flow from deoxyribonucleic acids (DNAs) to messenger ribonucleic acids (mRNAs), then to proteins and metabolites catalyzed mostly by enzymes. Metabolomics aims to achieve a comprehensive analysis of all metabolites in a biological system at the cellular level (Oliver et al., 1998). It has been applied in several research areas, including environmental and biological stress studies, functional genomics, biomarker discovery, and integrative systems biology (Khoo and Al-Rubeai, 2007; Goodacre et al., 2004). Metabolomics analyses focus on the investigation of low-molecular-weight biomolecules and correlating their phenotypic and genotypic characteristics. In particular, plant metabolomics is one of the important parts of metabolomics research because of the tremendous diversity in the secondary metabolite content. Medicinal plants or herbal medicines typically consist of mixtures of phytochemicals that can be identified, as well as characterized with metabolomics-driven approaches (Yuliana et al., 2013). Metabolomics analyses are typically categorized into two complementary methods: targeted and untargeted. The targeted approach focuses on identifying and quantifying a specific subset of metabolites in a sample, whereas the untargeted metabolomics approach enables us to carry out comprehensive qualitative and quantitative evaluation of the phytochemical content present in the plant (Quansah and Karikari, 2016). In the targeted approach, the chemical properties of the investigated compounds are known, usually hypothesis-driven, whereas untargeted analysis can generate a new hypothesis for further tests by measuring all the metabolites of a biological system. The workflow aims at comparing multiple chemical groups to identify metabolites that are significantly altered. It starts with an untargeted analysis to screen potential and putative metabolites of interest. These metabolites are then subjected to a targeted analysis for metabolite identification, quantitation, functional interpretation, and pathway analysis (Zhou et al., 2012). A metabolomics study involves the extraction of metabolites, analysis in a suitable platform (typically MS or NMR), data acquisition, collection, preprocessing, and statistical evaluation (Quan et al., 2014). There are various factors that affect the quality and standards of food and herbal products, including their genetic condition, cultivation, collection, storage, and milling and processing for final products.

Metabolomics has been used as a powerful tool for quality control and standardization of herbal medicine by utilizing several chromatographic and spectroscopic techniques. Thus, it is able to generate a standardized "metabolic fingerprint" of the specific HM product (Lu et al., 2008).The most common hyphenated techniques used in metabolomics are LC–MS, GC–MS, and NMR in combination with univariate and multivariate data analysis. In this way, metabolomics techniques are widely used for the identification and characterization of herbal medicine, and thus ensure their quality, safety, and efficacy. The Strengths, Weaknesses, Opportunities, and Threats (SWOT) of metabolomics are shown in Fig. 17.1.

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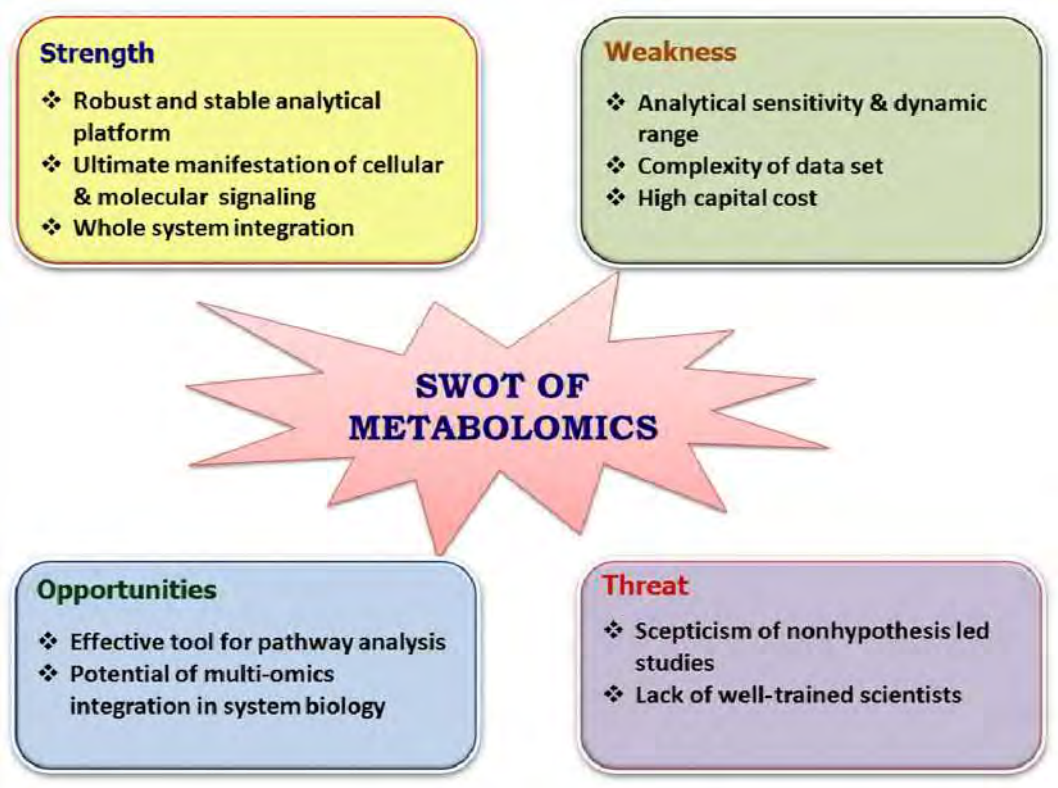


FIG. 17.1 SWOT of metabolomics study.

It also helps to observe the metabolite levels for assessing the synergistic effects of the phytochemical mixtures on various cellular pathways. The major goal of metabolomics analysis can be divided into four major areas:

(i) Targeted compound analysis—quantification of specific metabolites.
(ii) Metabolic profiling—quantitative and qualitative determination of a group of related compounds or of specific metabolic pathways.
(iii) Metabolomics fingerprinting—sample classification by rapid global analysis.
(iv) Metabolomics analysis—this involves the quantitative and qualitative analysis of "all" metabolites. The role of metabolomics in TM research is illustrated in Fig. 17.2.

The applications of metabolomics in the quality evaluation of herbal medicine may be highlighted as follows (Heyman and Meyer, 2012).

- Simultaneous identification and quantification of chemical compounds present in HM by using both targeted and nontargeted approaches.
- Quality assurance of herbal medicines to ensure their efficacy and safety.
- To ensure the content uniformity of different batches of herbal medicine as there are several factors (e.g., plant growth environment, collection/harvesting season, preparation and extraction process) affecting the chemical composition of herbal medicine.
- Classification and identification of adulteration of plant products using metabolomics analysis.
- Understanding the genetic variability of the medicinal plants for their secondary metabolite content.
- Establishment of the fundamental nature of the plant phenotypes in relation to development of metabolite content.
- Rapid, efficient, and high-throughput techniques in the drug discovery process, considered more efficient than traditional bioactivity-guided isolation techniques.

17.2 TARGETED AND UNTARGETED METABOLOMICS

The application of targeted metabolomics addresses specific subsets of compounds in a biological system. These analyses are designed to extract information selectively, related to a group of related metabolites from the complex mixture of

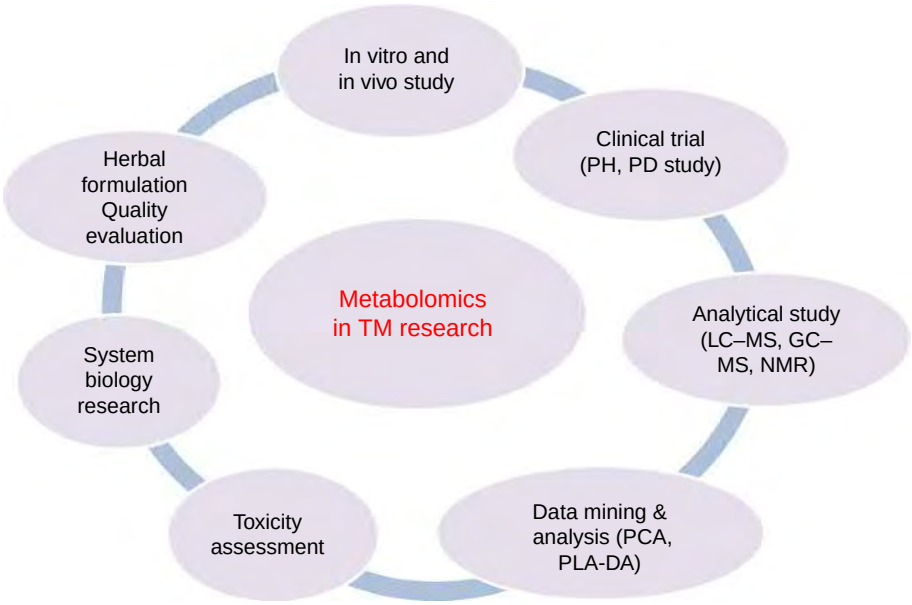


FIG. 17.2 Role of metabolomics investigation in TM research.

biomolecules present in the plant extract (Dudley et al., 2010). Targeted metabolomics can be defined as the measurement of some specific groups of chemically characterized and biochemically annotated metabolites, whereas untargeted metabolomics are intended to analyze all the measurable analytes. In contrast with a targeted metabolomics experiment, which measures ions from known metabolites, an untargeted metabolomics experiment registers all ions within a certain mass range, including ions belonging to structurally novel metabolites. Targeted studies analyze a relatively small and specific number of metabolites, which are chemically characterized with established biological importance. It offers greater selectivity and sensitivity than untargeted methods. Quantification of the metabolites is performed through the use of internal standards and authentic chemical standards of each metabolite. The targeted analysis is very useful for the discovery of new biomarkers and also for confirming the findings from profiling analysis. It mainly focuses on a specific group of metabolites associated with a specific pathway. Untargeted approaches mainly aim to identify the maximum number of metabolites present in a biological system. Due to lack of proper chemical standards, this approach is mostly dependent on data acquisition and analysis to identify the major metabolites.

The major differences between untargeted and targeted studies are the level of sample preparation required, the number of metabolites detected, and the level of quantification of the metabolites. Another major aspect of untargeted metabolomics is to generate the hypothesis, whereas targeted metabolomics focuses on the testing of the hypothesis (Lu et al., 2008; De Vos et al., 2007). Untargeted metabolomics has become a useful approach for carrying out comprehensive qualitative and quantitative evaluation of herbal products. While the targeted approach targets a specific subset of metabolites in a sample, the untargeted approach examines the complete metabolome. Analytical techniques employed in metabolomics studies include liquid chromatography–mass spectroscopy (LC–MS), gas chromatography–mass spectroscopy, capillary electrophoresis-mass spectroscopy, thin-layer chromatography, Fourier-transformed infrared spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. Among these, the most popular analytical methods are those based on MS and NMR spectroscopy. Progress in the field of analytical chemistry regarding the detection and characterization of small molecules has resulted in highly efficient platforms for comprehensive evaluation of metabolite data (Quansah and Karikari, 2016). The details of LC–MS-, GC–MS-, and NMR-based techniques will be discussed in the following sections.

17.2.1 LC–MS-Based Techniques

LC–MS is becoming a method of choice for profiling metabolites in complex plant matrices/herbal mixtures. Most metabolomics studies utilize the chromatographic separation method before mass spectrometric analysis, by which it can reduce sample complexity and alleviate matrix effects during ionization. In LC–MS, reverse-phase liquid chromatography, normally using C18 columns, is widely used to separate semipolar compounds, such as phenolic acids, flavonoids, glycosylated steroids, alkaloids, and other glycosylated species (Zhou et al., 2012). High-performance liquid chromatography (HPLC) is a versatile method that allows separation of compounds of a wide range of polarity through either isocratic

elution or a gradient elution. A mass spectrometer is typically composed of three major parts: ion source, mass analyzer, and detector. The ion source converts sample molecules into ions, the mass analyzer resolves these ions, either in a time-of-flight tube or in an electromagnetic field, before they are measured by the detector.

Several options are available for ion sources, including electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), and fast atom bombardment (FAB). Among them, ESI is the most preferred method in LC–MS-based metabolomics studies because of its "soft ionization" capability for producing a large number of ions. APCI and APPI are also complimentary to ESI for the analysis of nonpolar and thermally stable compounds, such as lipids. In every cases, both the positive and negative ionization modes are used under a wide scan range (*m/z* 50–1000) to maximize the metabolome coverage. A major functional component of LC–MS is the mass analyzer, which can be categorized as: quadrupole, ion trap, time-of-flight, Orbitrap, and Fourier transform ion cyclotron. High-resolution mass spectrometers (HRMS), such as FTICR, Orbitrap, and TOF, can provide accurate mass measurements to facilitate metabolite identification and also provide accurate metabolite quantitation. The mass analyzers resolve ions based on their *m/z* values, estimate their molecular masses, and further aid in metabolite identification by acquiring highly resolved and accurate MS/MS spectra. The methodologies and other aspects of LC–MS based studies have been highlighted in Chapter 11.

The ions are fragmented by applying collision-induced dissociation (CID) energy in either quadrupole-based tandem-in-space instruments, such as triple quadrupole, quadrupole TOF, or ion trap-based tandem-in-time instruments, such as quadrupole-ion trap. Some of the major advantages of LC–MS based metabolomics include the ability to perform positive and negative ion switching with the purpose of determining the molecular weight of the unknown compounds. The UV spectrum (usually LC–MS is equipped with a UV detector) is also able to determine the characteristics of the probable compound with a known molecular weight (Zhou et al., 2012).

Fig. 17.3 describes the workflow of an LC–MS-based metabolomics study, including sample collection and extraction, data acquisition, data processing and analysis, and metabolite identification. The sample collection and extraction stage is critical, as the collection of plant material needs to consider several factors that can influence sample integrity, including collection time, season, weather, and soil, among others. In addition, the extraction method and solvents used can influence the metabolite yield and the range of metabolites extracted (Quansah and Karikari, 2016). Several LC–MS based methods were developed for targeted and nontargeted analysis of medicinal plants. Metabolite profiling of *Centella asiatica* leaves was performed by Shen et al. using LC–MS/MS techniques in which asiatic acid (**1**), asiaticoside (**2**), and madecassoside (**3**) were identified as major secondary metabolites (Shen et al., 2009). Some therapeutically important

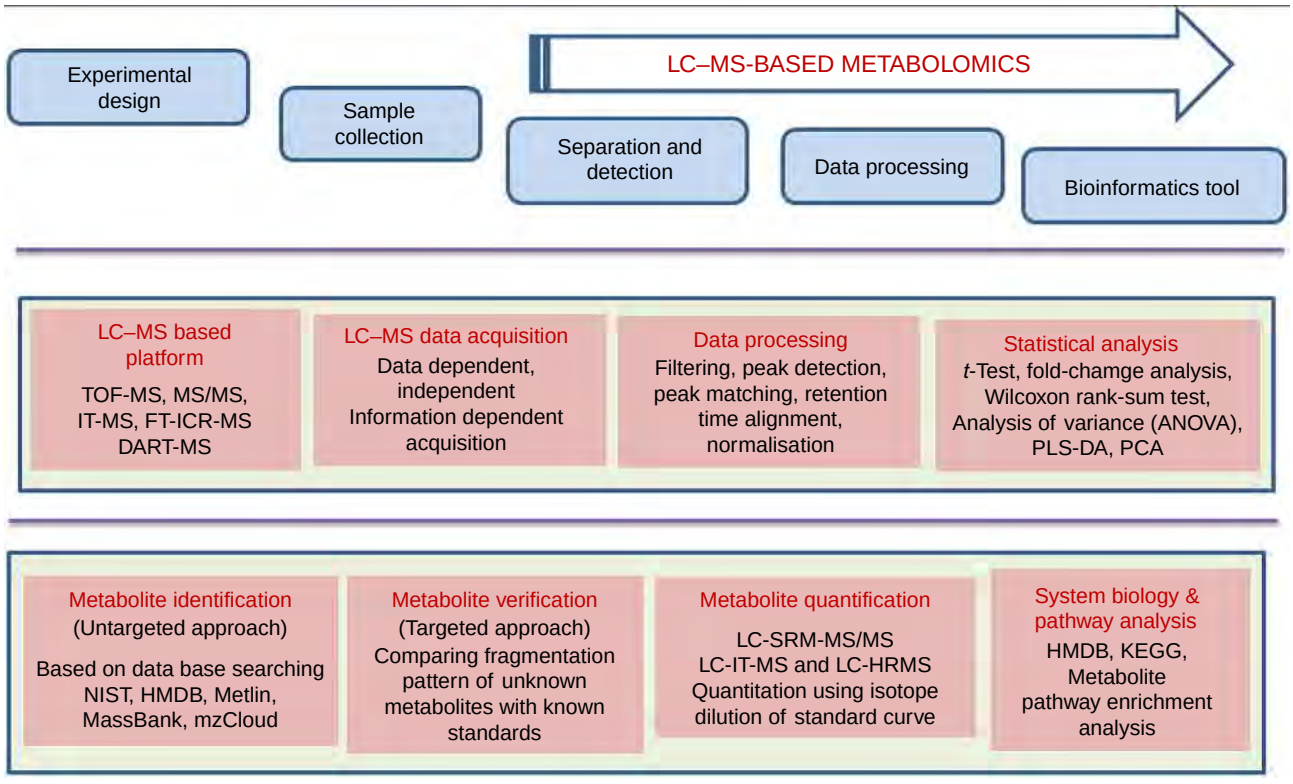
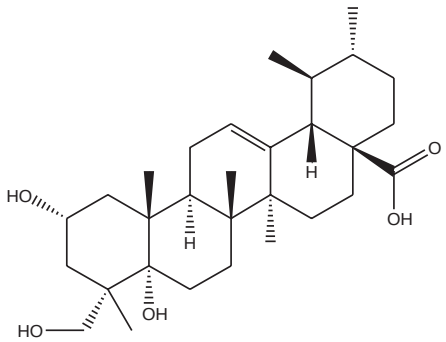


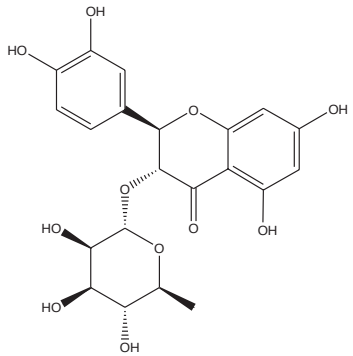
FIG. 17.3 The workflow of an LC–MS based metabolomics study of herbal medicine.

metabolites, namely, Bacopaside III, Bacopasaponin G, and Bacopasides A (**4**)**,** were reported from the whole plant of *Bacopa monnieri* through a LC-QTOF-MS-based method (Hou et al., 2002). In 2011, Montoroa et al. reported the presence of flavonoid compounds, liquiritin (**5**) and liquiritigenin (**6**)**,** in *Glycyrrhiza glabra* through an LC–MS-based metabolomics study (Montoroa et al., 2011).

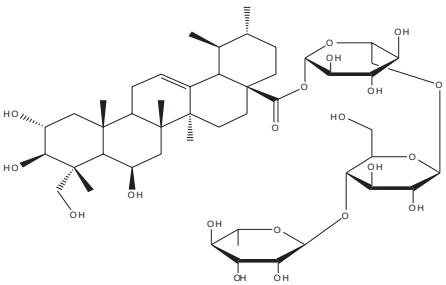
The alkaloid compounds reserpine, ajmalicine, ajmaline, serpentine, and yohimbine have been characterized in *Rauwolfia serpentina* roots by Kumar and his coworkers (Kumar et al., 2016). Another LC–MS-based study revealed the presence *of* withaferin A (**7**), withanolide D (**8**), withanoside IV or VI, and withanolide sulfoxide in *Withania somnifera roots* (Trivedi et al., 2017).



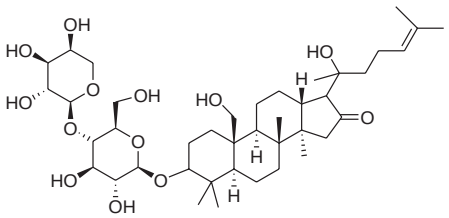
Asiatic acid (**1**)



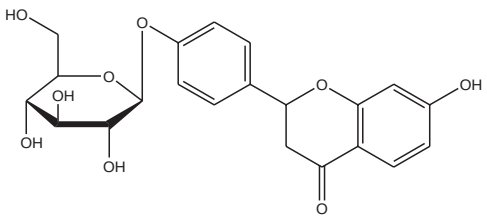
Asiaticoside (**2**)



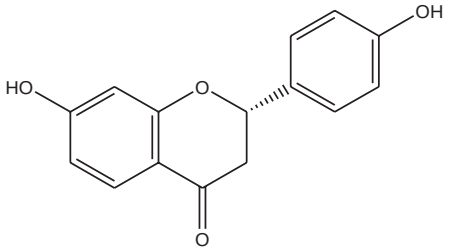
Madecassoside (**3**)



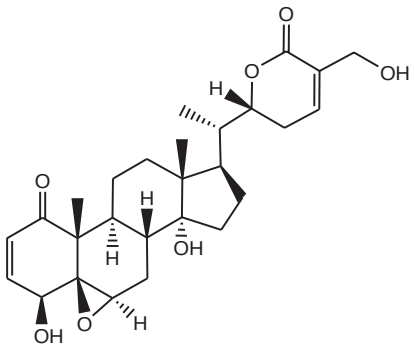
Bacoside A (**4**)



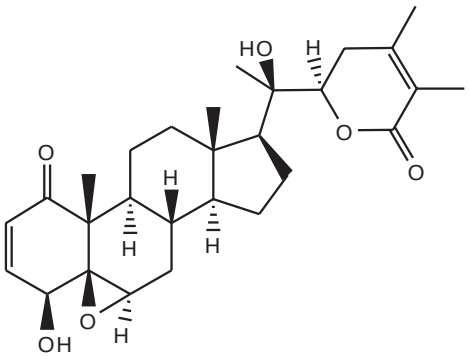
Liquiritin (**5**)



Liquiritigenin (**6**)



Withaferin A (**7**)



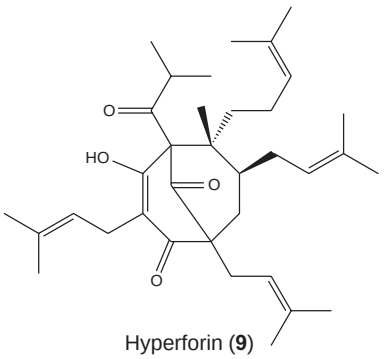
Withanolide D (**8**)

Several oxo-proto-berberine alkaloids, such as jatrorrhizine, berberine, palmatine, tetrahydro-berberine, and 20-hydroxyecdysone 8-oxoberberine, were reported from the *Coscinium fenestratum* stem (Deevanhxay et al., 2009). An LC–MS-based study revealed the presence of umbelliferone, psoralene, marmin, imperatorin, and skimmianine in *Aegle marmelos* (L.), root and stem bark (Gajbhiye et al., 2016). In 2009, Jiang et al. reported the presence of two pyrrolizidine alkaloids, senkirkine and senecionine, in *Tussilago farfara* flower extract (Jiang et al., 2009). Several phenolics and flavonoids, namely, lawsone, kaempferol, quercetin, and their glycosylated compounds, were found in *Impatiens balsamina* leaves (Chu, 2016). Table 17.1 summarizes the details of some secondary metabolites present in some well-known medicinal plants through LC–MS based studies. The structures of hyperforin (**9**) and three related polyprenylated acyl-phloroglucinols was elucidated by LC-DAD, -MS, and -NMR from St. John's wort (Fig. 17.4) (Charchoglyan et al., 2007).

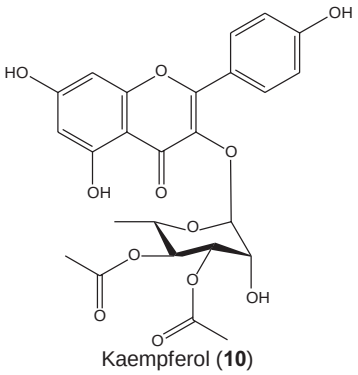
The identification of some novel antioxidants, metanephrine, benazepril, and prometone, from *Cajanus cajan* seeds was performed by high-resolution mass spectrometry analysis (Tekale et al., 2016). A large number of phenolic compounds, namely, kaempferol (**10**), catechin (**11**), syringic acid (**12**), quinic acid (**13**), sinapic acid (**14**), caffeic acid (**15**), chlorogenic acid (**16**), *p*-coumaric acid (**17**), gallic acid (**18**), and myricetin (**19**), were quantified in the Amaranthaceae plants by LC/ESI-MS/MS analysis. The biological activities of phenolic compounds extracted from Amaranthaceae plants were identified and characterized by LC/ESI-MS/MS, as represented in Fig. 17.5 (Yasir et al., 2016).

TABLE 17.1 LC-MS Profiling of Some Medicinal Plants

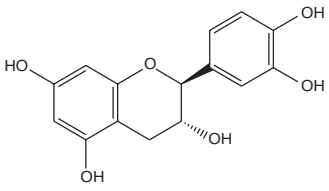
Plant Name	Secondary Metabolites	Parts Used	References
Centella asiatica	Asiatic acid, asiaticoside, madecassoside	Leaves	Shen et al. (2009)
Panax ginseng	Ginsenosides, pseudoginsenoside malonyl ginsenoside and ginsenoside	Rhizome	Xie et al. (2008)
Tussilago farfara	Senkirkine and (B) senecionine	Flower	Jiang et al. (2009)
Hypericum perforatum	Hyperforin, polyprenylated derivatives	In vitro tissue culture	Charchoglyan et al. (2007)
Artemisia annua	Artemisinin, artemisitene, arteannuin B, dihydroartemisinic acid, artemisinic acid	Leaves	Suberu et al. (2016)
Glycyrrhiza glabra	Liquiritin, liquiritigenin, isoononin, glycyrrhizic acid	Roots	Montoroa et al. (2011)
Rauwolfia serpentina	Reserpine, ajmalicine, ajmaline, serpentine, yohimbine	Roots	Kumar et al. (2016)
Gymnema sylvestre	Gymnemagenin	Leaves	Kamble et al. (2013)
Aegle marmelos (L.)	Umbelliferone, psoralene, marmin, imperatorin, skimmianine	Root and stem bark	Gajbhiye et al. (2016)
Coscinium fenestratum	Oxoprotoberberine, alkaloids, jatrorrhizine, berberine, palmatine, tetrahydroberberine, 20-hydroxyecdysone, 8-oxoberberine	Stem	Deevanhxay et al. (2009)
Commiphora wightii	*E*- and *Z*-Guggulsterone	Resin	Haque et al. (2009)
Rhodiola rosea	Flavonoids (gossypetin, herbacetin glycosides)	Arial part	Petsalo et al. (2006)
Bacopa monnieri	Bacopaside III, bacopasaponin G, and bacopasides A	Whole plant	Hou et al. (2002)
Andrographis paniculata	Apigenin-7-O-β-D-glucuronide, 5,4′-dihydroxy-7,8-dimethoxyflavone, 14-deoxyandrographiside, andrographolide, isoandrographolide, neoandrographolide, 14-deoxyandrographolide	Leaves	Song et al. (2013)
Withania somnifera	Withaferin A, withanolide D, withanoside IV or VI, withanolide sulfoxide	Roots	Trivedi et al. (2017)
Fritillaria spp.	Steroidal alkaloids	Dried bulb	Zhou et al. (2008)
Impatiens balsamina	Lawsone, kaempferol, quercetin and their glycosylated	Leaves	Chu (2016)
Cajanus cajan	Anabasamine, bisdesethylchloroquine, swietenine, desmethyltrimipramine, securinine	Seeds	Tekale et al. (2016)



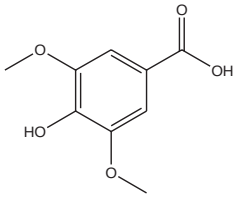
Hyperforin (**9**)



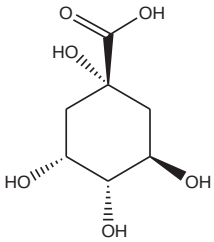
Kaempferol (**10**)



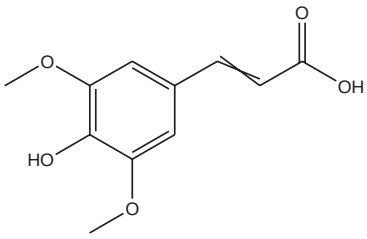
Catechin (**11**)



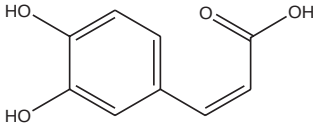
Syringic acid (**12**)



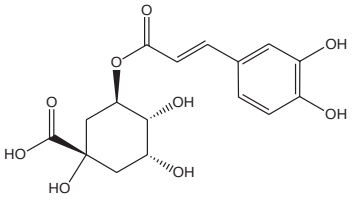
Quinic acid (**13**)



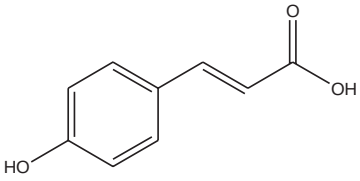
Sinapic acid (**14**)



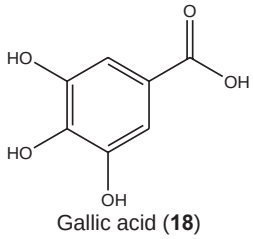
Caffeic acid (**15**)



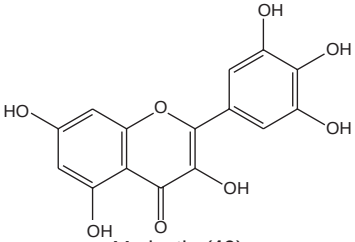
Chlorogenic acid (**16**)



p-Coumaric acid (**17**)



Gallic acid (**18**)



Myricetin (**19**)

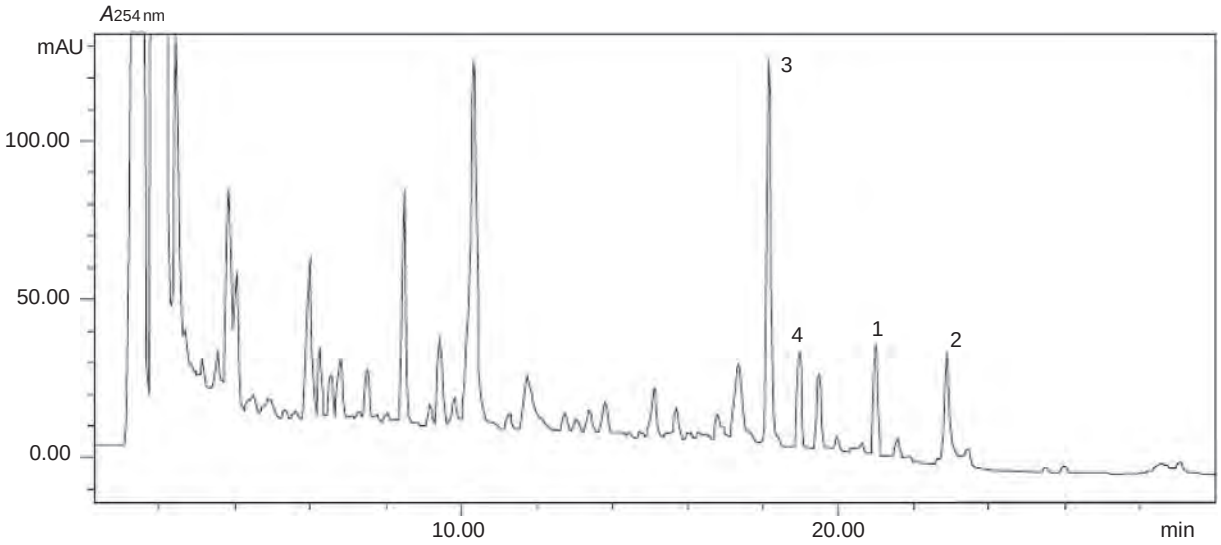


FIG. 17.4 LC–MS chromatogram of St. John's wort seco-hyperforin (**3**); Rt 18.85: adseco-hyperforin (**4**); Rt 21.04: hyperforin (**1**); Rt 21.53: adhyperforin (**2**). *Reproduced with permission from Charchoglyan, A., Abrahamyan, A., Fujii, I., Boubakir, Z., Gulder, T.A.M., Kutchan, T.M., Vardapetyan, H., Bringmann, G., Ebizuka, Y., Beerhues, L., 2007. Differential accumulation of hyperforin and secohyperforin in Hypericum perforatum tissue cultures. Phytochemistry 68, 2670–2677.*

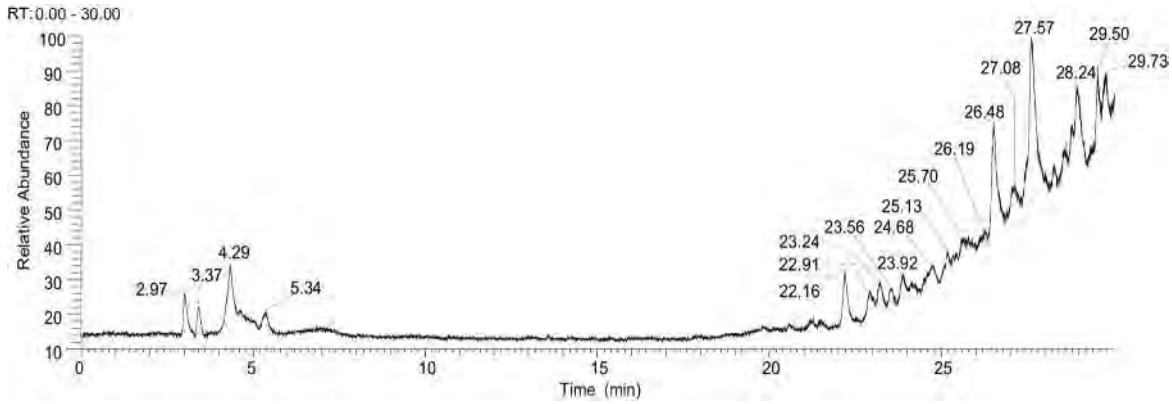


FIG. 17.5 Typical LC/MS chromatogram of *Achyranthes aspera* (Amaranthaceae plants). *Reproduced with permission from Yasir, M., Sultana, B., Amicucci, M., 2016. Biological activities of phenolic compounds extracted from Amaranthaceae plants and their LC/ESI-MS/MS profiling. J. Funct. Foods 26, 645–656.*

LC–MS/MS analysis was carried out to identify and quantify some hydrolysable tannins, flavonols, proanthocyanidins, and phenolic acids in *Quercus* leaf teas. A robust LC–MS-based method was developed for the determination of carotenoids in several plants (Fig. 17.6). This developed method was highly sensitive, selective, reproducible, and it reduced the analysis time (García-Villal et al., 2017).

17.2.2 GC-MS-Based Techniques

Gas chromatography–mass spectrometry (GC/MS) is one of the earliest techniques in metabolomics research. It is the most versatile tandem mass spectrometry technology, suitable for the analysis of metabolites with low polarity, low boiling point, or that are volatile after being derivatized. It has been one of the main analytical platforms in plant metabolomics due to its high resolution, high sensitivity, and good reproducibility (Qi et al., 2015). GC plays a role in separation and introduces target substances into an MS system by directly injecting analytes into a chromatographic column or introducing analytes into a chromatographic column after injecting and heating. The chromatographic column is heated thermostatically or is program-controlled. Each component is separated by the difference of thermodynamic properties (the difference of boiling points and the difference of selective absorption in the stationary phase) and the different distributions in stationary

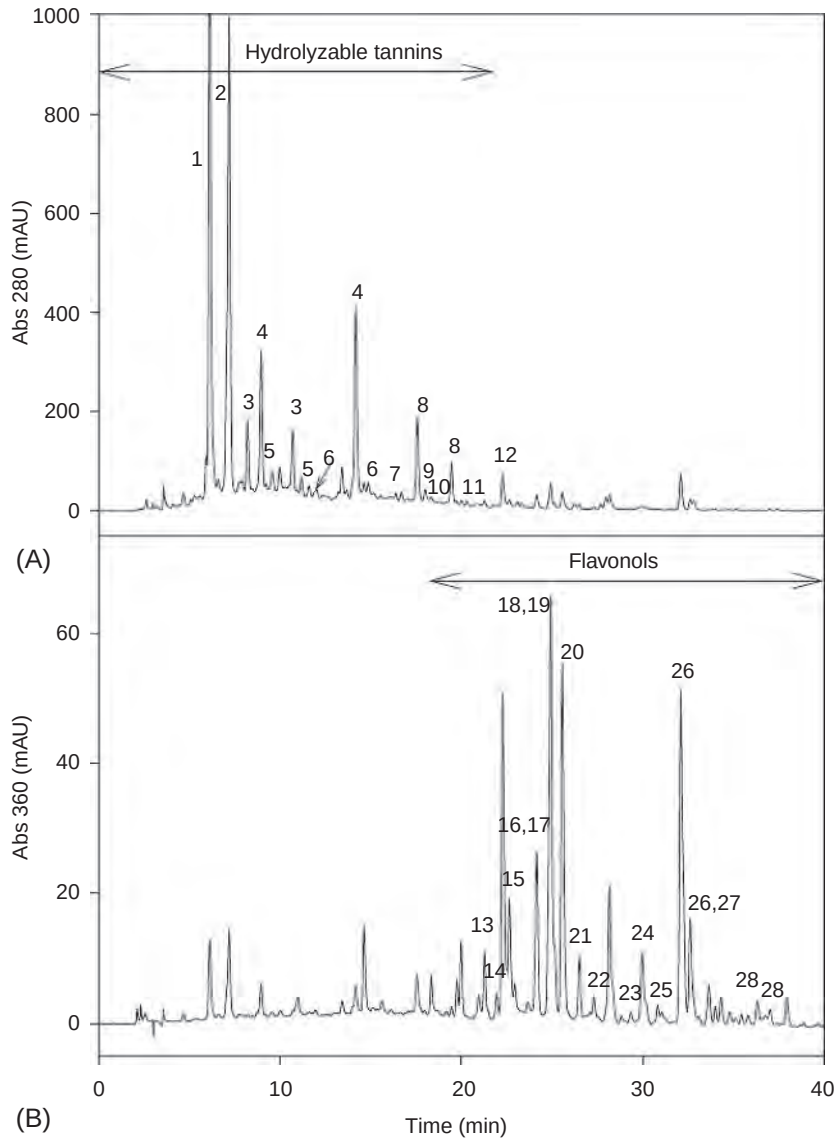


FIG. 17.6 LC–MS chromatogram of *Quercus* leaf teas. *Reproduced with permission from García-Villal, R., Espín, J.C., Tomás-Barberán, F.A., Rocha-Guzmán, N.E., 2017. Comprehensive characterization by LC-DAD-MS/MS of the phenolic composition of seven Quercus leaf teas. J. Food Compos. Anal. 1067, 34–37.*

phase and mobile phase (carrier gas). GC–MS represents the mass of a given particle (Da) to the number (z) of electrostatic charges (e) that the particle carries. GC–MS commonly uses electron impact (EI) and chemical ionization (CI) techniques. Target substances enter into MS through GC and are ionized into gaseous ions in the ionization source and then enter into the mass analyzer. Ions with different mass-to-charge ratios are sequentially separated and reach the electron multiplier, generating an electrical signal, in order to give information on the target substances, making qualitative analysis more accurate by using ion fragment information.

Although liquid chromatography-based methods offer distinct advantages, such as the broader range of metabolites detectable, they suffer from the lower reproducibility of retention times in liquid chromatography; in addition, owing to the predominant use of electron spray ionization, they are more susceptible to ion suppression effects, which render accurate quantification more difficult (Lisec et al., 2006). One of the advantages of GC–MS is that identification of detected species is based on both a retention time and a mass spectrum. Compounds produce reproducible fragmentation patterns when ionized by a fixed electron voltage (usually −70 eV). Thus, the fragmentation spectra obtained by GC–MS are not instrument dependent and allow for the creation of databases and the sharing of data between users, making the technique particularly valuable. In addition, GC–MS offers high sensitivity and reproducibility and permits working with standard libraries for the identification of detected species (Chauhan et al., 2014). In addition, some other features,

including the production of enhanced molecular ions, improved confidence in sample identification, significantly increased range for thermally labile and low-volatility samples, faster analysis, and improved sensitivity make GC–MS an ideal platform for plant metabolomics analysis. The major role of GC is to separate the analytes based on their polarity. After separation, the analytes are usually injected into the MS system directly. Helium is the commonly used mobile phase in GC/MS, which is provided by a compressed gas cylinder. In GC–MS, two types of columns are mainly used, packed and capillary columns. The chromatographic column is heated thermostatically or is program-controlled. An MS detector, consisting of an ionization source, mass analyzer, and electron multiplier tubes, is used. The target substances get ionized after entering the MS and finally offer qualitative and quantitative information of the target compounds by using the fragmentation pattern of the analytes (Xue et al., 2014). A schematic diagram of a GC/MS-based metabolomics study of medicinal plants is shown in Fig. 17.7.

The major drawback of GC–MS is that only volatile compound or compounds that can be made volatile after derivatization can be analyzed, and derivatization often requires extensive sample treatment. However, once the analysis is focused on low-molecular-weight metabolites, GC–MS is highly efficient, sensitive, and reproducible. Moreover, it is quantitative, and its compound identification capabilities are superior to other separation techniques because GC–MS instruments obtain mass spectra with reproducible fragmentation patterns (Xue et al., 2014). Moreover, coupled with a time-of-flight analyzer, GC–MS offers several advantages over the quadrupole technology, with faster scan times, improved deconvolution, reduced run times for complex mixtures, and higher mass accuracy (Lisec et al., 2006). Several therapeutically active essential oils and fixed oils have been identified and characterized through a GC–MS based platform. Most of them are classified into several chemical groups, namely, terpene hydrocarbons, monoterpene hydrocarbons, sesquiterpenes, phenols, alcohols, oxygenated compounds, sesquiterpene alcohols, esters, lactones, ketones, coumarins, ethers, monoterpene alcohols, aldehydes, and various oxides. GC–MS analysis revealed the presence of bicyclic monoterpenes and pinenes in the essential oil fraction of *Cuminum cyminum*, *Citrus limon*, and *Foeniculum vulgare* (Iacobellis et al., 2005). The aromatic phenol, eugenol, has been reported in *Eugenia aromatica*, *Zizyphus jujuba*, and *Ocimum sanctum* oils through a GC–MS based metabolomics study (Al-Reza et al., 2010). The GC–MS based phytochemical analysis of *Acorus calamus* rhizome revealed the presence of α- and β-asarone as a major secondary metabolite present in it (Kumar et al., 2010). The presence of ketonic terpenoids, such as carvone, was found in a significant amount in *Mentha spicata* and *Carum carvi* through GC–MS studies (Iacobellis et al., 2005; Boukhebti et al., 2011).

Setty et al. (2011) reported the presence of several secondary metabolites, namely, α-zingiberene, β-seiquphell andrene, α-curcumen, cyclohexane, and α-fernesene, in *Zingiber officinale* rhizome (Setty et al., 2011). GC–MS analysis was able to identify the presence of carvacrol (**20**), thymol (**21**), γ-terpinene, *p*-cymene, and limonene (**22**) in *Origanum vulgare*

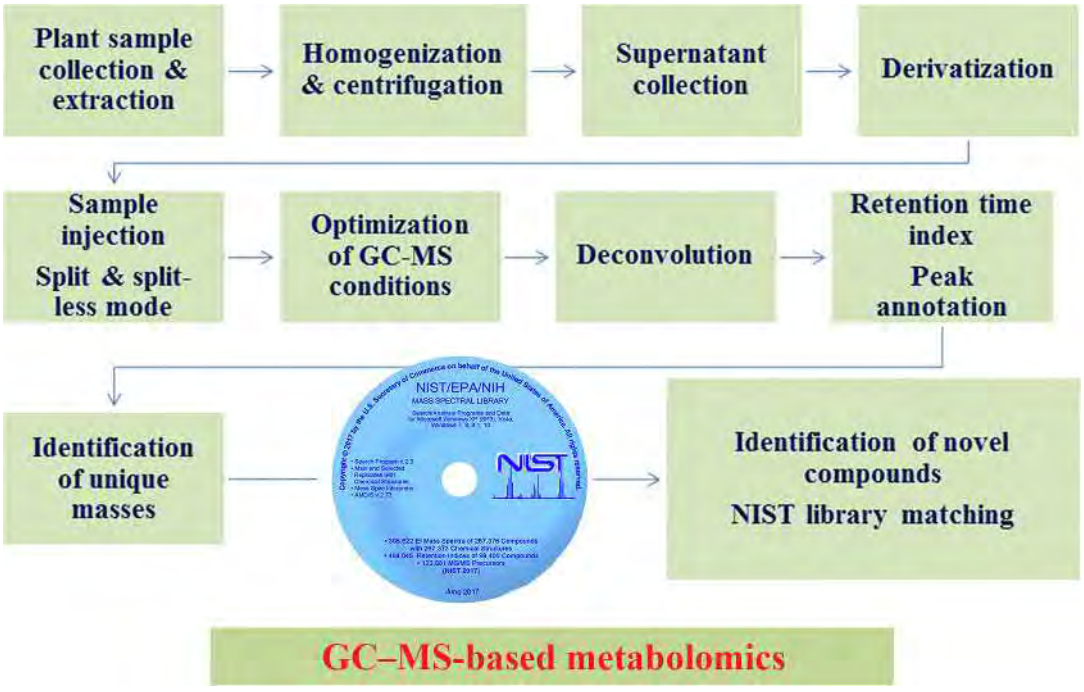
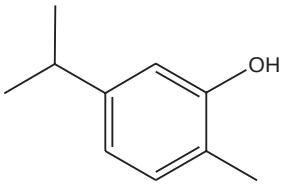
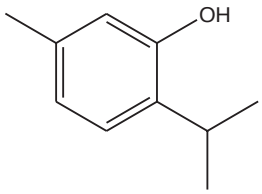


FIG. 17.7 The workflow of a GC–MS based metabolomics study of herbal medicine.

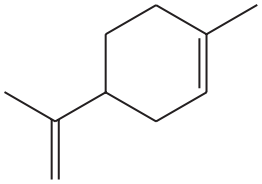
leaves (Derwich et al., 2010). Another GC–MS study was carried out for the determination of α-thujone (**23**), 1,8-cineole, α-pinene (**24**), camphor (**25**), and camphene in the aerial part of *Ricinus communis* (Kadri et al., 2011). A list of some important volatile/fixed oils obtained from medicinal plants may be found in Table 17.2. Several monosaccharides, polysaccharides, polyols, triterpenoids, phenolics, organic acids, fatty acids, alkaloids, and quinones were identified in *Artemisia herba alba* as major constituents (Fig. 17.8) for their antimicrobial and cytotoxic properties (Bourgou et al., 2017).



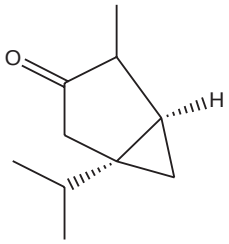
Carvacrol (**20**)



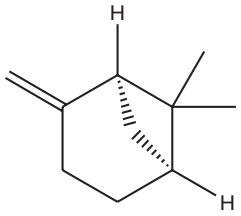
Thymol (**21**)



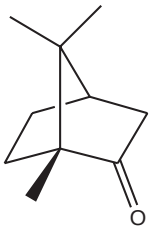
Limonene (**22**)



α-Thujone (**23**)



α-Pinene (**24**)



Camphor (**25**)

GC–MS analysis is also found to be very efficient for pesticide analysis in herbal plant extracts. Several efficient methods have been developed for the determination of 160 pesticides in herbal plant extracts (Fig. 17.9) (Taha and Gadalla, 2017).

A comparative metabolomics study of an Ayurvedic polyherbal formulation, Pancharishta, was undertaken by a group of researchers using a GC–MS platform in which 144 metabolites were identified (Fig. 17.10). In addition to that, 26 metabolites were found as common metabolites present at different stages of herbal formulation development (Khan et al., 2017). GC–MS profiles of some medicinal plants, as reported by different workers, are shown in Table 17.2.

17.2.3 LC-NMR-Based Techniques

NMR spectroscopy is another major analytical platform available for plant metabolomics studies. It offers several advantages over mass spectrometry by offering high reproducibility, quantitative analysis in a wide dynamic range, and the capability of determining structures of unknowns. NMR is capable of tracing metabolic pathways and fluxes using isotope labels and can quantify all the abundant compounds present in biological fluids, cell extracts, and tissues without the need for elaborate sample preparation or fractionation. It also offers advantages for compounds that are difficult to ionize or require derivatization for MS. NMR allows the identification of compounds with identical masses, including those with different isotopic isomer distributions. It also can be used to elucidate the dynamics and mechanisms of metabolite transformation and to explore the compartmentalization of metabolic pathways through the use of stable isotope labels. NMR-based metabolomics promises to improve the identification and quantitation of compounds in mixtures (Markley et al., 2017). As plant metabolomes are very complex due to their different polarity, chemical behavior, stability, and concentration, it is very

TABLE 17.2 GC–MS Profiling of Some Medicinal Plants

Plant Name	Chemical Constituents	Parts Used	References
Cuminum cyminum	α-Pinene, limonene, 1,8-cineole, linalool, α-terpineol	Seeds	Mohammadpour et al. (2012)
Mentha spicata	Carvone, limonene, germacrene-D, 1,8-cineole, β-caryophyllene	Leaf	Boukhebti et al. (2011)
Eugenia aromatica	Eugenol, eugenyl acetate, caryophyllene oxide, nootkatin, Phenol-4-(2,3-dihydro-7-methoxy-3-methyl-5-(1-propenyl)-2-benzofurane)	Buds	Nassar et al. (2007)
Tagetes minuta	*trans*-Tagetenone, *cis*-tagetenone, dihydrotagetone, *trans*-pinocarvyl acetate, carvone	Fruit	Vázquez et al. (2011)
Carum carvi	Carvone, limonene, germacrene D, *trans*-dihydrocarvone, carvacrol	Seed	Iacobellis et al. (2005)
Mentha piperata	Linalool, carvone, 3-octanol, *trans*-caryophyllene	Leaves	Sartoratto et al. (2004)
Nigella sativa	*trans*-Anethole, thymoquinone, *p*-cymene, longifolene, limonene	Seed	Gerige et al. (2009)
Citrus limon	Limonene, β-pinene, γ-terpinene, α-pinene, sabinene	Fruit	De Rodríguez et al. (1998)
Ricinus communis	α-Thujone, 1,8-cineole, α-pinene, camphor, camphene	Arial part	Kadri et al. (2011)
Origanum vulgare	Carvacrol, thymol, γ-terpinene, *p*-cymene, limonene	Leaves	Derwich et al. (2010)
Mentha spicata	Carvone, limonene, germacrene-D, 1,8-cineole, β-caryophyllene	Leaves	Boukhebti et al. (2011)
Piper longum	β-Caryophyllene, 3-carene, eugenol, D-limonene, zingiberene	Seed	Liu et al. (2007)
Cedrus deodara	α-Terpineol, linalool, limonene, anethole, caryophyllene	Needles	Zeng et al. (2012a,b)
Foeniculum vulgare	α-Pinene, limonene, *p*-cymene, fenchone, methyl chavicol	Root and schizocarps	Radulović and Blagojević (2010)
Ocimum sanctum	Eugenol, caryophyllene, cyclopentane, cyclopropylidene, benzene methanamine, octadecane	Leaves	Devendran and Balasubramanian (2011)
Zingiber officinale	α-Zingiberene, β-sesquiphellandrene, α-curcumen, cyclohexane, α-farnesene	Rhizome	Setty et al. (2011)
Acorus calamus	β-Asarone, 9,12-octadecadienoic acid, *n*-hexadecanoic acid, shyobunone, α-asarone	Rhizome	Kumar et al. (2010)
Ocimum basillicum	Borneol, naphthalene, α-cubene, eugenol, vanillin	Leaves	Dev et al. (2011)

difficult to analyze all the metabolites in one single experiment. NMR is a very suitable method to carry out such an analysis because it allows the simultaneous detection of diverse groups of secondary metabolites (flavonoids, alkaloids, terpenoids, and so on) besides abundant primary metabolites (sugars, organic acids, amino acids, and so on).

^{1}H NMR or one-dimensional (1D) is the most widely used NMR approach in metabolomics, which is able to generate the metabolomics data of a sample within a relatively short time. A typical ^{1}H NMR spectrum of plant material reflects a number of all the metabolites in the extract because the NMR signals are directly proportional to the molar concentration, independent of the characteristics of a compound. The absolute concentration of metabolites can be calculated by comparison of the peak intensity with an internal standard. In addition, NMR is a very useful technique for structure elucidation. By using various two-dimensional NMR measurements, many signals can be identified without the need for further fractionation of the extract (Kim et al., 2010).

The hyphenated and two-dimensional (2D) NMR techniques play a key role in compound identification, as well as fingerprinting. The 2D NMR method offers improved approaches for unambiguous identification of metabolites in mixtures. These 2D methods include ^{1}H–^{1}H COSY (correlated spectroscopy), ^{1}H–^{1}H TOCSY (total correlation spectroscopy), and ^{1}H–^{13}C HSQC (heteronuclear single-quantum correlation) (Markley et al., 2017). The NMR-based techniques also utilize ^{13}C NMR spectroscopy, which is capable of assessing ^{13}C enrichment for each nonequivalent carbon atom of a metabolite under study. On the basis of these ^{13}C NMR coupling signatures observed for every single (nonequivalent) carbon atom in a ^{13}C-labeled compound, information on the abundance of ^{12}C and ^{13}C at the respective neighboring carbon atoms is obtained. The metabolites are quantified on the basis of the signal intensities in terms of deconvolution of the complex

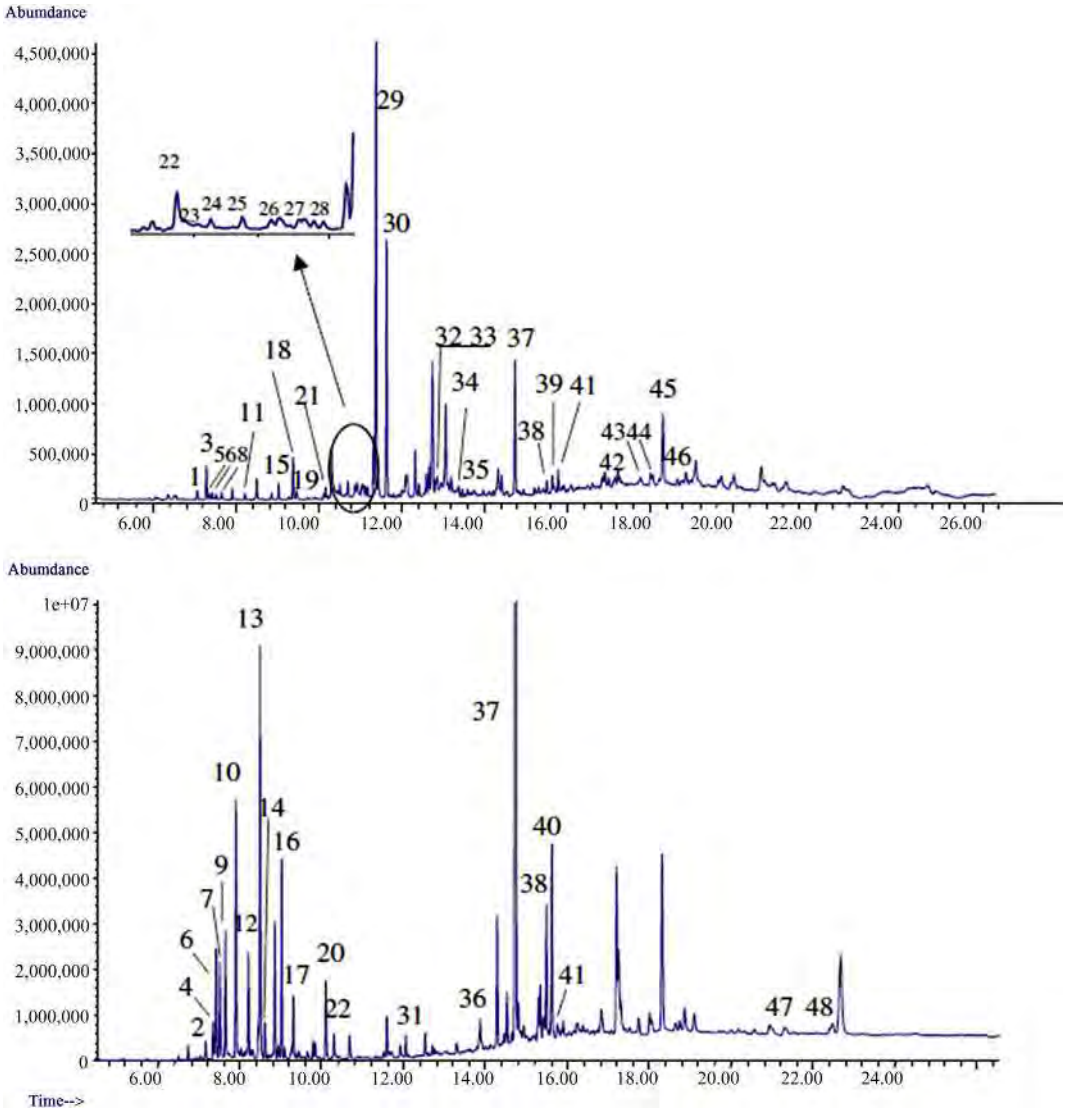


FIG. 17.8 GC–MS total ion chromatogram of *Artemisia herba alba. Reproduced with permission from Bourgou, S., Bettaieb, I., Mkadmini, K., Isoda, H., Ksouri, R., Ksouri, W.M., 2017. LC-ESI-TOF-MS and GC-MS profiling of Artemisia herba-alba and evaluation of its bioactive properties. Food Res. Int. 99, 702–712.*

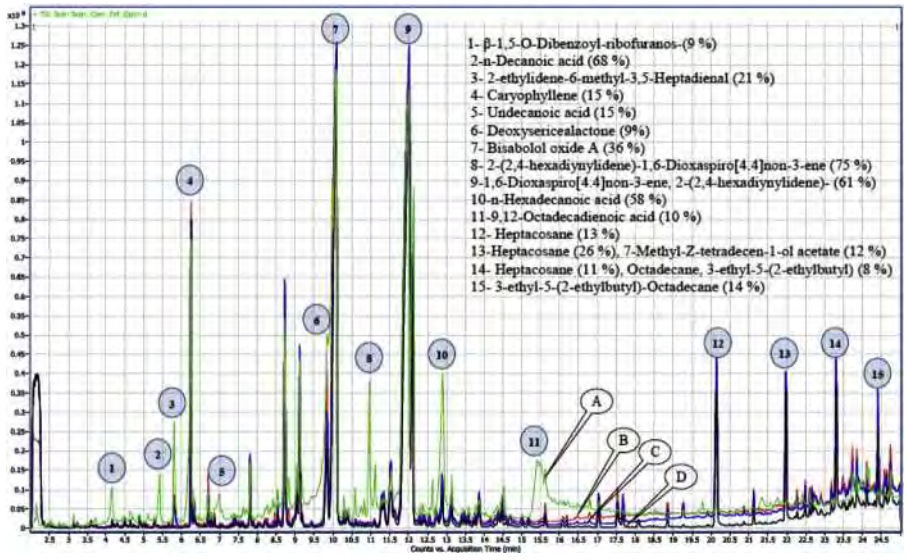


FIG. 17.9 GC–MS chromatogram of pesticides in herbal plant extract. *Reproduced with permission from Taha, S.M., Gadalla, S.A., 2017. Development of an efficient method for multi residue analysis of 160 pesticides in herbal plant by ethyl acetate hexane mixture with direct injection to GC-MS/MS. Talanta 174, 767–779.*

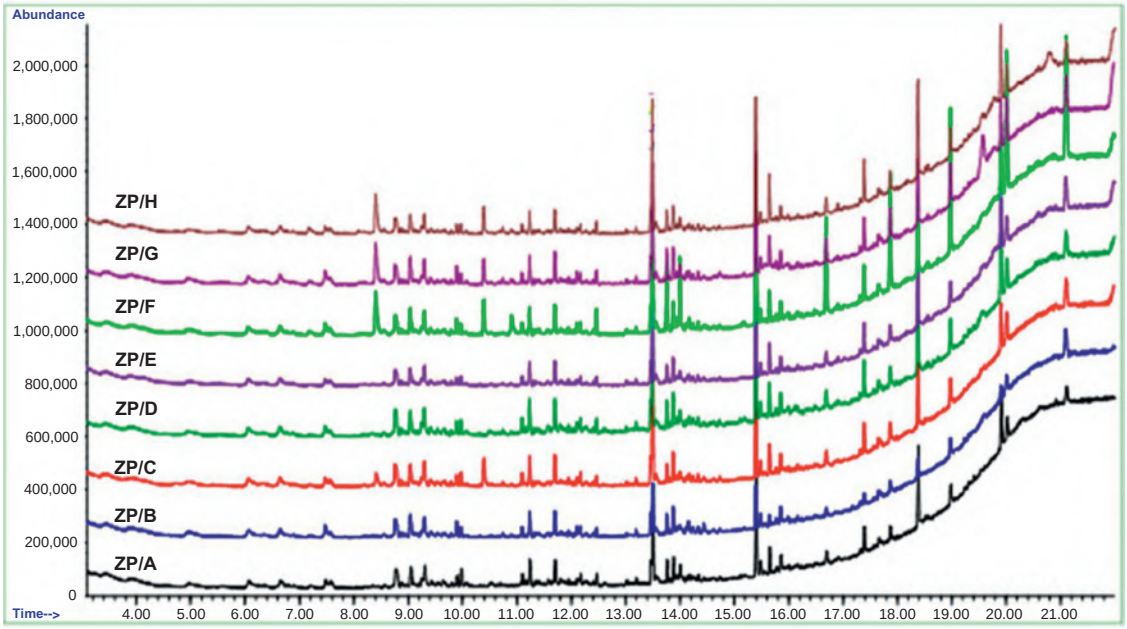


FIG. 17.10 GC–MS chromatogram of different Pancharishta formulations. *Reproduced with permission from Khan, W., Chester, K., Anjum, V., Ahmad, W., Ahmad, S., Narwari, A., Kumar, D.P., Katiyar, C.K., 2017. Chromatographic profiling of Pancharishta at different stages of its development using HPTLC, HPLC, GC–MS and UPLC–MS. Phytochem. Lett. 20, 391–400.*

coupling patterns for each component in ^{13}C NMR multiplets. The general workflow of an NMR-based metabolomics study involves sample preparation (freeze drying followed by extraction by ultrasonication with deuterated solvents), NMR analysis (standard ^{1}H, *J*-resolved, ^{1}H–^{1}H COSY, and heteronuclear multiple bond correlation [HMBC]), and chemometric methods, such as PCA and PLS-DA (Kim et al., 2010). A schematic diagram of an NMR-based study is shown in Fig. 17.11.

NMR metabolomics is gaining popularity in the fields of plant science and natural product chemistry, including species discrimination, quality control of medicinal plants, monitoring the biochemical response of plants to stress conditions, and investigations of alterations in genetically modified plants (Weckwerth and Kahl, 2013).

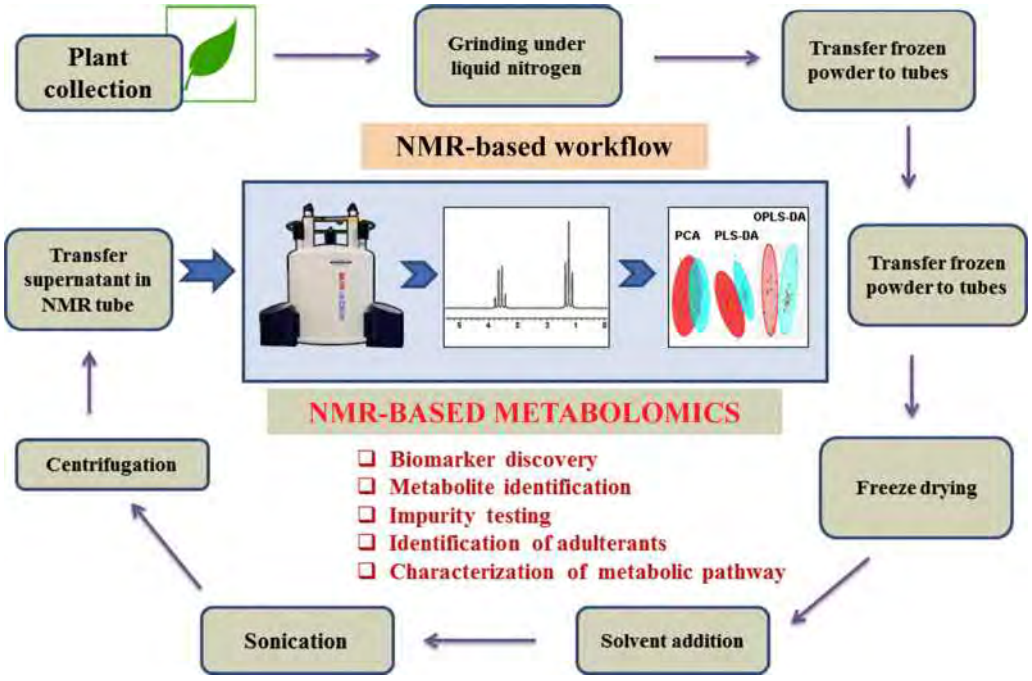


FIG. 17.11 NMR-based metabolomics for herbal medicine.

An LC-NMR based study has been reported for understanding the phyto-constituents and chemical variability of different curcuma species (Brooker et al., 2014). An authenticity and quality control study of *Rhodiola rosea* products was reported through an NMR-based metabolomics study (Booker et al., 2016a). An LC-NMR-based study was also found important for discriminating the major phyto-constituents of the root bark and stem bark of *Strychnos nux-vomica* (Frederich et al., 2004). A metabolomics study of Ephedra species was reported by Kim and his coworkers using ^{1}H NMR spectroscopy in addition to principal component analysis (Kim et al., 2005). A comparative study of the presence of phenyl-propanoids and flavonoids among three batches of *Farfarae flos* was carried out using an NMR-based metabolomics approach (Li et al., 2017a,b). An NMR-based metabolomics study was also found to be an effective quality control tool for the Chamomile flower (*Matricaria recutita* L.) (Wang et al., 2004). The differences between the artemisinin content in *A. annua* and *A. afra* was carried out for quality evaluation of *Artemisia* sp. (Van der Kooy et al., 2008). A metabolomic characterization of *Peucedani radix* was carried out for the determination of praeruptorin A (**26**) and praeruptorin B (**27**) using an NMR-based platform (Song et al., 2014). Some examples of LC-NMR based metabolomics are highlighted in Table 17.3.

TABLE 17.3 Metabolomics Profiling of Some Plants Through LC-NMR Studies

Plant Species	Work Performed	Outcome	Chemical Constituents	References
Ilex species	Metabolomic fingerprinting using nuclear magnetic resonance	Determination of a large number of metabolites makes the chemotaxonomical analysis of *Ilex* species possible; determination of adulterants	Arbutin, caffeine, phenylpropanoids, and theobromine	Choi et al. (2005)
Chamomile flower (*Matricaria recutita* L.)	Quality control of phytomedicine	Provides an efficient tool for the quality control and authentication of phytomedicines		Wang et al. (2004)
Strychnos nux-vomica	Metabolic profiling of three *Strychnos* species	Major compounds responsible for the discrimination of root bark and stem bark samples from *S. nux-vomica* from different origins	Brucine, loganin, fatty acids, and *Strychnos icaja* alkaloids, such as icajine and sungucine	Frederich et al. (2004)
Farfarae flos	Three batches of *Farfarae flos* were compared by an NMR-based metabolomic approach	Three batches of FFs differed from each other both in the primary and secondary metabolites, and there also existed in vivo differences among three groups of FFs	Phenylpropanoids and flavonoids	Li et al. (2017b)
Tree peony cultivars	Distribution of metabolites in the root barks of different tree peony cultivars for quality assessment	16 metabolites, from their methanol extracts, were simultaneously identified and quantified	Acetophenones, phenolics, monoterpene glycosides, flavonoids, and unsaturated fatty acids	Wang et al. (2017)
Rhodiola rosea	Authenticity and quality of *Rhodiola rosea* products Approximately 40 commercial products (granulated powders and extracts) were sourced from different suppliers	Adulteration with different species was identified	Rosavin, salidroside	Booker et al. (2016a)
Gingko biloba	Adulteration and poor quality of *Ginkgo biloba* supplements	Identification of rutin or quercetin as adulterants in *Ginkgo* supplements	Quercetin, rutin, kaempferol	Booker et al. (2016b)
Curcuma longa	Chemical variability along the value chains of turmeric (*Curcuma longa*)	Metabolomic composition of products derived from *Curcuma longa*	Bisdemethoxycurcumin tumerone	Brooker et al. (2014)

Continued

TABLE 17.3 Metabolomics Profiling of Some Plants Through LC-NMR Studies—cont'd

Plant Species	Work Performed	Outcome	Chemical Constituents	References
Peucedani radix	Metabolomic characterization of *Peucedani radix*	Determination of praeruptorin A and praeruptorin B is a promising means for evaluating medicinal samples of *Peucedani radix*	Praeruptorin A and praeruptorin B	Song et al. (2014)
Leontopodium franchetii	Metabolic fingerprinting of *Leontopodium* species	Metabolic patterns of 11 different *Leontopodium* species to determine their taxonomic relationship	Fatty acids, sucrose A, ent-kaurenoic acid, diterpenes, sesquiterpene	Safer et al. (2011)
Ephedra sp.	Metabolic fingerprinting of *Ephedra* species using ^{1}H NMR spectroscopy and principal component analysis	Discrimination of three different species of *Ephedra*	Ephedrine and benzoic acid analogue	Kim et al. (2005)
Artemisia sp.	Metabolomic quality control of *Artemisia afra* and *A. annua*	Differences between *A. annua* and *A. afra* based on their artemisinin content	Artemisinin	Van der Kooy et al. (2008)

NMR-based metabolomic analyses of three batches of *F. flos* were carried out by Li and his research group, as represented in Fig. 17.12 (Li et al., 2017a,b). NMR-based metabolomic fingerprinting was undertaken (Asteraceae) by means of a ^{1}H NMR study to find the taxonomic relationship between some *Leontopodium* species; a chromatogram is shown in Fig. 17.13 (Safer et al., 2011).

In 2014, Song et al. proposed an NMR-based method to characterize praeruptorin A and praeruptorin B in *P. radix* simultaneously. This method, coupled with principal components analysis (PCA), was also implemented for carrying out a complete metabolomic characterization of the *P. radix* species; a chromatogram is shown in Fig. 17.14 (Song et al., 2014).

17.3 METABOLOMICS IN SYSTEMS BIOLOGY

Metabolome analysis is an important tool in functional genomics, revealing the roles of genes from the comprehensive analysis of metabolomes. It is an emerging tool in systems biology for the quantitative study of an organism, DNA, mRNA, proteins metabolites, and their environment. It is also found very useful for studying relationships among active molecular components of the cell and their predicting behaviors. The metabolomics-driven system biology approach promises to transform the practice of medicine by facilitating drug discovery, treating disease, and improving bioprocesses. It mainly

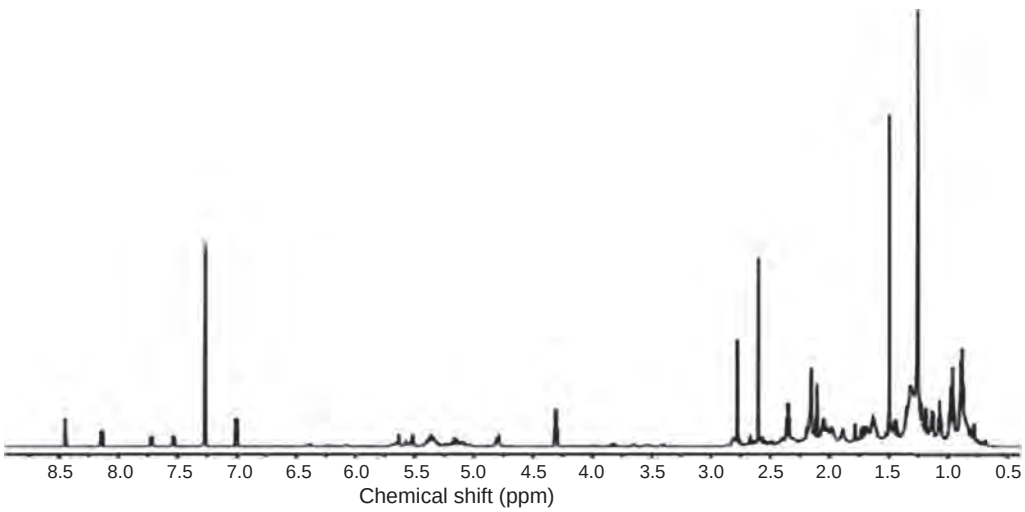


FIG. 17.12 Typical ^{1}H NMR spectra of *Farfarae flos*. *Reproduced with permission from Li, Z.Y., Li, J., Zhang, Z.Z., Mi, X., Du, G.H., Qin, X.M., 2017b. NMR-based metabolomic analyses for the componential differences and the corresponding metabolic responses of three batches of Farfarae flos. Chemom. Intell. Lab. Syst. 165, 1–10.*

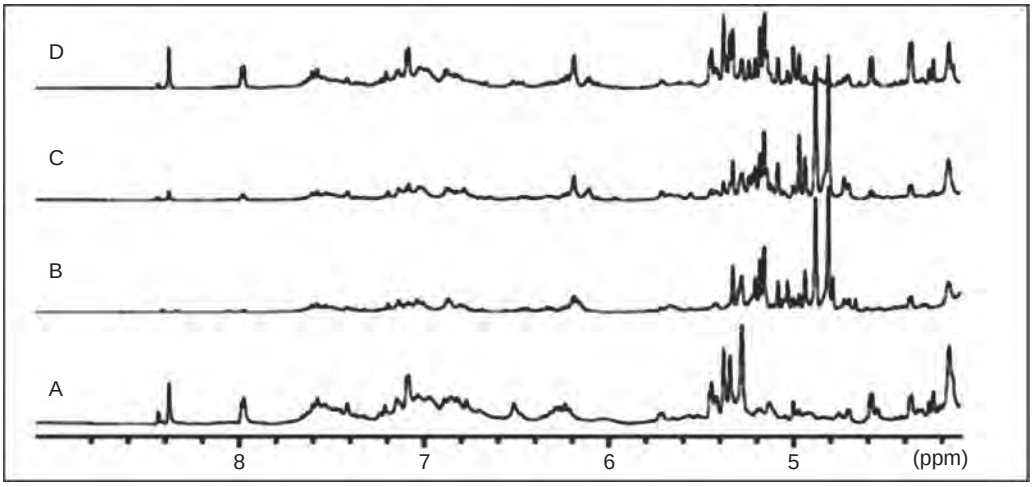


FIG. 17.13 Typical ^{1}H NMR spectra of different *Leontopodium* species closely related to *L. sinense* (D), *L. franchetii* (B), *L. dedekensii* (A), and an unidentified species, *L.* sp. (C). *Reproduced with permission from Safer, S., Cicek, S.S., Pieri, V., Schwaiger, S., Schneider, P., Wissemann, V., Stuppner, H., 2011. Metabolic fingerprinting of Leontopodium species (Asteraceae) by means of 1H NMR and HPLC–ESI-MS. Phytochemistry 72, 1379–1389.*

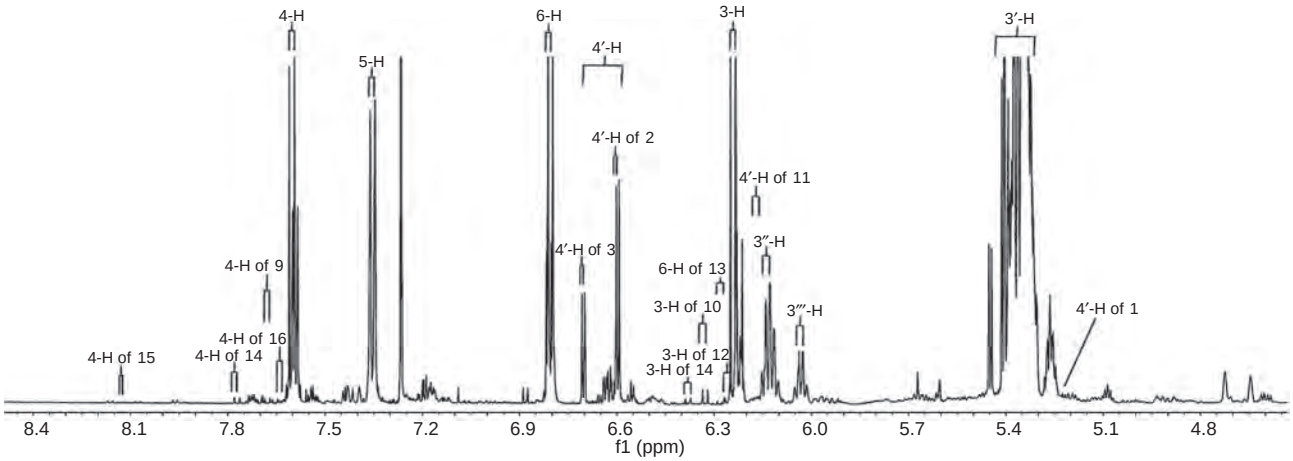


FIG. 17.14 ^{1}H NMR spectra of *Peucedani radix. Reproduced with permission from Song, Y.-L., Jinga, W.-H., Chen, Y.-G., Yuan, Y.-F., Yana, R., Wang, Y.-T., 2014. 1H nuclear magnetic resonance based-metabolomic characterization of Peucedani radix and simultaneous determination of praeruptorin A and praeruptorin B. J. Pharm. Biomed. Anal. 93 (2014) 86–94.*

focuses on quantifying metabolite levels and flows in primary metabolism. The major role of metabolomics in systems biology involves bridging the gap between genetic sequences and metabolites (Nielsen and Jewett, 2007; Wang et al., 2004). The use of LC–MS has been exploited to explore the metabolic activity and flexibility through dynamic analysis of intracellular metabolites. Apart from quantification of the biomolecules involved in central metabolism, target analysis of several metabolite pools is the key focus area of system biology-driven research.

The study of different metabolite levels from different biological conditions has also helped to integrate system biology with omics technology-driven research (Nielsen and Jewett, 2007). This approach can integrate the *endo*-metabolome (intracellular metabolites) and the *exo*-metabolome (extracellular metabolites) for identifying functional plant secondary metabolites in a targeted manner (Kell, 2004; Ward et al., 2007). With the development of omics technology, the system biology and network pharmacology approaches have been extensively implemented in the exploration of polypharmacological effects in herbal medicine. In 2017, Li and his coworkers evaluated the interaction of Qishenkel (QSKL), a Chinese herbal formula, with the specific pharmacological targets related to chronic myocardial ischemia (Li et al., 2017a,b). Additionally, the system toxicology approach was found to be an effective tool for evaluating the safety and quality parameters of Chinese herbal medicine. For example, the alkaloid profiling of *A. radix* in (mass spectroscopic) revealed that the toxicity of *A. radix* might be due to the presence of aconitine, mesaconitine, and hypaconitine (Wang et al., 2009a,b).

The system pharmacology-based in silico approach was applied to understand the pharmacological properties of Licorice by analysis of drug–target and drug–target–disease networks (Liu et al., 2013). The application of transcriptomics also plays a vital role in the quality control of herbal medicines by linking their gene expression profiles with biological, therapeutic, and toxicological potentials. A high-throughput microarray analysis of curcumin (**28**) has been reported for exploration of its antimetastatic activity in lung adenocarcinoma cell lines (Chen et al., 2004). The development of a system pharmacology approach will accelerate the evidence-based research of herbal medicine by integrating genomics, proteomics, and metabolomics platforms.

17.4 APPLICATION OF METABOLOMICS IN HERBAL MEDICINE

Medicinal plants contain a diverse array of metabolites, which have been used throughout history for preventing or treating disease. In this context, the standardization, quality control, and biological evaluation of herbal medicine are necessary for validating the ancient claims for healthcare. In turn, to improve the accuracy and consistency of herbal/medicinal plant-based preparations worldwide, it has become mandatory to develop new analytical methods for the standardization of herbal drugs. Therefore, utmost attention is necessary for the promotion and development of HM through metabolite profiling, which not only identifies the metabolites relative to the distribution of compounds with each other but also compares the nature of the compounds (Mukherjee et al., 2016).

Metabolomics is an important emerging technology in the natural product-based drug discovery process based on several hyphenated analytical methods, such as GC–MS, LC–MS, and NMR spectroscopy with data mining tools. It has been proven to be very rapid and superior to any other postgenomics technology for pattern recognition analyses and thus plays a significant role in fundamental plant biology and applied agricultural biotechnology. It is also applicable to assess the quality of crops, to plant improvement, to ensure the safety of botanical dietary supplements, to evaluate genetically modified crops, and to characterize metabolic responses to abiotic stresses (e.g., water deficiency, salt, and drought stress). In particular, metabolite profiling has been proven to be useful to the quality assurance of herbal medicines in order to ensure their safety and efficacy. Nontargeted metabolite profiling with large-scale data collection, followed by chemometric evaluation, has been proven to be a powerful tool for classifying several morphological characteristics of herbal ingredients (Hanhineva and Pasanen, 2017). There are several metabolomics studies available for characterization of plant metabolites with nutritional importance linked with the complex relationships between the dietary intake and its metabolism in the human body. Additionally, bioinformatics plays a key role in facilitating the storage, dissemination, and interpretation of metabolomics data obtained from complex mixtures of herbal medicine (Weckwerth and Kahl, 2013).

17.5 QUALITATIVE AND QUANTITATIVE STUDY OF HERBAL MEDICINE

Quality control of herbal medicine continues to be a challenge and restricts the development of herbal medicine throughout the world. The MS- and NMR-based metabolic fingerprinting techniques have been used for studying the quality of herbal medicines by characterizing the chemical constituents of phytopharmaceuticals (van der Kooy et al., 2009). The fingerprint analysis technique has been introduced and accepted by the World Health Organization (WHO), the Food and Drug Administration (FDA), and the European Medicines Agency (EMEA) as a strategy for assessing consistency between batches of botanical drugs. One of the main difficulties of the quality assessment of herbal drugs is the lack of an analytical method for scientifically evaluating the complex chromatograms of herbal medicines. The fingerprint analysis technique does not correct the chromatographic shifts among different runs or from different experimental conditions and cannot compare the fingerprints of herbal medicines from different species of herbs, grown at different locations, from different harvesting seasons, or extracted and processed using different methods. To overcome the difficulties, LC–MS/GC–MS-based metabolomics has been found to be an effective approach for the quality control of herbal medicine to ascertain the consistency/variations in multiple batches of herbal medicines. In combination with different data analysis methods, such as principle component analysis and OPLS-DA, it can reduce the dimensionality of multivariate data without losing important information. Thus, LC–MS/GC–MS-based metabolomic studies are very useful in qualitative and quantitative profiling of the major phyto-constituents present in herbal medicine, in addition to multidrug target screening for improvement of therapeutic activity. An integrated metabolomic-based quality control strategy was reported in Shengmai Injection (traditional Chinese medicine) for the evaluation of its quality, efficacy, and safety (Li et al., 2013a). In 2012, Zhang and his coworkers performed a quality control study of white and red ginseng using UPLC-QTOF-MS/MS-based techniques (Zhang et al., 2012a,b). The application of direct analysis in real time mass spectrometry and multivariate data analysis was reported for quality evaluation of traditional Chinese medicine by Zeng et al. The emergence of the "Herbalome" concept in traditional Chinese medicine has introduced several insights in the quality control process by integrating new separation

techniques for compound identification, high-throughput screening, and the construction of an herbal resource library and system biology research (Zhang et al., 2012a,b). Another comprehensive approach of "Phytomics QC" was found very applicable in the quality control of different batches of Huangqin Tang (a traditional Chinese medicine). This approach was able to combine the chemical and biological fingerprints of botanical extracts based on their LC/MS and qRT-PCR profiles (Tilton et al., 2010). In 2015, Heinrich and his group proposed an interdisciplinary approach between chemical variability and value chains of herbal medicines with the application of metabolomics in qualitative and quantitative analysis of biomarkers present therein for an effective quality control tool for the determination of adulterants (Heinrich, 2015).

17.6 IN VITRO, IN VIVO, AND CLINICAL STUDIES OF HERBAL MEDICINE THROUGH METABOLOMICS

The metabolomic approach is becoming a useful tool for investigating changes in the metabolome of biological systems, providing information on the small molecules in cells or tissues, and defining the metabolic signature, thus leading to enhanced understanding of disease mechanisms. It mainly assesses the final downstream products of gene transcription, reflecting the phenotypic characteristics of the system. The potential applications of metabolomic profiling techniques for evaluating bioactivity and the possible nutraceutical properties of diverse foods and/or bioactive compounds have created unprecedented opportunities. Metabolomics, transcriptomics, and proteomics also offer the possibility of linking food with health and diseases, thus emerging as a new omics platform called "foodomics" (Garcia-Cañas et al., 2012). However, in vitro metabolomics can focus on a small number of components, providing practical global knowledge about the metabolic pathways in which natural products are involved. In this context, Catalán et al. (2017) highlighted the biological effects of some phenolic compounds through in vitro metabolomic approaches for understanding disease mechanisms. The conventional approach of disease development can be understood in in vitro cellular studies based on some specific disease molecules at the protein and/or RNA level (Catalán et al., 2017). Metabolomics was also applied in herb–drug interaction studies to explain the role of plant secondary metabolites in the inhibition of CYP3A4 by using LC–MS and NMR-based platforms. Metabolomics cell-based studies have emerged as a promising technique to elucidate the mechanisms of action of herbal drugs in the cellular systems, thus establishing safety margins for herbal medicines (Hanhineva and Pasanen, 2017).

The analysis of the biological/metabolic alteration in herbal treatment often involves the use of animal experiments. This offers the possibility to examine the metabolic events perturbed with the herbal treatment in various organs, rather than solely with biofluid samples as is the case in human trials. In this regard, the nontargeted metabolite profiling approaches are useful in providing detailed information about the herb-derived compounds that are potentially deposited in various organs, as well as monitoring the effect on the endogenous metabolite levels. Several metabolic differences were observed with the herbal treatment reflected in the alterations of various pharmacological activities in terms of impaired amino acids, carbohydrates, and lipid metabolism. In this way, LC/GC–MS and NMR-based metabolomic analyses are useful to understand the role of several biomarkers involved in the disease pathway (Hanhineva and Pasanen, 2017).

The systematic investigation of drug metabolism has become an indispensable part of drug development, clinical therapy, and the mechanistic study of drug-induced toxicity. Many drugs are transformed into more polar and stable metabolites by hepatic enzymes, which facilitate their elimination from the body. Reactive metabolites can be generated during the process of drug metabolism that can covalently bind to protein, RNA, and DNA, resulting in toxicity. The identification of reactive metabolites helps researchers to minimize the formation of reactive metabolites by optimizing the structure of the parent drug. This knowledge also provides valuable information on the mechanism of drug-induced toxicity. Retrometabolic drug design is a strategy for the design of safer drugs using either a soft drug or targeted drug delivery approaches in the field of drug discovery. Retro-metabolic drug design has been found to be a noble approach based on drug metabolism, which can improve the bioavailability of poorly bioavailable drugs (mostly natural products) by metabolism-based structural modification of active pharmacophores (Bodor and Buchwald, 2008). Compared with traditional methods, an LC–MS-based metabolomic strategy can provide a more comprehensive profile of both regular and reactive metabolites and is capable of handling the large data-sets generated from the bio-matrix. It also results in metabolite identification and straightforward visualization of the metabolic changes after the administration of herbal medicine (Li et al., 2012).

Metabolomics approaches have been used to estimate the herbal constituents found in urine or plasma, and also for evaluating the alterations of various biomarkers related to endogenous metabolism. The therapeutic efficacy is monitored in response to the responsiveness of patients/subjects to endogenous metabolism, including carbohydrates, lipids, micronutrients, and vitamins in blood and/or urine samples. One of the major goals is to identify the potential biomarkers to be considered as an endpoint for pharmacodynamics outcomes. Several clinical trials were undertaken to observe the therapeutic response to herbal therapy by analyzing the biomarkers present in the plasma and urine collected from patients (Hanhineva and Pasanen, 2017). From the clinical viewpoint, pharmaco-metabolomics has emerged as a novel translational

"omics" tool that has the potential to accelerate drug development by identifying the efficacy and safety of herbal medicine. Pharmaco-metabolomics complements the genomic, transcriptomic, proteomic, and epigenomic approaches, leading to a comprehensive and holistic understanding of drug effects, considering the interindividual variation in drug response (Burt and Nandal, 2016).

Metabolomics also plays a vital role in the development and exploration of the concept of "precision medicine." Current metabolomic technologies are capable of analyzing hundreds to thousands of metabolites followed by characterization of metabolic phenotypes. It can be applied for the characterization of metabolic derangements that underlie the discovery of new therapeutic targets and biomarkers that may be used to either diagnose disease or monitor the activity of therapeutics. These LC–MS, GC–MS, and NMR-based clinical metabolomics approaches targeting PK–PD analysis can be integrated in order to increase the clinical relevance of the identified biomarkers and precision medicine (Kohler et al., 2017; Clish, 2015).

The ^{1}H NMR- and MS-based metabolomics approaches were implemented to explore the therapeutic effect of *Cortex fraxini* on rats and also for predicting hyperuricemia (Wang et al., 2016). In 2016, Zhang and his coworkers carried out a metabolomics study of urine samples for the prediction and diagnosis of *Polygonum multiflorum*-induced hepatotoxicity by using an integrated LC–MS-based metabolic approach, followed by a pattern recognition approach and pathways analysis (Zhang et al., 2016). The LC–MS-based targeted and untargeted metabolomics approach was also applied for exploring the memory-improving effect of ginseng (Feng et al., 2016). Further bioactivity-guided metabolite profiling, along with a multivariate data analysis approach, was able to identify some major target compounds in plant extracts (Abdallah et al., 2015). An NMR-based metabolomics study can correlate the pharmacological activity of the bioactive constituents of herbal medicine. For example, one research group in China identified chlorogenic acid, 3,5-dicaffeoylquinic acid, and rutin as the major constituents present in *Tussilago farfara* and related them with their antitussive and expectorant activities. This work was carried out through ^{1}H NMR spectrometry together with PCA and partial least squares discriminant (PLS-DA) analysis (Li et al., 2013b). LC–MS- and ^{1}H NMR-based metabolomics analyses were used in the in vitro toxicological assessment of the *Aristolochia* species for understanding the mechanism of toxicity (Michl et al., 2016). Thus, it can be concluded that in vitro metabolomic studies are able to identify the mechanism of action of several phyto-molecules at the cellular level. It has been found that in vitro assays are helpful for studying drug safety through the CYP-mediated metabolism pathway. In this context, several studies were performed with *Echinacea purpurea, Ginkgo biloba*, and *Serenoa repens* on human CYP2C9, 2D6, and 3A4 in metabolomic and system biology platforms for exploration of their potential herb–drug interaction (Yale and Glurich, 2005). Another group of researchers found that coadministration of St. John's wort and clopidogrel increased the antiplatelet effect by enhancing CYP3A4 activity (Lau et al., 2011). In a study, Catalán and his colleagues elucidated the mechanisms of action of some polyphenolic compounds by using a cell-based metabolomics approach to understand their potential biological effects. This study highlighted the application of in vitro MS-based assays in a human cell line to understand the biological pathways involved. The active constituents of rosemary extract, carnosol, and carnosic acid were employed in a nontargeted metabolomics study for evaluation of the biological pathways involved in neuro-transmitter degradation, polyamine metabolism, terpenoid metabolism, and others (Catalán et al., 2017). Thus, metabolomics approaches will provide future directions in natural product-based drug development and discovery.

17.7 STATISTICAL ANALYSIS OF METABOLOMICS DATA

In earlier days, biochemical approaches typically focused on a very limited number of metabolites, keeping the results manually interpretable by researchers. However, being a very active field of research, metabolomics has made rapid progress allowing modern instrumentation to measure thousands of metabolites simultaneously. This growing complexity of high-throughput small-molecule measurements now constitutes a substantial challenge to researchers. The challenges arise in a derivation of biologically meaningful results given the thousands of chemically distinct metabolites measured in a specific experiment. In order to answer this question, robust statistical methods are suitable for analysis and functional interpretation of the complex interactions between the analytes. The analyses used to interpret high-throughput metabolomics data can be categorized as univariate and multivariate. The univariate techniques, including *t*-test, fold-change analysis, Wilcoxon rank-sum test, analysis of variance (ANOVA), and others, are used to assess the statistical significance of each peak separately. They are used to find the significantly altered metabolites in either unpaired or paired studies. *P*-Values are usually assessed in univariate methods, either through parametric approaches or permutation tests. However, univariate methods fail to discriminate between groups if there are only minor differences at the single molecule level, even if multimolecule combinations would delineate them on a systems level. Therefore, multivariate analysis methods seek to capture not only changes of single metabolites between different groups, but also to utilize the dependency structures between the individual molecules.

The most prominent multivariate analysis techniques applied in the field of metabolomics are PCA, cluster analysis, and partial least squares (PLS) regression. Multivariate analysis considers the combinatorial effect of multiple variables. It can be further categorized into unsupervised and supervised techniques. One of the most popular unsupervised techniques in LC–MS-based metabolomics studies is PCA, which finds a series of orthogonal projection directions that maximize the variance of the projected data. PCA has been extensively used in multiple studies to elucidate the metabolomics consequences in the exploration of phenotypic and genotypic relationships in disease pathophysiology. Other unsupervised techniques, such as self-organizing map (SOM) or two-mode clustering, have also been found suitable for the analysis of metabolomics data.

An extension to the PLS repository is the orthogonal-PLS (OPLS) method. OPLS-DA is a more powerful data analysis method, which not only can reflect the differences between species but also can find substances causing such differences between species. OPLS has drawn attention in metabolomics research with a broad variety of applications, including molecular epidemiology, alternative medicine, and the monitoring of kidney transplant patients (Bartel et al., 2013; Zhou et al., 2012). There are several commercial software packages available for processing MS metabolomics data, such as Markerlynx, Marker View, Mass Hunter, Metabolic Profiler, Metabolyzer, metAlign, and Phenomenome Profiler (Want and Masson, 2011). The workflow of processing metabolomics data is presented in Fig. 17.15. The application of a PLS-DA model was able to analyze the metabolic variations in mixtures of Kansui and licorice by a UHPLC-QTOF/MS-based metabolomics study. Chemometrics and multivariate data analysis were found useful for the authentication and batch control of herbal medicine (Shen et al., 2017). The data analysis tools of PCA and HCA/SIMCA-P were applied for NMR-based identification and quantitation of chemical constituents of some Vietnamese herbal medicines against rheumatic diseases. Some exploratory data analyses through CDA/AMIX and the Random forest/ R computing system were also reported in an NMR-based metabolomics study for authentication of medicinal plants (Kazi et al., 2013; McKenzie et al., 2011).

One of the main challenges of plant metabolomics studies is the enormous complexity and diversity of the plant metabolome and the incomplete knowledge of plant metabolic pathways. As the plant contains a large number of primary and secondary metabolites, the analysis of the metabolomes requires a versatile tool for data interpretation with wide dynamic ranges. A second challenging aspect is the unavailability of metabolite-specific libraries and known reference compounds for identification and/or structural elucidation of bioactive metabolites. Fortunately, a number of strategies, such as advancement in/and complementary use of technology (LC-NMR-MS, GC×GC-TOF-MS, highly improved MS instrumentation, etc.) and metabolomics databases, are increasingly being brought forward to assist in metabolite annotations and compound identification (Tugizimana et al., 2013).

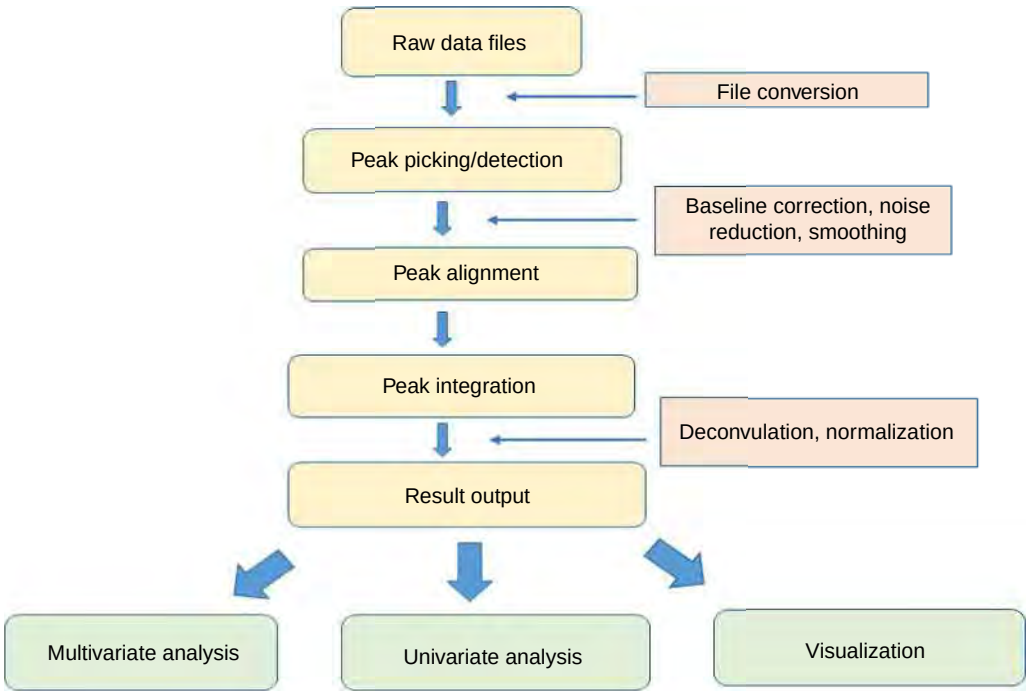


FIG. 17.15 Workflow for LC–MS- and GC–MS-based metabolomics data processing.

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Chapter 18

Safety-Related Quality Issues for the Development of Herbal Drugs

Chapter Outline

18.1 SAFETY CONCERNS FOR HERBS

Herbal medicines have an extensive and assorted variety of multidimensional chemical structures. The utility of natural products as organic capacity modifiers has also won major consideration. They have been efficiently utilized in the discovery of new drugs and have been used in healthcare in chemico-biology. Considering their unique synthetic assorted variety and novel means of activity, natural products have continued to play a significant role in numerous drug improvement and research programs. With time, these natural products have experienced intriguing and significant improvements in their capacity to interface with various changed natural targets, and some have turned into the most vital drugs in a social insurance framework. For instance, plants, microorganisms, and creatures produce small molecules, which have assumed a noteworthy part in drug discovery (Krause and Tobin, 2013a, b).

Plant-derived products in healthcare are very commonly used not only in developing countries but also in developed countries, either alone or in combination with conventional medicine. Numerous herbal prescriptions are there for utilization of medicinal plants that are used for avoidance and treatment of different ailments. It ranges from the customary and well-known solutions of each nation to the utilization of institutionalized and titrated herbal concentrates (Mukherjee and Wahile, 2006). Herbal-inferred cures require an intense and profound evaluation of their pharmacological characteristics and safety that can be achieved by new biologic innovations, such as pharmacogenomic, metabolomic, and microarray methodologies (Firenzuoli and Gori, 2007). Further, without the knowledge of the prescriber, a consumer tends to consume the herbal products, along with prescription medicine which may lead to herb–drug interaction (Isnard Bagnais et al., 2004).

The increased usage of herbs as dietary supplements and over-the-counter products suggests the need for the development of clinical and scientific data for quality and safety evaluation (Mukherjee, 2002a, b). Due to their long-term use, many of the herbal medicines being used today are time tested. With specific scientific backup, the complex mixture of herbal formulations should be addressed for their safety and efficacy and documented with proper experimental outcomes (Mukherjee et al., 2015).

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There are several regulatory requirements in different countries relating to safety and for assessing the potential interaction between the phytoconstituents and other conventional medicines (Mukherjee, 2003). The regulatory agencies require documentation on the interactions of herbal medicine involving CYP isoforms before marketing the product. Several phytoconstituents have been identified as inhibitors or inducers of cytochrome, resulting in herb–drug interactions. Similarly, drug interactions between phytoconstituents and conventional medicines have also been reported. There are several examples of herb–drug interaction reported. This usually occurs when the use of the herb is not disclosed by the patient to physician. In some cases, it may be caused due to the absence of proper scientific documentation and awareness. Phytocompounds, such as allicin, quercetin, and silymarin, have been reported for their extensive undesirable effects in patients (Mukherjee et al., 2015).

Sometimes, the patients are unknowingly using different herbal products or dietary supplements along with prescribed medicines. So, to obtain maximum benefit and minimize the undesired side effects, we should have detailed knowledge about the pharmacology, mechanisms of action, drug interactions, safety, and efficacy of herbal medicines. Awareness regarding drug interactions with herbal medicines and their secondary metabolites has increased. It is evident that natural products in combination can interact and alter the therapeutic benefits of these commonly used medicaments (Strandell et al., 2004). The concurrent use of herbal medicines with over-the-counter products may cause serious adverse effects, which may be due to their interaction with drug-metabolizing enzymes (Mukherjee et al., 2015; Takanaga et al., 2000a, b). Studies on drug-metabolizing enzymes enhance our understanding of the possibilities for herb–drug interactions (Mukherjee et al., 2011).

18.2 QUALITY-RELATED SAFETY ISSUES OF HERBAL MEDICINES

Plants contain pharmacologically active compounds and they may also contain toxic substances. Herbal toxicity may occur for many reasons, such as incorrect or improper identification of the plant. This may occur when plants are contaminated with other drugs, hormones, or heavy metals. Safety is a fundamental principle in the provision of herbal medicines and herbal products for healthcare, and a critical component of quality control. These safety concerns are compiled from literature reports and include adverse effects and potential or suspected drug interactions. It is reasonable to assume that these effects could occur in patients taking herbal medicines, particularly over a long period of time and with high doses.

The efficacy and safety of herbal medicinal products are directly dependent on the quality of raw herbs. However, the phytochemical profiles are qualitatively and quantitatively variable as is the definition of quality of medicinal herb raw materials. Therefore, it is important to specify the quality parameters that are intricately linked to the constancy. The chemoprofile and quality evaluation of herbal drugs has been described in Chapter 12. Strict adherence to the harmonized quality assessments will enable consistent pharmacological activity and facilitate meaningful comparison and metaanalysis of clinical data. The details of bioassay-guided isolation and evaluation of herbal drugs has been discussed in Chapter 13. The ingredients used in commercially marketed traditional and modern herbal medicines are today mostly assessed for quality to ensure their safety. However, there is a need for a quality assurance paradigm for botanicals that will address both the safety and efficacy of herbal drugs (Govindaraghavan and Sucher, 2015).

Globally, a number of terms, definitions, and quality parameters are being used, based on the intended use of herbal products and the types of health claims associated with them. Herbal products are marketed as complementary/alternative/nonconventional medicines, which are often based on traditional medicines, such as traditional Chinese medicines (TCMs), Indian Ayurvedic, and Siddha medicines, and Japanese Kampo medicines (Ekor, 2013).

Still today, the medicinal herb industry and herbal ingredient manufacturers rely on the quality control specifications as described in pharmacopeial herbal monographs, in which the identification descriptors start with dried, unprocessed, whole or fragmented plants or plant parts. The identity of the ingredients is based on macroscopic, microscopic, and organoleptic analysis of sorted and dried plant parts. On the other hand, whole plant specimens (including the flowering and fruiting parts) provide information on leaf shape, size and type, phyllotaxy, floral characters, and arrangement in inflorescence that may be irretrievably lost when the material is fragmented or sorted. Hence, these monographs provide an identification tool, which is relevant only when the whole plant material is authenticated prior to sorting (Govindaraghavan and Sucher, 2015).

18.3 TOXICITY EVALUATION OF HERBAL MEDICINE

As the ubiquity and demand for traditional medicine is growing quickly, there is increasing concern for the quality and the safety of drug products. A common concept related to the safety profile of herbs is that these products are to be considered safe because they have had a long history of traditional use, without considerable experience and knowledge about their toxic effects. The traditional experience is a powerful tool for the identification of adverse effects, which occur in the majority of users and develop rapidly after the start of therapy (Delgoda and Westlake, 2004). In this context, there were incidents in 1991 and 1992 in Brussels in which 30 women were treated with a Chinese herbal slimming preparation and died due to renal failure caused by the presence of aristocholic acid, which is toxic in nature (Abdullah et al., 2017). Another example

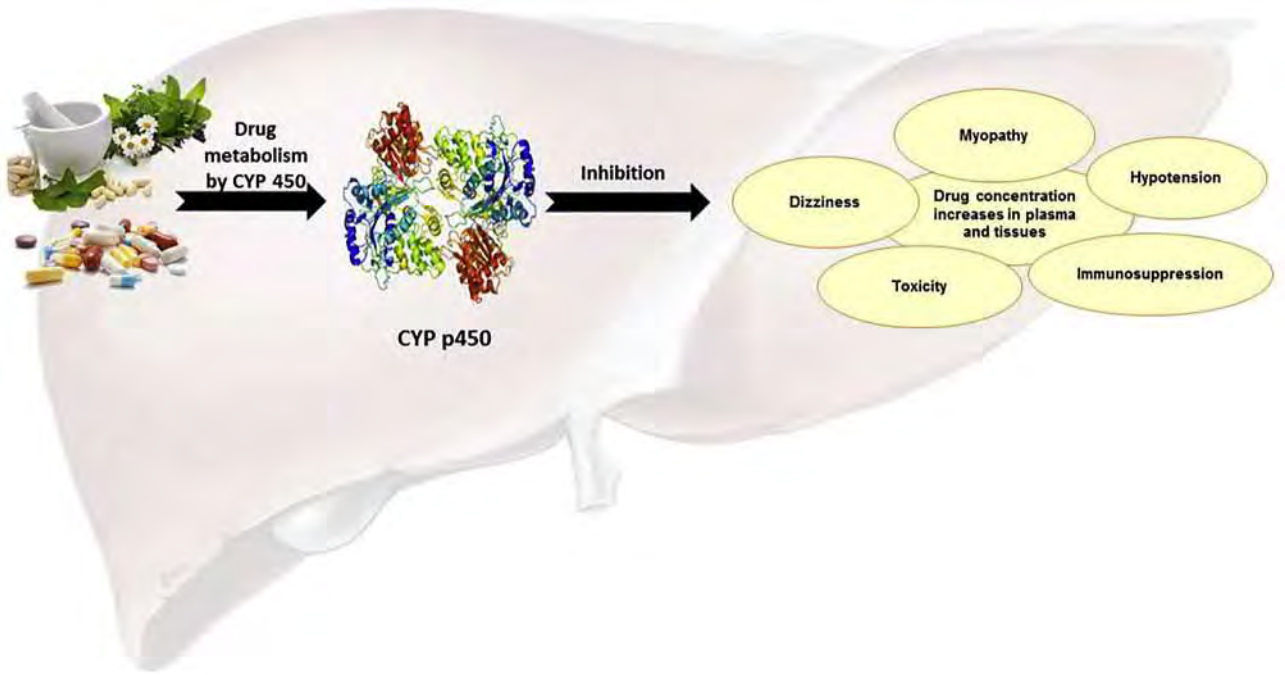

FIG. 18.1 CYP450 inhibition and herb–drug interactions.

is the induction of anticholinergic symptoms, such as palpitations, dryness of the mouth, and dilatation of the pupils, by herbal medicines rich in belladonna alkaloids (Hung and Hung, 2015). Such reactions are pharmacologically predictable and dose dependent. This could be prevented by dose reduction. So, the importance of controlling the correct identification of herbal preparations should be considered. In addition to the problem of incorrect plant identification, some mixtures may be toxic, particularly if they are misused. The message that consumers believe is that herbal products are natural and natural is safe. It is very much necessary to continually review and assess the safety of most commonly consumed botanicals with an emphasis on surveillance and use of these products to identify unknown hazards or risks and address them expeditiously (Mukherjee et al., 2002). This is due to the effect of the drug-metabolizing enzymes, specifically cytochrome enzymes (Takanaga et al., 2000a, b). Herb–CYP450 interactions can occur in two different ways. Herbal products can induce CYP450 enzymes and increase the metabolism of medications, possibly leading to therapeutic failure. They can also inhibit CYP450 enzymes, decreasing the metabolism of medications, and possibly increasing therapeutic effects and toxicity, as represented in Fig. 18.1 (Tovar and Petzel, 2009).

Traditional herbal remedies have been prevalent in many countries from Asia, Africa, and Europe. They need to undergo regulatory approval to access the markets of other countries. Several traditional medicinal systems, including TCM and the Indian System of Medicine, including Ayurveda, have been generally utilized throughout the world as an essential treatment procedure in many countries.

18.3.1 Methods for Toxicological Evaluation of Herbs

Toxicity testing of herbal drugs is aimed at the toxicological measurement of herbs or herbal preparations to identify their adverse effects and to determine the limits of dose exposure at which such effects occur.

18.3.1.1 Preclinical Toxicity Test

Crude plant extracts or purified compounds obtained by fractionation from the crude extract can be evaluated in these tests. This includes a wide range of toxicity tests performed using experimental animal models before conducting clinical tests for toxic effects in humans. Generally, these experiments are performed using different in vivo and in vitro experimental models (Ifeoma and Oluwakanyinsola, 2013).

18.3.1.2 Toxicity Evaluation by Cell-Based Cytotoxicity Tests

This involves short-term exposure of cultured cells with varying concentrations of test substances. It can provide insight toward the cytotoxic and genotoxic dispositions of plant extracts and derived compounds. The ability of the test material

to inhibit cellular growth and viability is used to evaluate its toxicological effects. Different parameters, such as inhibition of cell proliferation, cell viability markers (metabolic and membrane), and morphologic and intracellular differentiation markers, are accessed (O'Brien and Haskings, 2006; Malinin, 1973).

18.3.1.3 Determination of Toxicokinetic Properties of Herbs

In this case, the toxicological effects of herbs or purified phytomolecules are accessed for the prediction of toxicity due to pharmacokinetic disposition from potential herb–drug interactions. For evaluation of herbal toxicokinetics, the test material is assayed for CYP450 enzyme inhibition or induction using different human liver microsomal Cytochrome P450 isoforms at different concentrations. This helps to identify the metabolites that are known to cause toxicological modulation at any level of cellular organization (Bush et al., 2007; Wienkers and Heath, 2005).

18.3.1.4 Tools for Toxicogenomic Screening

The toxicogenomic screening tool is a collective term that refers to the measurement of potential toxicological interactions of herbs using different "*omics*" tools at submolecular (epigenomics, transcriptomics), molecular (proteomics), cellular, and tissue and organ (metabonomics) levels (Youn et al., 2010).

18.3.1.5 High-Throughput DNA Sequencing

The development of DNA sequencing is a helpful tool as next-generation sequencing (NGS). It has also been applied in the creation of large genetic databases of plants, which can serve in the identification of potentially toxic plants, or those that may contain these types of toxic substances (Schuster, 2008). For example, NGS technology has already been applied in unraveling the genome of Gingko, which holds potential for biomarking toxicity in the 21st century (Lin et al., 2011).

18.3.1.6 Using In Vivo Animal Models

18.3.1.6.1 Determination of Systemic Acute Toxicity

For determination of acute toxicity, animals were exposed to a fixed dose of the test material according to the Organization for Economic Cooperation and Development (OECD) guidelines. The method was first adopted in 1996 and extensively validated against LD50 (lethal dose 50%). Different toxic signs and the severity, onset, progression, and reversibility of the signs were observed and recorded in relation to dose and time for at least 7–14 days. If necessary, a histopathological examination should be conducted on any organ or tissue showing macroscopic changes at autopsy.

18.3.1.6.2 Determination of Subacute or Subchronic Toxicity

This is a repeat-dose-dependent study performed using suitable animal models to observe any damaging changes in an organ through hematological and biochemical parameters that may arise in the course of administration of the test substance. Other tests, such as organ functionality tests, ECG, and visual and auditory tests were also performed (Lorke, 1983). The long-term effects of the test material under investigation were observed for varying time periods, as mentioned in Table 18.1 (Abdulrahman et al., 2007).

18.3.1.7 Determination of Carcinogenicity or Chronic Toxicity

This is similar to a subchronic study except that it is conducted with a larger number of animals to reveal the toxicity of the test substance that may arise during exposure for 24 months or for an entire lifespan. Here, mutagenic or

TABLE 18.1 Time Period for Toxicological Evaluation of Test Materials With Respect to Their Clinical Use

Time period for clinical use of test material	Time period for the toxicity study
Repeated administration <1 week	2 weeks to 1 month
Repeated administration, 1–4 week(s)	4 weeks to 3 months
Repeated administration, 1–6 month(s)	3–6 months
Repeated administration >6 months	9–12 months

carcinogenic susceptibility of test substances and the likely organs in which they may accumulate are revealed (Ifeoma and Oluwakanyinsola, 2013).

18.3.1.8 Evaluation of Specific Toxicity

Special toxicity tests, such as mutagenicity, carcinogenicity, and reproductive and developmental toxicity tests, have also been performed for toxicological evaluation of herbs or herbal drugs. For mutagenicity tests, different methods have been developed, such as the reverse mutation test in bacteria, the chromosomal aberration test with mammalian cells in culture, and the micronucleus test with rodents (Ifeoma and Oluwakanyinsola, 2013).

18.3.1.9 Tests for Evaluation of Local Toxicity

Herbal formulations intended for dermatological preparations should go through different test methods for the determination of local toxicity, such as the Adjuvant and patch test, Buehler test, Draize test, Freund's complete adjuvant test, Maximization test, Open epicutaneous test, Optimization test, and the Split adjuvant test. After application, the skin reaction of each animal is noted and evaluated according to the assessment standard of the test method used. Other local toxicity tests, such as vaginal, rectal, and respiratory irritation tests, were also included if the herbal medicine which are intended for such use (Balls et al., 1995; Takahashi et al., 2008).

18.3.1.9.1 Determination of Neurotoxicity

In some cases, neurological adverse effects may arise from acute systemic exposure to some herbs, while cerebrovascular accident, encephalopathy, and psychosis can become evident in subacute, subchronic, and chronic tests for toxicity. It is important to note that the presence of high levels of metals in the herbal medicine or herbal formulations can contribute to neurotoxicity (Choi, 2005).

18.3.1.10 Determination of Genotoxicity

Genotoxicity may be defined as a chemically induced mutation or alteration of the structure and/or segregation of genetic material. Recently, a guidance document on the assessment of genotoxicity of herbal preparations has been drafted by the European Medicines Agency. More reliable tests, such as the mouse micronucleus test and the mouse lymphoma assay, can be used (Ifeoma and Oluwakanyinsola, 2013).

18.3.1.11 Determination of Reproductive Toxicity Development

Thalidomide was used in the treatment of leprosy and multiple myeloma. But soon it was discovered that thousands of the offspring of women who had taken thalidomide to treat morning sickness were born with serious birth defects (Botting, 2002).

In designing a suitable model for reproductive developmental toxicity, a large number of animals are exposed to the test material at varying doses before mating, during gestation, and after delivery, up to the entire lifetime of the new offspring, to detect the effects of the herb on reproductive performance and/or developing offspring. Toxicity end points include spontaneous abortion, premature delivery, and birth defects (Stephens et al., 2000).

Both the safety and viability of herbs depend on their restorative sign; on a fundamental level, a substance has no clinical usefulness in the event that it is "protected." The unwanted effects of several well-used herbs have been reported, as discussed below.

(a) *Aconite*: *Aconitum variegatum* (Fam: Ranunculaceae) Alkaloids from the root part of the species Aconitum have analgesic as well as antiinflammatory properties and are often administered for the treatment of rheumatoid, neuralgia, and cardiovascular-related problems. But in spite of its beneficial effects, this plant (especially the roots and root tubers) is extremely cardiotoxic and neurotoxic (Chan, 2009).

(b) *Alfa-alfa*: *Medicago sativa* (Fam: Fabaceae) The herb is widely used in Homeopathy and is a huge source of different vitamins, such as A, C, E, and K, and minerals, such as calcium, potassium, phosphorus, and iron. But eventually, this herb was found to cause syndromes, such as systemic lupus erythromatosus, in patients previously exposed to this condition (Alcocer-Varela et al., 1985).

(c) *Aloe vera*: *Aloe barbadensis* (Fam: Asphodelaceae/Liliaceae) It is the most extensively used plant for the management of skin-related problems (e.g., skin discoloration and bruises) and, apart from its external use, it is consumed internally because of its laxative property, which may cause serious electrolyte imbalance (decreased level of potassium) if the treatment continues for 7–14 days. So, it is better to avoid the long-term use of laxatives containing anthraquinone glycosides (Saka et al., 2012).

(d) *Comferey*: *Symphytum officinale* (Fam: Boraginaceae) In the first century, Greek physicians used to give a tea of this plant in the form of a poultice for wound healing and to join broken bones. Later, in the 1970s, the herbal formulation was found to cause hepatotoxicity due to the presence of the pyrrolizidine class of alkaloids (Adeneye, 2014).

(e) *Ephedra*: *Ephedra sinica* (Fam: Ephedraceae) has been used since ancient times to treat respiratory disorders in TCM. It has also been reported that it is used as a mood elevator and to manage obesity. It has been observed that nowadays manufacturers have started including this herb in different slimming products, eventually producing severe health problems. This is because Ephedra contains an alkaloid, namely, ephedrine, which causes several complications related to the cardiovascular system, such as high blood pressure and hepatic damage (Fleming, 2008).

(f) *Ginkgo biloba*: *Ginkgo biloba* L. (Fam: Ginkgoaceae) For thousands of years Ginkgo fruits and seeds have been used as a supplement with the medicinal property of enhancing mental alertness and memory. However, there is substantial scientific proof that its extract inhibits platelet-activating factor. Therefore, long-term use causes enhanced bleeding time, hemorrhage, and subdural hematomas (Bent et al., 2005).

(g) *Ginseng*: *Panax ginseng* (Korean), *Panax japonicas* (Japanese), *Panax notoginseng* (Chinese), *Panax vietnamensis* (Vietnamese), *Panax quinquefolius* (American) (Fam: Araliaceae). It is one of the commonly used herbs in the TCM system as an all-purpose medicine, especially related to stress and strength along with high blood pressure, diabetes, and depression-like symptoms. Later, it was found to cause several side effects, such as hypertension, mastalgia, and blood clotting (Wang et al., 2015). It also interacts with blood thinners, causing problems in blood clotting.

(h) *Ispaghul*: *Plantago ovata*, *Plantago indica* (Fam: Plantaginaceae) has been widely used for centuries due to its commendable laxative and demulcent effects. Apart from its therapeutic use, it was found to cause bronchospasm, asthma, and intestinal obstructions. It may also produce esophageal obstruction if swallowed in dry form (Cock, 2015).

(i) *Licorice*: *Glycyrrhiza glabra* (Fam: Leguminosae/Fabaceae) The pattern of licorice use in Western culture has changed in the last 3000 years. Generally, its root has been utilized to treat different therapeutic complications, such as asthma, ulcers, and shingles (caused by Herpes infection), joint pain, chronic depression, and so on. It has demulcent and expectorant properties, and additionally invigorates mucous discharges of the trachea. Prior research reports have demonstrated that glycyrrhizin invigorates the discharge of hormones by the adrenal cortex. Recently, it has been seen that one of the dynamic ingredients, glycyrrhizinic acid, when taken in large amounts, can advance sodium and water maintenance and also potassium consumption, making it dangerous for individuals with circulatory strain, kidney, or coronary illness. Potential undesirable impacts incorporate edema and hypertension because of unnecessary use (Nazari et al., 2017).

(j) *Sassafras*: *Sassafras albidum* (Fam: Lauraceae). In the traditional medicine of North America this plant was used to treat joint pain, rheumatism, cold, and flu. When taken in excessive amounts for a long time, it causes hepatotoxicity due to the presence of a potent carcinogen (Hausner and Poppenga, 2013).

(k) *Senna*: *Cassia angustifolia* (Fam: Caesalpiniaceae) is one more example of an herb traditionally used for weight loss and to treat constipation. It may exert heart problems if used daily due to a reduction in the potassium level of the body. It was also found to cause circulatory failure, seizures, high blood pressure, and anaphylactic reactions (Tamokou and Kuete, 2014).

(l) *Milk thistle*: *Silybum marianum* (Fam: Asteraceae) Silymarin is obtained from this herb and widely used as a liver tonic; it has been associated with cerebral hemorrhage, hepatic coma, and neuropathy (George, 2011).

(m) *St. John's Wort*: *Hypericum perforatum* (Fam: Hypericaceae) is a widely used plant for its antidepressant activity. It has a huge market potential, mostly in Europe and the United States. It significantly decreases the bioavailability of Indinavir due to a drug interaction when used concurrently (George, 2011).

18.4 SYNERGISTIC EFFECTS OF HERBAL MEDICINES

For designing new multitarget drugs and drug combinations, one should study the synergism and the mechanism behind it. This involves interactions with many pathways and targets, which may be influenced by the different environmental, behavioral, and genetic profiles (Bahadur et al., 2017; Mukherjee et al., 2015). Therefore, herbal ingredients or drugs of lower potencies need to be appropriately combined in accordance with these profiles and probably in a personalized manner to achieve sufficient levels of efficacy. These synergistic features are unique to phytotherapy and they contribute both to efficacy and safety. It is claimed that combinations of herbs have synergistic effects. There are several pieces of in vitro and/or in vivo evidence that supports synergism occurring between phytoconstituents in herbal extracts; however, the clinical evidence is quite a bit weaker. Synergy is also taken to mean an attenuation of undesirable effects, which is another key principle of herbalism for the toxicity of plant extracts, and is less than that of a single isolated constituent (Barnes, 1999).

Synergism has a major role in the therapeutic efficacy of medicinal plants and plant-derived formulations. It is assumed to happen when the effective concentration of ingredients in combination is significantly reduced or the effects of ingredients in combination are significantly increased with respect to an individual ingredient. It is hard to differentiate synergistic effects from additive effects and this generally depends on high ranges of dissimilarity. In several cases, crude herbal extracts are found to exert better therapeutic activity than an isolated compound at the same dose. The mechanism of individual components and their combinations need further research (Wagner, 1999). In this instance, the synergistic effects may be beneficial with low doses, which should be explored cautiously without admixing with other herbs or medicaments (Mukherjee et al., 2015).

Piper nigrum contains the alkaloid piperine. It increases the bioavailability of many prescribed medicines, such as rifampicin. Many herbs also contain tannins, which delay the absorption of many alkaloidal drugs and proteins. They also increase the expression of different drug-metabolizing enzymes, such as cytochrome P450, resulting in increased drug metabolism and low therapeutic effects (Mandal et al., 2018). The increasing consumption of herbal extracts along with prescription medicine and issues concerning the safety of herbs have become major topics and researchers are now more focused on the effects of phytomolecules on different drug-metabolizing enzymes. This will be helpful to understand the extent of interactions between herbs and drugs (Mukherjee et al., 2015).

India has several systems of traditional medicine, which have been practiced together for years. They include Ayurveda, Yoga, Unani, Siddha, and Homeopathy (AYUSH). Promoting these systems is the major thrust of the Ministry of AYUSH, Government of India. Ayurveda, the traditional medicine of India, focuses on the idea of synergy, in which knowledge of multicomponent action is a key concept. It is called "samyoga," or the knowledge of synergistically combining ingredients for maximum safety and efficacy. Synergism plays a major role in therapeutic efficacy of herbs or herbal formulations. The Ayurvedic literature "Sarangdhar Samhita" highlighted the concept of polyherbalism to achieve greater therapeutic efficacy. Synergism plays a major role in the therapeutic efficacy of herbs or herbal formulations. The multicomponent nature of these medicinal herbs and traditional formulations makes them particularly suitable for treating complex diseases and offers great potential for exhibiting synergistic actions (Mukherjee et al., 2017).

Based on the nature of the interaction, there are two mechanisms of synergism (pharmacodynamics and pharmacokinetic). Pharmacokinetic synergism is the ability of the herb to facilitate the absorption, distribution, metabolism, and elimination of the other herbs. Other than that, the doses, time of intake, and "Anupana" (the carrier with which the herbal medicines are prescribed, such as hot water, milk, and honey) are also emphasized in the study of herbals under Ayurveda. The ancient texts, such as Charaka, Sushruta, and Vagbhata, have defined anupana to be limited to food only. Later, Yoga Ratnakara further elaborated that the nature of Anupana is not only limited to food, but anything that is administered with oushadha or medicine. The medicine becomes more potent when given with suitable Anupana, which may be personalized according to the avastha (stage) and bala (strength) of the rogi and roga (patient and disease) (Mukherjee et al., 2018).

Pharmacodynamic synergism can be observed in "Trikatu," which is a combination of *Zingiber officinale* (ginger) with *P. nigrum* (black pepper) and *Piper longum* (long pepper) that enhances their heating and mucous-reducing effects, as well as pharmacokinetic stability. "Triphala," which is a combination of *Embellica officinalis* (Amla), *Terminalia chebula* (Haritaki), and *Terminalia bellerica* (Bahera), is also an example of synergy that is useful in various therapeutic situations as mentioned in Ayurveda. Synergy is also observed in Ayurvedic single herbs showing polypharmacological activity. Arjuna (*Terminalia arjuna*), a well-known cardiotonic, contains saponin glycosides, which accounts for its primary activity in improving cardiac muscle function and the pumping activity of the heart, whereas the flavonoids afford antioxidant action and vascular strengthening. Another example is lemongrass (*Cymbopogon citratus*), an essential oil that contains three major phytoconstituents, geranial, neral, and myrcene, of which both geranial and neral have in vitro antibacterial action. However, when mixed with myrcene, their activity is increased due to synergy (Mukherjee et al., 2018).

Pharmacokinetic synergism in Ayurveda can be elaborated by the concept of bioenhancers or biopotentiators first described to explain the increase in the antiasthmatic effects of *Adhatoda vasica* (vasaka) leaves by the addition of *P. longum*. The concept of a bioenhancer is called Yogvahi in Ayurveda and it enhances the bioavailability, tissue distribution, and efficacy of drugs, especially with poor oral bioavailability, and decreases the adverse effects in the process. Specific yogvahis or bioenhancers are called Anupaan and Sehpaan. Sehpaan implies that the vehicle is used during the manufacture of the medicine to increase its effect, for example, for panchgavya ghrita and brahmi ghrita, clarified butter is used. General yogvahis routinely used in many Ayurvedic preparations are trikatu, sesame/til, gold/swarna bhasma, and cow urine distillate. Examples of interactions of some herbs with different classes of medicine, along with their synergism antagonism effects are listed in Table 18.2.

Knowledge from traditional medicine inspired the identification and isolation of piperine from *P. longum*—as a bioenhancer molecule. Recently, Risorine, a composition of rifampicin, isoniazid, and piperine, has been marketed for the management of tuberculosis, resulting in a decrease of rifampicin dose from 450 to 200 mg, with 60% improvement in its

TABLE 18.2 Interactions of Some Herbs With Different Classes of Medicine

Herb	Type of Interaction With Class of Medication		References
	Synergism	Antagonism/Inhibition	
Aloe vera	Insulin, topical hydrocortisone, zidovudine		Ulbricht et al. (2007)
Belladonna (*Herbae pulvis standardisatus*)		Cisapride, Tacrine	Ulbricht et al. (2004)
Bitter melon (*Momordica charantia*)	Hypoglycemic agents		Basch et al. (2003)
Black cohosh (*Cimicifuga racemosa*)	Antihypertensive, antilipidemic, antineoplastic agents	Antihistamines, oral agents	Barrette et al. (2012)
Blue cohosh (*Caulophyllum thalictroides*)	Nicotine, Cocaine		Ceurvels et al. (2012)
Boswellia (*Boswellia serrata*)	Antineoplastic agents	Leukotriene inhibitors	Basch et al. (2004)
Butterbur (*Petasites hybridus*)	Anticholinergics		Giles et al. (2005)
Calendula (*Calendula officinalis*)	Sedatives, antihypertensives		Basch et al. (2006)
Cinnamon (*Cinnamomum* spp.)	Antibiotic, anticoagulant, antidiabetic, antifungal, antilipemics, antineoplastic agents, antiretroviral agents, anxiolytics, estrogens, hepatotoxic agents, sympathomimetics		Armbruester et al. (2012)
Dandelion (*Taraxacum officinale*)	Anticoagulants, cytochrome P450 1A2 and 2E-metabolized agents	Ciprofloxacin	Sweeney et al. (2005)
Devil's claw (*Harpagophytum procumbens*)	Antiarrhythmic agents	Inotropic agents	Brendler et al. (2006)
Fenugreek (*Trigonella foenum-graecum*)	Antidiabetic agents, laxatives	Anticoagulants and antiplatelets	Ulbricht et al. (2007)
Ginkgo biloba		Paracetamol (acetaminophen), anesthetics, aspirin, ibuprofen	Ernst et al. (2005)
Ginseng (*Panax ginseng*)	DHT, antidiabetics, antilipemics	Anticoagulant, digoxin, diuretics	Ulbricht et al. (2009)
Green tea (*Camellia sinensis*)	Analgesics, anticoagulants and antiplatelets, antilipemics, antivirals, hepatotoxic agents, antiseizures, β-adrenoceptor agonists	Antiandrogens, cytochrome P450-metabolized agents, estrogen, sedatives, *P*-glycoprotein modulators	Basch et al. (2012)
Guggul (*Commifora mukul*)	Thyroid agents, anticoagulants, antiplatelet agents	Propranolol, Diltiazem	Ulbricht et al. (2005)
Gymnema (*Gymnema sylvestre*)	Antidiabetic agents, antilipemic agents		Ulbricht et al. (2011a)
Kava (*Piper methysticum*)	Sedatives/CNS depressants	Cytochrome P450 substrates, dopamine agonists and antagonists, monoamine oxidase inhibitors	Ulbricht et al. (2005)

TABLE 18.2 Interactions of Some Herbs With Different Classes of Medicine—cont'd

Herb	Type of Interaction With Class of Medication		References
	Synergism	Antagonism/Inhibition	
Lavender (*Lavandula angustifolia Miller*)	Sedatives, anticoagulants, NSAIDs, antiplatelet agents, antiseizures, cholesterol lowering agents		Basch et al. (2004)
Lemon balm (*Melissa officinalis*)	Barbiturates, sedatives	SSRIs	Ulbricht et al. (2005)
Noni (*Morinda citrifolia*)	Antiangiogenic drugs, antibiotics, anticoagulants, antiinflammatory agents, hepatotoxic agents	Anticoagulants, immunosuppressants	Brendler et al. (2012)
Peppermint (*Mentha piperita*)	Antibiotics, calcium channel blockers, oxytetracycline	Benzoic acid, ciclosporin, cytochrome P450-metabolized agents	Keifer et al. (2007)
Red clover (*Trifolium pratense*)	HRT and OCPs	Cytochrome P450-metabolized agents	Nelsen et al. (2002)
Rosemary (*Rosmarinus officinalis*)	Anxiolytics, antibiotics, anticoagulants, or antiplatelets		Ulbricht et al. (2010)
Saffron (*Crocus sativus*)	Alzheimer's agents, SSRIs, MAOIs, fertility agents, antihypertensive	Anticoagulants or antiplatelets	Ulbricht et al. (2011a)
Saw palmetto (*Serenoa repens*)	Antiandrogenic drugs, anticoagulants and antiplatelets, antibiotics, antiinflammatory agents, immunomodulators	Androgenic drugs	Ulbricht et al. (2006)
Senna (*Cassia senna*)	Digoxin, anticoagulant and antiplatelet agents, antibiotics, antineoplastics		Ulbricht et al. (2011b)
Spearmint (*Mentha spicata, Mentha viridis*)	Nephrotoxic agents, hepatotoxic agents	Cytochrome P450-metabolized agents	Ulbricht et al. (2010)
St John's Wort (*Hypericum perforatum*)		Immunosuppressants, antiretrovirals, CVD drugs, anticancer drugs, CNS drugs, antimicrobials	Whitten et al. (2006)
Thyme (*Thymus vulgaris*)	5-Fluorouracil		Basch et al. (2004)
Turmeric (*Curcuma longa*)	Acetylcholinesterase inhibitors, amiloride, antibiotics, anticoagulants and antiplatelets, antidiabetic agents, antiinflammatory agents, antilipemic agents, antineoplastic agents, Celecoxib, erythromycin, erythropoietin, hormonal agents, Norfloxacin, Oxaliplatin, P-glycoprotein-regulated drugs, retinol, Talinolol, Warfarin	Paracetamol (acetaminophen), cytochrome P450-metabolized agents, hormonal agents, P-glycoprotein-regulated drugs, Talinolol	Ulbricht et al. (2011b)

bioavailability. Piperine acts by suppressing the P-gp and cytochrome P450 enzymes, which counteract the metabolism of rifampicin via these proteins, thus enhancing the oral bioavailability of rifampicin. It also decreases the intestinal production of glucuronic acid, thus allowing more substances to enter the body in active form. It was found to increase the bioavailability of various drugs from 30% to 200%. *P. nigrum* extract (10 mg/kg orally) significantly enhanced the analgesic activity of diclofenac sodium and pentazocine. The extract alone did not show any significant analgesic activity, whereas in combination with diclofenac sodium it produced a significant increase in analgesic activity. Piperine showed a dose-dependent synergistic effect on nimesulide-induced antinociception. It has been found that coadministration of *Carum carvi* (Jeera) seed extracts increases bioavailability (25%–300%) of some antibiotic, antifungal, antiviral, anticancer, cardiovascular, antiinflammatory/antiarthritic, anti-TB, antileprosy, antihistaminic, and antiulcer drugs, along with some corticosteroids and immune-suppressants (Atal et al., 1981). Novel bioactive nitrile glycosides, niaziridin and niazirin, obtained from the leaves, pods, and bark of *Moringa oleifera*, hinder tumor promoter–induced Epstein–Barr virus activation and enhance the bioactivity of rifampicin, tetracycline, and ampicillin (Atal and Bedi, 2010; Mukherjee et al., 2017).

Network pharmacology research focuses on excellent systems biology techniques, such as protein interaction, genomic expression, and mRNA expression data, which are employed to gain insight into the mechanism of action (MoA) prediction and validation. The exploration of synergistic mechanisms of herbal ingredients with network pharmacology is not only helpful for researchers to discover new phytomedicines or drug combinations, but also to help avoid possible negative synergy. Network pharmacology can also be utilized to validate the synergistic interaction of different bioactive phytoconstituents of botanical drugs. Synergistic polypharmacology in Ayurveda and other Indian systems of medicine reveals the contribution of the constituents to the therapeutic effect and also the mitigating potential side effects in combination. Several aspects of the existing synergy of traditional drugs are very important and are the integral part for deciphering the Indian Systems of Medicine (Mukherjee et al., 2017).

18.5 HERB–DRUG INTERACTIONS

The efficacy of drug therapy depends on many factors that are related to the pharmacokinetic and pharmacodynamic properties of the drugs, which may be tailored by differences in age, gender, genetic polymorphisms, circadian rhythms, pathophysiological conditions, pharmaceutical dosage form, and xenobiotics. On coadministration of herbal products, such as dietary supplements for treatment or prevention of ailments, this may cause unpredicted adverse drug reactions. Various studies have indicated that 14%–31% of prescription drug users take herbal products as nutritional supplement (Colalto, 2010).

The major cause of clinically relevant herb–drug interactions is the modulation of cytochrome-mediated metabolism, which may lead to altered pharmacokinetics. The induction of cytochrome enzyme results in a decrease in drug efficiency, whereas inhibition causes an increase in plasma drug concentration and toxicity (Mukherjee et al., 2015) The mechanism of herb–drug interaction is shown in Fig. 18.2. There are many drug substances capable of interacting with the CYP enzymes in several ways, along with a variety of naturally occurring dietary or herbal components (Colalto, 2010). The evaluation of herb–drug interactions is also essential because nowadays multidrug therapy is most commonly used by patients without consulting their physician. The most common cause of clinically significant drug–drug or herb–drug interactions is CYP450 inhibition. The manufacturer's evaluation of these supplements for toxicology in preclinical and clinical data is not compulsory and is not subject to standard pharmaceutical criteria for safety (Mukherjee et al., 2008). There are several regulatory requirements in different countries relating to safety and for assessing potential interactions between the phytoconstituents and other conventional medicines (Mukherjee and Saha, 2003). The regulatory agencies require documentation on the interactions of the herbal medicine involving CYP isoforms before licensing using different in vitro, in vivo, and in silico techniques. Establishing the safety of herbs using cytochrome-modulating enzymes will attract herbal drug manufacturers' attention to the potential marketing benefit (Mukherjee et al., 2010).

The traditional preparations, which have been in practice for several hundreds of years, comprise medicinal plants, minerals, organic matter, and other materials. Herbal drugs constitute only those traditional medicines that primarily use medicinal plant preparations for therapy. It has been estimated that about three quarters of the world population currently use herbs and other forms of traditional medicines to treat disease. Even as we entered into the new century with its exciting prospect of gene therapy, herbal medicines remained one of the common forms of therapy available to the world population. Much research is still required in herbal therapy to examine individual plant constituents and to determine how plants interact with drugs and food. Some researchers suggest that herb–drug interactions occur less often than predicted. If an interaction between an herb and a drug does occur, conventional drugs are usually the culprits because they are more pharmacologically active. Several examples of some herb–drug interactions and their effects with the most common drugs are shown in Table 18.3.

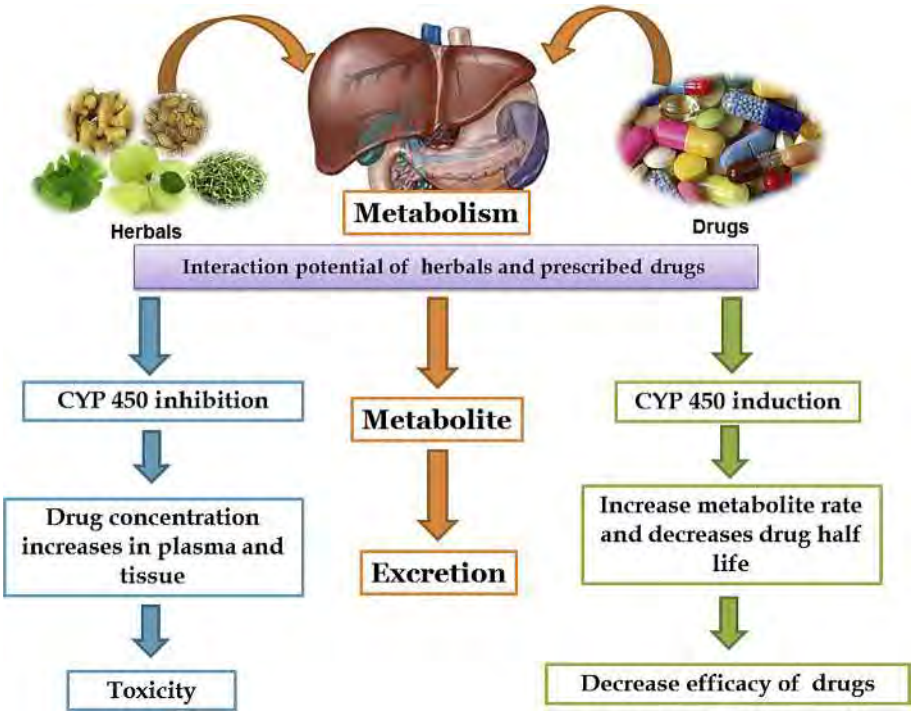

FIG. 18.2 Herb–drug interaction.

18.6 VALIDATION OF HERBAL THERAPIES

It is generally believed that conventional drugs are xenobiotics, with several side effects, while herbal products are natural and very safe. In fact, some herbs can also be dangerous and even cause serious diseases leading to death, if used inappropriately. The major sciences supporting traditional medicine and dietary supplements are in the midst of dynamic change, so opportunities to improve this appalling healthcare situation are certainly available. Adequate funding, as well as corporate and regulatory commitment to a significantly higher ethical standard for dietary supplements, are required (Mukherjee et al., 2002). A safety assessment of herbal products in contrast to new chemical entities is essential.

Many medicinal herbs have a long history of use in therapy. These herbs have been reported for their respective drug interaction activities and these have been documented with significant evidence. There are even published toxicological data on those plants, leading to development of monographs with epidemiological examinations and clinical experience. This helps in development of the facts accumulated in postmarketing surveillance (Mukherjee et al., 2015).

Health risks concerned with the use of herbal products may have several causes, but in general, three major categories can be distinguished. The first can be designated as extrinsic or nonplant-associated toxic effects or adverse reactions arising from accidental or deliberate contamination or adulteration or substitution of the plant material described on the label or from poor quality control. The second category is more intrinsic or plant-associated in nature (Fong, 2002). In this category, the plant material acts as an active ingredient in the health risks of an herbal product, for example, because it also contains some toxic constituents, or constituents that are known to affect the bioavailability and pharmacokinetic or pharmacodynamic interaction of other compounds or drugs, or it has been subjected to specific manufacturing processes resulting in highly concentrated or otherwise nonconventional extracts. The third category are "consumer-dependent" causative factors, that is, health risks associated with users or patients who show, for instance, hypersensitivity reactions or belong to a specific population that is much more prone to toxic effects and/or adverse events (De Smet, 2004). Another problem associated with the use of herbal products is underreporting of observed adverse reactions and herb–drug interactions. One study by the US Department of Health and Human Services found that the adverse event reporting system revealed only a few of the events associated with dietary supplements, including herb–drug interactions. It has been reported that 69% of United Kingdom and 61.7% of Italian people that use herbal products do not consult their physician. Another study found that 58% of users do not inform their physician when they buy any herbal medicinal products (Colalto, 2010).

With the help of an easy registration process for these data, it would be helpful as a replacement for in vivo animal experiments and would decrease the rate of clinical trials. It has proven to be difficult to associate other slighter symptoms of

TABLE 18.3 Common Herb–Drug Interactions and Their Effects

Herb	Drug	Action	Reference
Actaea racemosa, *Larrea tridentata*, Symphytum, *Piper methysticum*	Hepatotoxic drugs: acetaminophen, amiodarone, atorvastatin, azathioprine, carbamazepine, isoniazid, methotrexate	May increase risk of developing liver damage	Padiyara and Khan (2006), Escher et al. (2001), and Furbee et al. (2006)
Allium sativum	Isoniazid	Decreased concentration of isoniazid	Tovar and Petzel (2009)
Aloe vera, *Eucalyptus Globu*, *Panax ginseng*	Glimepiride, glyburide, insulin, metformin, pioglitazone, rosiglitazone	May potentiate hypoglycemia	Padiyara and Khan (2006)
Aloe vera, *Glycyrrhiza glabra*	Digoxin, furosemide, hydrochlorothiazide, other thiazide diuretics	May cause hypokalemia, altering drug effects	Dasgupta et al. (2000) and Tovar and Petzel (2009)
Andrographis paniculata	Etoricoxib	Reduced etoricoxib activity	Balap et al. (2017)
Andrographis paniculata	Naproxen	Synergistic antiarthritic activity	Balap et al. (2017)
Carica papaya	Amiodarone	Increase the drug bioavailability	Rodrigues et al. (2014)
Caulophyllum thalictroides	Glimepiride, glyburide, insulin, metformin, pioglitazone, rosiglitazone	May cause hyperglycemia leading to decreased efficacy	Tovar and Petzel (2009) and Silva et al. (2003)
Caulophyllum thalictroides, *Glycyrrhiza glabra*	Antihypertensive drugs: metoprolol, verapamil, diltiazem	May cause hypertension leading to decreased efficacy	Steenkamp et al. (2000) and Shintani et al. (1992)
Citrus aurantium	Amiodarone	Increase the peak plasma concentration of amiodarone	Rodrigues et al. (2013)
Echinacea purpurea, *Panax ginseng*	Azathioprine, cyclosporine, mycophenolate, tacrolimus, sirolimus, corticosteroids	May interfere with immune suppressing effects	Tovar and Petzel (2009), Banerjee et al. (1994), Kim and Park (2003) and Salgueiro et al. (1997)
Epimedium sagittatum	Sildenafil	Decrease the drug bioavailability	Hsueh et al. (2013)
Fucus vesiculosus	Amiodarone	Decrease bioavailability of amiodarone	Rodrigues et al. (2013)
Fucus vesiculosus, *Oenothera biennis*, *Tanacetum parthenium*, *Vitis vinifera*, *Allium sativum*, *Ginkgo biloba*, *Panax ginseng*	Warfarin, aspirin, clopidogrel, enoxaparin	Increased bleeding	Padiyara and Khan (2006), Cohosh (2006), Bent et al. (2005), Vale (1998), and Heck et al. (2000)
Ginkgo biloba and *Evodia rutaecarpa*	Theophylline	Decreases theophylline concentration	Tang et al. (2007) and Jan et al. (2005)
Gymnema sylvestre	Glimepiride	Significant increase in antihyperglycemic activities	Kamble et al. (2016)
Hypericum perforatum	Paroxetine, trazadone, sertraline, nefazodone, triptans, dextromethorphan, MAOIs	Lethargy, incoherence, increased risk of serotonin syndrome	Linde et al. (1996), Philipp et al. (1999), and Patel et al. (2002)
Hypericum perforatum	Nonnucleoside reverse transcriptase inhibitors, protease inhibitors, Irinotecan, imatinib	Decreased effects reduced efficacy	Gaster and Holroyd (2000), Calapai et al. (2001), and Shelton et al. (2001)

TABLE 18.3 Common Herb–Drug Interactions and Their Effects—cont'd

Herb	Drug	Action	Reference
Hypericum perforatum	Digoxin	Decreased digoxin concentration	Johne et al. (1999) and Dasgupta et al. (2000)
Hypericum perforatum	Simvastatin	No effects of simvastatin	Tovar and Petzel (2009)
Momordica charantia	Rosiglitazone	Slightly potentiate the hypoglycemic effect	Nivitabishekam et al. (2009)
Morus alba, *Glycyrrhiza glabra*, *Artemisia capillaris*, *Astragalus membranaceus*, and *Carthamus tinctorius*	Atazanavir	Enhances the inhibition potentials against HIV protease activity	Cheng et al. (2015)
Panax ginseng	Thiazide diuretics	Hypertension	Vuksan et al. (2000) and Tovar and Petzel (2009)
Panax ginseng	Glimepiride, glyburide, insulin, metformin, pioglitazone, rosiglitazone	May alter insulin release and metabolism	Tamaoki et al. (2000) and Sievenpiper et al. (2004)
Paullinia cupana	Amiodarone	Decrease the drug bioavailability	Rodrigues et al. (2012)
Piper methysticum, *Passiflora*, *Valeriana officinalis*	Benzodiazepines, sedatives, barbiturates, zolpidem	May potentiate sedative effects	Russmann et al. (2001), Dhawan et al. (2001), and Weiss et al. (2005)
Rheum palmatum	Phenytoin	Decrease the drug bioavailability	Chi et al. (2012)
Salvia miltiorrhiza	Human serum albumin	Potentiate the anticoagulant effect	Shao et al. (2016)
Scutellariae baicalensis	Mefenamic acid	Potentiated the antiinflammatory effects	Fong et al. (2015)
Trifolium pratense	Contraceptive drugs, estrogens, Tamoxifen	Competitive inhibition may interfere with binding at estrogen receptor	Tovar and Petzel (2009) and Marcus and Snodgrass (2005)
Zingiber officinale	Glimepiride, glyburide, insulin, metformin, pioglitazone, rosiglitazone	May increase insulin release causing additive hypoglycemic effects	Miyoshi et al. (2003) and Backgon (1991)
Zingiber officinale	Nifedipine, verapamil, diltiazem, felodipine	May have effects at calcium channels and potentiate hypotension	Tovar and Petzel (2009) and Kuichi et al. (1992)

toxicity or long-term unfavorable effects with the use of a particular herb and these may therefore have easily been missed (Mukherjee et al., 2015). However, the data on traditional usage cannot provide information on carcinogenic, teratogenic, chronic toxicity, and mutagenic effects. Hence, traditional data alone does not confirm safety before marketing authorization of the herbal products. These issues have been acknowledged by many legislative bodies and insisted upon for supplementary safety testing by national authorities (Zhou et al., 2003).

18.7 EFFECTS OF HERBS ON CYP450 ENZYME INHIBITION AND INDUCTION

Herbs are common components in CAM. These plant-derived medicines are used alone or in combination with other conventional drugs. There should be an integrated approach to the use of these medicinal plants based on their quality, safety, and efficacy (Mukherjee and Wahile, 2006). In this context, more focus should be put on their therapeutic properties, interaction with other drugs, MoA, efficacy, and safety to minimize adverse effects. The safety issues regarding the use of herbs have become important due to the growing consumption of herbal extracts together with prescription drugs. The disposition of conventional drugs may be affected by herbal therapies and different herbal phytoconstituents (Strandell et al., 2004).

The risk of serious adverse effects concerning the metabolism of drugs has been reported by different scientific and medical articles supported by different in vivo and in vitro data (Takanaga et al., 2000a, b; Mukherjee et al., 2007, 2011).

Multidrug therapy has now become very common. Many people take herbal medicine without consulting their physicians. But these herbs can cause either potentially dangerous side effects and/or reduced benefits from the medications when taken along with the prescribed medicines, through alteration of the physiological effects of drugs. Due to the fact that herbs are composed of a multitude of ingredients whose interactions with the body are exceedingly complex, a high level of sophistication in research methodology is necessary to describe these interactions (Mukherjee et al., 2015; Naveen et al., 2011). The herb–drug interactions can impact health and the effectiveness of treatments in the following respects:

- increase the side effects of drugs, possibly leading to toxicity;
- decrease the therapeutic effect of drugs, possibly leading to treatment failure;
- modify the action of drugs, possibly leading to unexpected complications;
- enhance the therapeutic effect of drugs, possibly leading to over medication.

Some drugs have been withdrawn from the market due to safety issues. The drug mibefradil (CYP3A inhibitor) was withdrawn from the market due to toxicity related to CYP3A enzyme inhibition. CYP3A substrate drugs, such as astemizole, cisapride and terfenadine, may produce severe drug-induced toxicity by increasing the drug concentration in the blood. Coadministration of mibefradil (CYP3A inhibitor) with hypolipidemic drugs, such as lovastatin and simvastatin, may cause rhabdomyolysis in the patient (Izzo and Ernst, 2001). *Echinacea purpurea* (Fam. Asteraceae) has been used for the treatment of the common cold, influenza, respiratory tract infections, and immunomodulatory agents (Modarai et al., 2009). The extract has an inhibitory effect on CYP3A4 isozymes. The compound alkylamide, which is responsible for an immunomodulatory effect, also has a mild inhibitory effect on CYP enzymes, such as the CYP3A4 and CYP2D6 isozymes (Modarai et al., 2007). Chamomile extract has been widely used for the treatment of indigestion and inflammation. The essential oil of chamomile and its major constituents (chamazulene, *cis*-spiroether, and chamazulene) have an inhibitory effect on recombinant human cytochrome P450 enzymes, such as CYP2D6 and CYP3A4 (Ganzera et al., 2006).

Grapefruit juice inhibits CYP1A2, CYP2C9, and most strongly intestinal CYP3A4 (Greenblatt et al., 2003). St. John's Wort is widely used for the treatment of concussion depression. However, it has been reported to cause several herb–drug interactions through induction of the drug transporter P-glycoprotein and CYP3A4 (Scott and Halpert, 2005; Wang et al., 2002). St. John's Wort inhibits CYP1A2, 2C9, 2C19, 2D6, and 3A4 (Gordon, 1998). There are many other medicinal herbs that may induce or inhibit CYP450, such as chamomile (*Matricaria recutita*), peppermint (*Mentha piperita*), dandelion (*Taraxacum officinale*), Siberian ginseng (*Eleuthercoccus senticosus*), milk thistle (*S. marianum*), saw-palmetto (*Serenoa repens*), echinacea (*E. purpurea*), black cohosh (*Cimicifuga racemosa*), valerian (*Valeriana officinalis*), soya (*Glycine max*), and goldenseal (*Hydrastis canadensis*) (Nowack, 2008; Strandell et al., 2004). Ginkgo has been reported to inhibit CYP1A2, 2C9, 2C19, 2D6, and 3A4 in vitro and is responsible for the mechanism-based inhibition of CYP3A in rats (Zhou et al., 2003). Herb–drug interactions have also been reported for Indian herbs and herbal formulations, such as *T. chebula* (Ponnusankar et al., 2011a), *Emblica officinale*, *T. bellerica* (Ponnusankar et al., 2011b), *Acorus calamus* (Pandit et al., 2011a), *G. glabra* (Pandit et al., 2011b), *Trigonella foenum-graceum* (Ahmmed et al., 2015) *Z. officinale*, *Capsicum annuum*, *Murraya koenigii* (Pandit et al., 2012), *Morus alba* (Kar et al., 2015), Triphala and Trikatu formulation (Ponnusankar et al., 2011b; Harwansh et al., 2014), *Gymnema sylvestre* (Kamble et al., 2016), *Aegle marmelos* (Bahadur et al., 2015), and *Swertia chirata* (Ahmmed et al., 2016). Detailed descriptions of several plant species and their various CYP450 isozymes inhibition potential are given in Table 18.4, along with the respective methodology used for the individual plant.

18.7.1 Cytochrome P450: Its Role in Metabolism

This is a class of vital drug-metabolizing enzyme under hemoproteins and is responsible for the metabolism of drugs and drug-like substances. On the basis of heritable genetic characteristics and environmental factors, the expression of this enzyme varies in each individual. In the case of multidrug therapy, this leads to serious adverse effects and even serious interactions when the pharmacokinetic parameter of one drug is altered by another. However, the CYP enzymes 1A2, 2C9, 2C19, 2E1, and 3A4 are considered to be the most important among them. Drug interactions involving the CYP isoforms generally result in enzyme induction or inhibition. The repeated administration of drugs can induce CYP enzymes by enhancing the rate of enzyme synthesis (Mukherjee et al., 2011).

Based on the enzymatic mechanism, the inhibition of CYP enzyme may be reversible or irreversible. In the case of a reversible inhibition, the substrate and the inhibitor compete with each other for binding with the active site. When the inhibition is due to the reactive metabolites produced from CYP catalyzed reactions, it is known as an irreversible type (Fugh-Berman and Ernst, 2001). The mechanisms of inhibitions are related to three factors: dosage, the inhibition time

TABLE 18.4 Examples of Some Herbal Medicines Evaluated for Inhibition Potential Through CYP450 Enzymes

Plant Name (Family)	Part(s) Used	Study Method	CYP Isoforms Used	Reference
Achillea millefolium (Asteraceae)	Leaf and flower	Spectro fluorimetry	Human CYP3A4, CYP2C19	Scott et al. (2006)
Acorus calamus (Acoraceae)	Root	Fluorimetry	Human CYP3A4 and CYP2D6	Pandit et al. (2011a)
Aegle marmelos (Rutaceae)	Fruit	Fluorescence	Human CYP3A4, CYP2D6, CYP2C9, CYP1A2	Bahadur et al. (2015)
Alstonia scholaris (Apocynaceae)	Bark	Radiometry	Rat CYP2D6	Usia et al. (2006)
Andrographis paniculata (Acanthacae)	Whole plant	HPLC	In vivo study on rats	Balap et al. (2017)
Arctium lappa (Asteraceae)	Root	Spectro fluorimetry	CYP3A4, CYP2C19	Scott et al. (2006)
Bacopa monnieri (Scrophulariaceae)	Aerial parts	Fluorescence	Human CYP3A4, CYP2D6, CYP2C9, CYP1A2	Kar et al. (2017)
Berberis aristata (Rutaceae)	Bark	Fluorescence	Human CYP3A4, CYP2D6, CYP2C9, CYP1A2	Bahadur et al. (2017)
Catharanthus roseus (Apocynaceae)	Aerial part	Radiometry	Rat CYP2D6	Usia et al. (2006)
Centella asiatica (Mackinlayaceae)	Whole plant	HPLC	Human CYP2C9, CYP2D6, CYP3A4	Pan et al. (2010)
Cinnamomum burmani (Lauraceae)	Bark	Radiometry	Rat CYP2D6	Usia et al. (2006)
Citrus aurantium (Rutaceae)	Fruit	HPLC	In vivo study in rats	Rodrigues et al. (2013a)
Curcuma longa (Zingiberaceae)	Rhizome	HPLC	Human CYP2C9 CYP2C19, CYP2D6 CYP3A4	Foster et al. (2003)
Fucus vesiculosus (Fucaceae)	Leaf	HPLC	In vivo study in rats	Rodrigues et al. (2013)
Gaultheria procumbens (Ericaceae)	Leaf	Fluorimetry	Human CYP3A4	Scott et al. (2006)
Ginkgo biloba (Ginkgoaceae)	Plant	HPLC	CYP3A4, CYP2D6, CYP2C9	Chandra and Ciddi (2011)
Glycyrrhizae radix (Fabaceae)	Root	LC–MS	In vivo study in rats	Shi et al. (2015)
Gymnema sylvestre (Asclepiadaceae)	Leaf	HPLC	In vivo study in rats	Kamble et al. (2016)
Hypericum perforatum (Hypericaceae)	Aerial part	HPLC	In vivo study in rats	Venkataramanan et al. (2015)
Matricaria recutita (Asteraceae)	Flower heads	Spectro fluorimetry	Human CYP1A2, CYP2C9, CYP2D6 and CYP3A4	Ganzera et al. (2006)
Moringa oleifera (Moringaceae)	Leaves	Fluorescence	Human CYP3A4, CYP2D6	Ahmmed et al. (2015)
Morus alba (Moraceae)	Leaf	HPLC	Human CYP3A4, CYP2D6, CYP2C9, CYP1A2	Kar et al. (2015)
Origanum vulgare (Lamiaceae)	Leaves	HPLC	Human CYP2C9, CYP2C19, CYP2D6, CYP3A4	Foster et al. (2003)

Continued

TABLE 18.4 Examples of Some Herbal Medicines Evaluated for Inhibition Potential Through CYP450 Enzymes—cont'd

Plant Name (Family)	Part(s) Used	Study Method	CYP Isoforms Used	Reference
Origanum vulgare (Lamiaceae)	Leaves	HPLC	Human CYP2C9, CYP2C19, CYP2D6, CYP3A4	Foster et al. (2003)
Origanum vulgare (Lamiaceae)	Leaves	HPLC	Human CYP2C9, CYP2C19, CYP2D6, CYP3A4	Foster et al. (2003)
Panax notoginseng (Araliaceae)	Bark	LC–MS	In vivo study in rats	Dai et al. (2017)
Petiveria alliacea (Phytolaccaceae)	Both root and whole plant	Fluorescence	Human CYP3A4, CYP2D6, CYP1A2, CYP2C9, CYP2C19	Murray et al. (2016)
Phyllanthus amarus (Euphorbiaceae)	Aerial part	Fluorescent spectrophotometry	Rat CYP1A1, CYP1A2, CYP2B1, CYP2B2	Harikumar and Kuttan (2006)
Piper nigrum (Piperaceae)	Fruit	Radiometry	Rat CYP3A4 and CYP2D6	Usia et al. (2006)
Rhodiola rosea (Crassulaceae)	Root	Spectro fluorimetry	CYP3A4	Scott et al. (2006)
Schisandra chinensis (Schisandraceae)	Leaves	Fluorescence, HPLC	Rat liver microsomal CYP1A2, 2C6, 2C11, 2D2, 2E1, and 3A1/2	Wang et al. (2014)
Scutellariae radix (Lamiaceae)	Roots	HPLC	In vivo study in rats	Fong et al. (2015)
Strychnos ligustriana (Loganiaceae)	Wood	Radiometry	Rat CYP2D6	Usia et al. (2006)
Swertia chirata (Gentianaceae)	Leaves	Fluorescence	Human CYP3A4, CYP2D6	Ahmmed et al. (2016)
Syzygium aromaticum (Myrtaceae)	Flower	Radiometry	Rat CYP3A4 and CYP2D6	Usia et al. (2006)
Syzygium aromaticum (Myrtaceae)	Flower buds	HPLC	Human CYP2C9, CYP2C19, CYP2D6	Foster et al. (2003)
Terminalia chebula (Combretaceae)	Fruit	Fluorimetry	Human CYP3A4 and CYP2D6	Ponnusankar et al. (2011b)
Thonningia sanguine (Balanophoraceae)	Root	Spectro-Photometry	Rat CYP1A1, CYP2B1, CYP2B2, CY1A2	Gyamfi et al. (2000)
Tinospora cordifolia (Menispermaceae)	Leaf	Fluorescence	Human CYP3A4, CYP2D6, CYP2C9, CYP1A2	Bahadur et al. (2016)
Trifolium pratense (Fabaceae)	Leaf	HPLC	Human CYP3A4, CYP2D6, CYP1A2	Arora et al. (2015)
Trigonella foenum-graceum (Leguminosae)	Seeds	Fluorescence	Human CYP3A4, CYP2D6	Ahmmed et al. (2015)
Valeriana officinalis (Valerianaceae)	Root	Fluorimetry	*CYP3A4*	Lefebvre et al. (2004)
Zingiber officinale (Zingiberaceae)	Rhizome	HPLC	In vivo study in rats	Weidner and Sigwart (2000)

period, and the lag time needed for inhibition. Sometimes strong inhibition requires time for mechanism-based inhibition through the formation of the active metabolites by metabolism of the parent compound. Therefore, mechanism-based inhibitions require a longer lag period of time (Fugh-Berman, 2000). Thus, the key factor to alter the pharmacokinetic parameters of a drug is modulation of hepatic and intestinal CYP450 (Mukherjee et al., 2011).

18.7.2 Induction of CYP P450-Mediated Drug Metabolism

CYP450 enzymes are induced mainly by novo RNA and protein synthesis. The inducer binds to the nuclear receptors of a cell, which leads to enhanced cytochrome transcription (Lehmann et al., 1998). Alternative mechanisms of induction are

the stabilization of enzymes and the inhibition of protein degradation (Yang et al., 2008). CYP450 induction may decrease the efficacy of drug therapy (Tirona and Bailey, 2006). Rifampicin, carbamazepine, phenobarbital, and St. John's Wort are well-known CYP3A inducers (Zhou, 2008). When CYP3A is induced, an inducing agent binds to the nuclear pregnane X receptor (PXR), forming a heterodimer with the retinoid X receptor (Lehmann et al., 1998). The heterodimer then binds to the regulatory region of the CYP3A4 gene.

18.7.3 Inhibition of Cytochrome P450-Mediated Drug Metabolism

This inhibition is the most common cause of the clinically significant drug–drug or herb–drug interactions of CYP450 inhibition. The inhibition of CYP450 leads to a decreased rate of drug metabolism. Consequently, it decreases the elimination of drug compounds from the body, which results in increased concentrations of the drug in the blood and thus leads to toxicity of the substrate (Lin and Lu, 2001). For prodrugs, the inhibition can result in decreased concentrations of the active metabolite responsible for the clinical effects. The CYP450 inhibition mechanism can be categorized as a reversible and time-dependent inhibition (Pelkonen et al., 2008).

18.7.3.1 Reversible Inhibition

In this mechanism, the substrate reversibly binds to the active site of the enzymes. Reversible inhibition is the most common mechanism responsible for drug–drug or herb–drug interactions. Reversible inhibition involves a rapid association and dissociation between drugs and enzymes. Further, reversible inhibitions can be divided into competitive, uncompetitive, noncompetitive, and mixed inhibitions. The competitive inhibition is the most common and the uncompetitive inhibition the rarest occurrence. In reversible inhibition, the binding occurs rapidly with weak bonds, which get broken easily without destroying the enzymes (Lin and Lu, 1998; Pelkonen et al., 2008).

18.7.3.2 Noncompetitive Inhibition

In this type of inhibition, the inhibitor binds to both free and substrate-bound sites of enzymes different from the substrate-binding site, resulting in a conformational change of the protein. In noncompetitive inhibition, it is assumed that the inhibitor binds to the unbound enzyme and ES complex with similar affinity (Lin and Lu, 1998).

18.7.3.2.1 Uncompetitive Inhibition

The inhibitors bind to the enzyme only when it has formed a complex with the substrate. However, unlike competitive and noncompetitive inhibition, the inhibitor cannot bind to the unbound enzyme (Thummel and Wilkinson, 1998).

18.7.3.3 Mixed Inhibition

In this type of inhibition, the inhibitor binding site is to the free and the substrate-bound enzyme with different affinities. A mixed inhibitor can bind at a site on the enzyme that is physically separate from the active site; however, it also can bind on enzyme–substrate complex (ES) and enzyme itself (Thummel and Wilkinson, 1998).

18.7.3.4 Time-Dependent Inhibition

Time-dependent inhibition can be defined as a phenomenon in which inhibitors display an increased intensity of enzyme inhibition upon prolonged duration of preincubation with the enzyme and NADPH. Time-dependent inhibition is also known as mechanism-based inhibition and involves the inactivation of the enzyme via the formation of metabolic intermediates that bind tightly and irreversibly to the enzyme. Hence, time-dependent inhibition is also referred to as irreversible inhibition. This is mechanism-based inhibition and characterized by NADPH, time- and concentration-dependent enzyme inactivation, and substrate protection. Mechanism-based inhibition results in unfavorable herb–drug interactions as the inactivated enzyme has to be replaced by de novo synthesis to recover its in vivo activity. Time-dependent inhibition can be further classified into (i) quasiirreversible inhibition and (ii) irreversible inhibition (Lin and Lu, 1998).

18.7.3.5 Quasiirreversible Inhibition

The quasiirreversible inhibition mechanism is characterized by the formation of a noncovalent bond between the metabolic intermediate and the prosthetic heme of the CYP450 enzyme. This metabolic intermediate complex is slightly reversible in vitro, but reversible in vivo, and is therefore called quasiirreversible (Pelkonen et al., 2008). In quasiirreversible inhibition, the inhibitors undergo metabolic activation by the CYP enzyme to generate reactive intermediates that coordinate with the ferrous form of the CYP heme in a tight noncovalent complex, resulting in the formation of a metabolic intermediate

TABLE 18.5 Some Clinically Used Drugs Metabolized by CYP3A4, CYP2D6, CYP2C9, and CYP1A2 Isozymes and Their Inhibitors and Inducers (Ogu and Maxa, 2000)

Isozymes	Substrates	Inhibitors	Inducers
CYP3A4	Buspirone, calcium channel blockers, carbamazepine, cisapride, doxorubicin, erythromycin, fentanyl, etoposide, felodipine, quinine, simvastatin, alprazolam, midazolam, triazolam, cyclosporine, indinavir, nelfinavir, ritonavir, saquinavir, astemizole, chlorpheniramine, terfenadine, amlodipine, lercanidipine, nifedipine, atorvastatin, hydrocortisone, progesterone, testosterone	Quinidine, omeprazole, grapefruit juice, indinavir, nelfinavir, ritonavir, clarithromycin, itraconazole, ketoconazole, itraconazole, nefazodone, saquinavir, aprepitant, erythromycin, diltiazem, fluconazole, verapamil, cimetidine, chloramphenicol, ciprofloxacin	Barbiturates, rifabutin, rifampin, ritonavir, carbamazepine, glucocorticoids, modafinil, nevirapine, oxcarbazepine, phenobarbital, phenytoin, pioglitazone, rifabutin, rifampin, St. John's Wort, troglitazone
CYP2D6	Codeine, amitriptyline, propafenone, venflaxine, tramadol, timolol, thioridazine, risperidone, paroxetine, metoprolol, imipramine, bufuralol, chlorpheniramine, chlorpromazine, debrisoquine, dextromethorphan, methoxyamphetamine, oxycodone, perhexiline, phenacetin, phenformin	Quinidine, fluoxetine, cimetidine, haloperidol, chlorpheniramine, thioridazine, cocaine, paroxetine, ticlopidine diphenhydramine, doxorubicin, indinavir, hydroxyzine, metoclopramide, moclobemide, perphenazine, sertraline, ranitidine, ritonavir	Dexamethasone, rifampin
CYP2C9	Phenytoin, omeprazole, nelfinavir, lansoprazole, imipramine, diazepam, cyclophosphamide, clomipramine, citalopram, amitriptyline	Amiodarone, fluconazole, fluoxetine, fluvastatin, isoniazid, metronidazole, paroxetine, phenylbutazone, sulfamethoxazole, trimethoprim, sulfaphenazole, ticlopidine	Phenobarbital, rifampin, secobarbital
CYP1A2	Caffeine, clozapine, cyclobenzaprine, fluvoxamine, mipramine, mexiletine, olanzapine, pimozide, propranolol, tacrine, theophylline, warfarin	Cimetidine, ciprofloxacin, citalopram, diltiazem, enoxacin, erythromycin, fluvoxamine, mexiletine, ofloxacin, tacrine, ticlopidine	Carbamazepine, tobacco, broccoli, brussel sprouts, char-grilled meat, insulin, methylcholanthrene, modafinil, nafcillin, β-naphthoflavone, omeprazole

complex. The intermediate complex is catalytically inactive and cannot bind to carbon monoxide, which is used as a diagnostic test to assess whether the CYP inhibition occurs. Therefore, drug interactions due to quasiirreversible inhibitors are prominent after multiple dosing compared with reversible inhibitors (Lin and Lu, 1998).

18.7.3.6 Irreversible Inhibition

The irreversible inhibition of a CYP enzyme involves the formation of a covalent bond between the metabolite and the CYP enzyme. Irreversible mechanism-based inhibition is also known as "suicide inhibition" and metabolic activation is required for enzyme inactivation. This type of inhibition depends on a number of factors, such as competing detoxification pathways, daily dose, dose regime, concentration, and NADPH. A reactive metabolite is formed within the CYP active site, which can cause irreversible inhibition. The CYP450 enzymes are inactivated by the formation of a tight binding between inhibitory metabolites and the heme protein of the CYP450 enzyme. These complexes are very stable and the biosynthesis of new enzymes is the only way to restore the function of CYP enzymes. This type of inhibition is most stable (Lin and Lu, 1998; Pelkonen et al., 2008). Examples of some clinically relevant CYP450 substrates, inducers, and inhibitors are given in Table 18.5.

18.8 PHARMACOVIGILANCE FOR HERBAL MEDICINES

Pharmacovigilance may be defined as the collective responsibility of all stakeholders involved in the production and use of herbal medicine, which includes the consumers, researchers, health professionals, media, academics, drug regulators, the pharmaceutical industry, and international and governmental organizations, for monitoring, communicating, and evaluating the effectiveness and safety of herbal medicines with intense implications with integrity. The main focus should be on

monitoring safety and distinguishing the adverse effects that were neglected during clinical evaluation (Mukherjee et al., 2015). Although these methods were developed for monitoring modern medicines, they are also applicable for evaluation of the safety and toxicity of herbal products. Unexpected toxicity is a common problem with herbal drugs due to the use of poor quality herbs, misidentification, wrong processing methods, and adulteration. In the United Kingdom, the Medicines and Healthcare Products Regulatory Agency (MHRA) specified several issues related to the regulation of herbal medicine (Kayne, 2006):

(i) lack of knowledge about the products being used,
(ii) limited use of the yellow card adverse drug reporting scheme; this represents underreporting rather than an indication of an absence of adverse reactions, and
(iii) no uniform manufacturing standards, mostly of unlicensed products, etc.

By using the models of conventional pharmacovigilance, herbal pharmacovigilance aims at the detection of serious adverse reactions and the quantification of their incidence and identification of contributive and modifying factors. A classic and inexpensive tool of pharmacovigilance is spontaneous reporting on a voluntary basis by health professionals and consumers who observe or experience a suspected or possible adverse reaction during daily practice (Guo et al., 2007).

Quality-related problems during manufacturing may be overcome with the help of improved standard regulations for good manufacturing practices (GMP). The essential tool for the development of consistent information on safe use of herbal medicine is known as pharmacovigilance. The existing system for synthetic drugs needs modification to deal with herbal medicines. For development of proper guidelines regarding safe and effective use of herbal medicines, pharmacovigilance is important (Debbie et al., 2012; Mukherjee et al., 2015).

18.8.1 Phytovigilance

The information associated with "phytovigilance" (the term used for pharmacovigilance of herbal drugs) raises the suspicion that there tends to be unequal treatment in the case of herbal medicine. In several countries, manufacturers do not require any regulation to demonstrate the safety and efficacy of herbal products in human trials before marketing and there are no specific warnings about known or unknown adverse drug effects on labels (Mukherjee et al., 2007). In the present situation, the importance of pharmacovigilance is growing gradually. Herbal toxicity is considerably underreported due to the lack of a surveillance system for monitoring. For documentation of adverse effects, there should be no discrimination between herbal drugs and synthetic drugs (Mukherjee et al., 2015). Botanicals are complex mixtures of multiple components or unknown active ingredients. That can change pharmacokinetic characteristics through various mechanisms of action. In many cases of herbal drugs, the product is defined by the process, which makes it impossible to extrapolate the scientific data on products from different manufacturers. The extent of herb–drug interaction depends on proper identification of the plant parts by authority, including Latin binomial names, extraction methodology and the isolation procedure for plant secondary metabolites (Mukherjee et al., 2015; Huang et al., 2004).

Concomitant administration of herbal medicine with approved conventional medications can result in therapeutic failures or adverse effects (Huang et al., 2004). Several research reports have suggested that *St. John's Wort* decreases the plasma levels of various other drugs (Sachse et al., 1999). There are no strict regulatory guidelines and there are gaps in the inefficient regulatory processes that have allowed entry of unsafe product into the market (Mukherjee et al., 1998). Self-medication with prescribed medicines is unsafe and, so far, it has not been easy to control because people are used to believe that herbal medicines are safe because they are natural and because of their traditional use. Herbal pharmacovigilance can promote safer medicines in the market and provide better benefits of drugs and their use. It can also lead to rational use of drugs and prevention of drug-related toxicities and other related adverse effects (Ponnusankar et al., 2007; Mukherjee et al., 2015).

18.8.2 Challenges in Pharmacovigilance: How Safe Are Your Herbs?

There are so much diversity in the regulation of herbal medicines and this diversity adds to the challenges of herbal pharmacovigilance, including basic questions, such as identification, standardization, and validation of the herbal medicine. These are not normally an issue with monitoring synthetic medicines. Some of these questions, such as naming issues or adulterations, do not fit easily into the existing systems of pharmacovigilance or the electronic data systems that were developed for pharmaceuticals. However, although some modifications may be needed, developing separate systems for herbals is not the answer as this is likely to add complications and cause confusion if different forms or systems are used, leading to the risk of further reducing reporting rates (Menniti-Ippolito et al., 2008). The regulation of herbal products may vary between the jurisdictions of different countries. In the United States, herbal products are used as dietary supplements,

not as medicines; therefore, pharmacovigilance reporting is not compulsory for manufacturers. There are subtleties in the legal differentiation between food supplements and herbal medicine. But broadly, a medicinal product is defined as any substance or combination of substances presented as having properties for treating or preventing diseases in human beings. In contrast, a food supplement cannot claim to treat or prevent disease, or contain a pharmacologically active substance. This can be a complex area with the same herb being supplied as an herbal medicine, but also as an ingredient in a dietary supplement. In Europe, herbal medicines are registered under "traditional herbal medicinal products." Quality control, GPM, and safety data are essential for their production. In most of the countries, food supplements do not have the same regulation for quality control. The description of an herbal product as a food or medicine may have considerable impact for pharmacovigilance (Debbie et al., 2012).

Herbal medicines are usually promoted in the market as natural and, therefore, are regarded as safe and harmless in many cases without any evidence. Quality control in the manufacture of many herbal products is far below the standards generally recognized as necessary for therapeutic drugs (Snodgrass, 2001). Similarly, the consistency of many herbal products prevents extrapolating the results of any high-quality clinical trial to other products or even to other batches of the same product from the same manufacturer. Standardization has been strongly recommended as the answer to the quality question (Mukherjee et al., 2006). For certain ingredients and products, monitoring marker compounds provides a positive control for production and confirmation that the product contains the correct amount of extract. Management of marker compounds ensures batch-to-batch consistency, but not necessarily the quality of the finished formulation. Manufacturing botanicals to meet analytical standards for marker compounds does not necessarily ensure product efficacy or generic equivalence with products that have shown efficacy. Compounds other than the marker compound may also contribute to the pharmacologic response and there may be differences in bioavailability (Amagase, 2001). As herbal medicines are a complex mixture of several active compounds, sometimes it is hard to predict how these compounds are responsible for the desired therapeutic activity. These active compounds may produce serious interactions with conventional drugs or herb–herb interactions. The drugs with low therapeutic index, such as digoxin and warfarin, may interact with herbal drugs and may produce considerable safety concerns (Zhou, 2007). The quality and effectiveness are usually established through data obtained from animal studies, preclinical and clinical trials involving humans, and in vitro testing to ensure compliance with acceptable standards (Yadav, 2008). It is a well-established fact that premarketing clinical trials do not have the statistical power to detect rare adverse reactions, nor do they have sufficient follow-up to identify delayed adverse effects or effects from long-term exposure. The pharmacovigilance program has been initiated to establish safety, which will help to understand and prevent adverse effects or other drug related issues. Botanicals should be regulated and the necessities include labelling, good manufacturing practices, packaging, marketing, and adverse effects reporting. In order to ensure the quality, safety, and reliability of herbal products, and to mainstream herbal products in the present healthcare system and gain public faith, regulatory agencies, researchers, and manufacturers should apply systematic methodologies for manufacturing, quality evaluation, and clinical trials (Mukherjee et al., 2015).

18.8.3 Steps to Commence Pharmacovigilance for Herbal Products

It is necessary to develop a pharmacovigilance for the practice of herbal medicines at several levels due to an increase in awareness. In connection with conventional medicines, several pharmacovigilance models and related tools have been developed to monitor the safety of herbal medicines (Barnes, 2003). The use of phytoconstituents or plant-derived products is increasing and there is a need to improve not only quality but also safety monitoring of such therapies so as to ensure the public is receiving a safe drug (Mukherjee et al., 2015). Several aspects of herbal pharmacovigilance has been explained in Fig. 18.3 shows some of the steps to be initiated to introduce herbal pharmacovigilance to monitor the safety of herbal medicines.

Good regulatory practice should be initiated with herbals and their formulations, such as single entity compounds. Although products are being registered at a faster rate, safety has not been compromised at the expense of speed. The drug control authority should provide caution when registering products with limited safety data and herbal formulations suspected to be associated with ADR. The inefficiency in providing such information on serious ADRs will lead to major unwanted effects (Mukherjee et al., 2015). The importance of controlling the correct identification of herbal preparations should be very much considered at the beginning step. In addition to the problem of incorrect plant identification, some mixtures may be toxic, particularly if they are misused. It is very much needed to continually review and assess the safety of most commonly consumed botanicals with an emphasis on surveillance of the use of these products to identify unknown hazards or risks and address them expeditiously.

It is necessary to have compliance with a good quality management system and good standard documentation of product information through computerization that allows efficient and quick retrieval of information when immediate action has

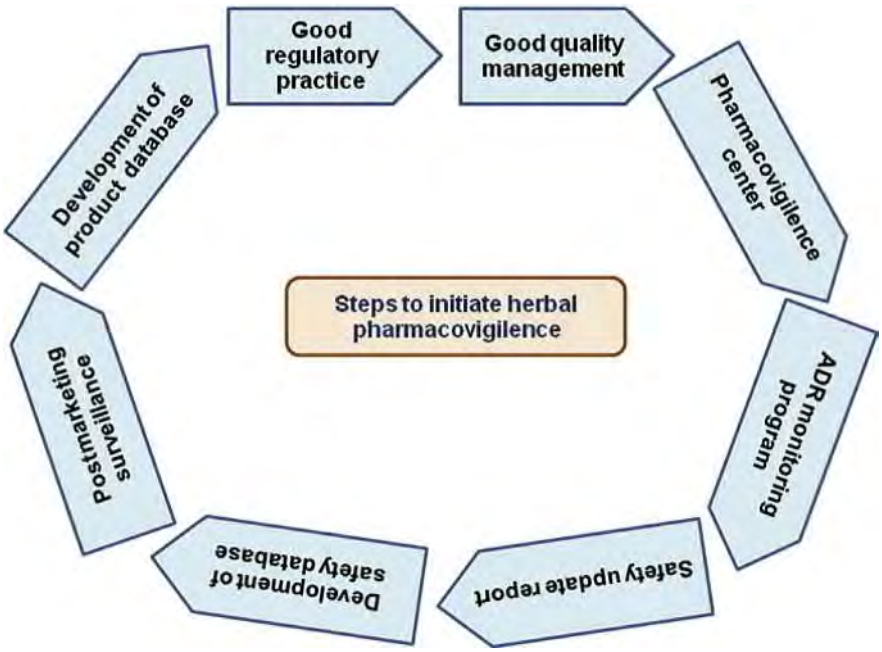

FIG. 18.3 Herbal pharmacovigilance.

to be taken. An adverse drug reaction monitoring program should be included in the good regulatory practice system, by which it will be easy for the national center to change safety issues into policies (Mukherjee et al., 2002).

Phytosurveillance can be a new concept to conventional medical practitioners; hence, they should be informed and trained in reporting ADRs through a spontaneous reporting system. Good regulatory practice should insist that the pharmaceutical industry give safety update reports and submit information pertaining to literature reports and the outcomes of postmarketing surveillance studies that will enable understanding of the recent ADRs of the product in various settings. During the product evaluation and registration, guidance to the pharmaceutical industry by the regulatory authority will assist in the development of a product database. A new enhanced computerized system assisted database should be developed, which will enable the ADR reports and information obtained from other sources to be linked with the product data base. Information on existing products or newly marketed products, the occurrence of adverse events, and drug utilization figures will be more readily available to hospitals and healthcare professionals. A technical working group should be established, including regulators, manufacturers, and healthcare professionals to observe how the herbal industry can improve pharmacovigilance practices (Mukherjee et al., 2015).

18.8.4 Postmarketing Surveillance

Conventional medicines are thoroughly tested before they are licensed and access is carefully controlled through the issue of prescriptions and dispensing through pharmacies. Unfortunately, such regulatory control does not favor the supply of herbals. Some adverse reactions may not appear until the herbal formulations are in general use. Therefore, it is very important to know the effects of these drugs after marketing, especially herbals that are not prepared based on traditional approaches, and they should be closely monitored under their usual conditions of use in daily practice. Importance should be given to continuous surveillance and of actively requesting information rather than just collecting reports and even this can be considered as a national program (Mukherjee et al., 2002). This process is commonly referred as postmarketing surveillance: the systematic and scientific evaluation of all intended and unintended effects of medicines on human health after their release for marketing (Mukherjee et al., 2015).

18.9 ADVERSE DRUG REACTIONS IN HERBAL MEDICINE

ADR reporting program should be initiated with good relations between professionals, industry, and local agencies. Feedback from opinion leaders, practitioners, and medical associations regarding the safety issues of registered products should be sought. In order to improve the rationale for prescribing and utilization, hospital pharmacists, and prescribers

should be encouraged to be involved in pharmacovigilance activities. Further, information on safety issues should be utilized in formulating decisions and policies made by the Drug and Therapeutics Committee (DTC) in hospitals. The metabolism of a drug can be altered by another drug or foreign chemical and such interactions can be clinically significant. Herbal medicines, such as St. John's Wort, garlic, piperine, ginseng, and gingko, which are freely available over the counter, have given rise to serious clinical interactions when coadministered with prescription medicines (Mukherjee et al., 2007). The observed induction and inhibition of CYP enzymes by herbal medicines in the presence of a prescribed drug has led to the general acceptance that natural therapies can also have adverse effects, contrary to the popular beliefs in countries in which there is an active practice of ethnomedicine (Mukherjee et al., 1998). A commonly heard argument related to the safety of herbal medicines is that these products have a longstanding history of traditional use, resulting in considerable experience with and knowledge about their wanted and unwanted effects. Herb–drug interactions have been increasingly reported but are under researched. The identification of drugs that may interact with bioactive phytoconstituents and the mechanism involved is a prerequisite for better clinical risk assessment of herbal drugs. A major safety concern is the potential for interactions of herbal drugs with prescribed drugs, especially important with respect to drugs with narrow therapeutic indexes (e.g., warfarin and digoxin).

Generally, it is hard to find an adverse reaction before the definitive approval of an herbal or synthetic drug. It requires about 10,000 patients to participate in a large pharmaceutical trial for 1 year. The incidence of severe drug reactions with a ratio of 1:20,000 can be missed. However, when it is marketed and used by people for the long term, adverse effects are more likely to be observed. ADRs are ranked as one of the top ten causes of morbidity, mortality, and illness in the developed world (Lazarou et al., 1998; Pillans, 2008). Still, there are no specific methods that have been developed for the determination of ADR. So, it is really important to establish a valid basis for real clinical knowledge and toxicity in HMs, which requires balancing two aspects of scientific validity: internal and external validity (Linde and Jonas, 1999; Ioannidis, 2005). Internal validity means that the research must have reliable test hypothesized relationships between an intervention and an outcome under controlled conditions. External validity refers to the practical applicability of research results to a target population outside the experimental conditions of the research study (Linde and Jonas, 1999).

18.9.1 Assessment of Severity and Seriousness of ADRs

For the assessment of ADRs, ICH has distinguished serious from severe ADRs. The grading of the degree of the reaction is defined as severity. The outcome of the reaction is known as seriousness (Edwards and Biriell, 1994). The WHO has defined a serious ADR as any inconvenient medical occurrence at any dose resulting in death or that requires inpatient hospitalization or prolongation of existing hospitalization. Severity, or a severe type of reaction, may be potentially life-threatening, and may cause permanent damage and require intensive medical care. But in the case of a moderate type of reaction, we can overcome the situation by changing the drug therapy or the specific treatment to prevent a further adverse reaction.

Manufacturers and researchers of herbal medicine are encouraged to fully comply with the available guidelines and to apply tools to evaluate potential herb–drug interactions. If drugs have to be used in combination with herbs, dose optimization may be needed and discontinuation of therapy is necessary when toxic herb–drug interactions leading to life-threatening or lethal adverse effects occur. Quality assurance of herbal products should be enforced by the cGMP guidelines for herbal products. Both patients and clinicians should be educated on the clinical significance of herb–drug interactions. With continuous improvement in our understanding of the mechanism of drug interactions, the risks associated with such interactions can be better predicted, evaluated, and managed in order to reduce the susceptibility of clinically significant adverse drug interactions.

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Chapter 19

Quality Assurance of Herbal Drugs and Stability Testing

Chapter Outline

19.1 QUALITY ASSURANCE AND STABILITY TESTING OF HERBAL DRUGS

Herbal drugs have turned out to be an important part of a next generation health system. It has been observed in that herbal products are substituting for conventional medicines in many countries. An increased demand for herbal products leads to growing demand in the global market (He et al., 2015) with an increase in the use of herbal medicine, there is also an increase in the reports of toxicity and adverse reactions. Such undesirable events can be due to:

i) side effects;
ii) reactions that result from overdose, overduration, tolerance, and dependence-addiction;
iii) hypersensitivity, allergic, and idiosyncratic reactions; and
iv) mid-term and long-term toxic effects, including liver, renal, cardiac and neurotoxicity effects, as well as genotoxicity and teratogenicity.

The herbal products being marketed are generally not thoroughly tested for their pharmacological and toxicological effects. In addition, there have been problems in the quality of herbal products relating to unexpected toxicity, which occurs due to the use of poor-quality raw materials, misidentified herbs, adulterations, and contaminations. With the

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help of good manufacturing practices (GMP), these quality issues can be addressed properly and the manufacture of herbal medicine can be improved. Although there are some issues that cannot be neglected, such as herbs that come from different countries and areas that have different standards and regulations, these matters will remain a problem. Due to several reports of serious effects, such as hepatotoxicity, renal failure, and allergic reactions, the regulatory authorities are facing questions regarding the safety of marketed herbal medicines. The World Health Organization has developed guidelines for the monitoring of herbal safety within the existing pharmacovigilance framework (Shaw et al., 2012).

As nearly every pharmaceutical product undergoes some kind of alteration process, the speed of which depends on the nature of the active substances, the properties of the excipients and additives, the nature and size of the contact surfaces, and many other factors, it is customary to take the stability limit at 90% of the declared active substance content. This assumes that, apart from loss of active substance, no other detrimental alteration has taken place and that the decomposition of the active substance has not led to the formation of toxic products. It is also customary to add a manufacturing or safety margin to medicinal products in which the active substances undergo relatively rapid decomposition and in which the therapeutic breadth (the margin between therapeutic dose and toxic dose) permits this, so that the product appearing on the market contains the declared active substance content, which is seldom considered for phytopharmaceutical preparations (Mukherjee, 2002).

19.1.1 Quality Assurance Measures

19.1.1.1 Good Agricultural and Collection Practice (GACP) and Species Authentication Using Classical Systematics

In many developed countries, such as Australia, Canada, Europe, and the United States, the quality assurance and quality control of herbal ingredients (raw materials) is primarily the job of the product license holder who should ensure the efficacy and compliance of the herbs with the respective national regulatory framework. There should be adequate measures taking care for quality compliance of ingredients (raw materials) across the supply (value) chain right from the field to the manufacturing process to assure quality. Vendor audit programs for quality compliance of herbal raw materials and manufacturing based on GMP protocols should be followed stringently (Govindrajan et al., 2015). The proper sourcing of authentic herbs is absolutely necessary for the quality assurance of herbal ingredients throughout the supply chain. To establish the purity of the ingredient, authenticating the starting material is the major requirement. Authentication is often difficult when the herbs are purchased from local markets with no traceability of their origin, medicinal herbs are sourced either from organized cultivation or by wild-crafting. Details on this subject are given in Chapters 12 and 21.

19.1.1.2 Genomic Profiling and DNA Barcoding in Species Authentication

Conventional methods to identify raw drugs and plant material at the species level are not always feasible due to limitations inherent in morphology-based systems and the dwindling pool of taxonomists. For the last decade, microgenomic identification systems have provided a promising approach toward the diagnosis of biological diversity, with DNA barcoding becoming popular. The diversity among DNA sequences used to identify taxa can be viewed as genetic barcodes. The plant mitochondrial genome has certain constraints, which precludes its use as a universal plant barcode. The quest shifted toward the plastid and nuclear-based regions, following initial in silico and laboratory-based evaluations of different coding and noncoding markers. The outcome of these trials proposed major individual candidate regions matK, rbcL, rpoB, rpoC1, and the intergenic spacers ITS, psbA-trnH, trnL-F, atpF-atpH, and psbK-psbI for use in plants based on their discrimination capacity (Govindrajan et al., 2015). Due to pitfalls and challenges associated with a single locus, the combination of loci emerged as a promising choice to obtain appropriate species discrimination. The details are described in Chapter 12.

19.1.1.3 Plant Authentication Based on Macroscopic and Microscopic Features

Authentication of medicinal plants based on macroscopic and microscopic characteristics and their phytochemical (metabolite) profiles is crucial, considering the pharmacopeial monograph standards. The identification of the species starts with morphological and anatomical characters of dried and sorted plant parts, such as roots, leaves, berries/fruits, bark, flowers, seeds, and husks, or derived products, such as gum/resins (Govindrajan et al., 2015). Details on this context have been provided in Chapter 5.

19.1.1.4 Phytochemical Profiling of Plant Parts as a Tool for Identification and Characterization

For species and plant part-specific identification, phytochemical profiling is increasingly being used. The main analytical components of phytochemical profiling are high-performance liquid chromatography and high-performance thin-layer chromatography (HPTLC) in addition to capillary electrophoresis (CE) and gas chromatography (GC). Commonly used detectors include the light-based photodiode array (PDA). Versatile detectors, such as mass spectrometry (MS)-based detectors, are also used. The primary techniques used in the medicinal herb industry for the generation of chemical fingerprints are thin-layer chromatography (TLC) and HPTLC, as well as qualitative and semiquantitative techniques (Govindrajan et al., 2015). Details on TLC, HPTLC, and semiquantitative techniques have been described in Chapter 9, 10, and 11, respectively.

19.1.1.5 Guidelines for Good Plant Authentication and Identification Practice (GPAIP)

An outstanding good practices guideline regarding plant identification for the herbal industry has been provided by Agriculture and Agri-Food Canada and is shown in Fig. 19.1. It has been recommended to the industries manufacturing herbal medicines that GPAIP should be followed as the herbal ingredients undergo change at every step of the process along the supply chain.

19.1.1.6 Assurance of the "Purity" of Botanical Raw Materials: Impurity Profiling

To obtain good quality herbal ingredients, the main focus should be on purity, that is, they should be free from impurities, which can ensure quality end-products. There are many components that fall under the category of impurities, such as heavy metal, pesticide residues, and aflatoxins/mycotoxins. Contaminated soil is the major source of heavy metal contamination in medicinal plant species. It has been reported that over 500 plant species have accumulated heavy metals. However, in some species, the aerial parts exceed critical toxicity levels due to high concentration of heavy metals (Govindaraghavan and Sucher, 2015).

19.1.2 Challenges in Quality Assurance of Herbal Medicine

Herbal medicines are not isolated as single products; they are complex and rich in nature (Shaw et al., 2012). A number of factors can influence the qualitative and quantitative chemical profile, including:

- Geographical origin, which includes climate, soil, photoperiod.
- Genotype of the plant.
- Parts of the plant (e.g., leaves, stems, root, root bark).
- Harvesting time (year, season, time of day) and conditions.
- Storage, processing, extraction.
- Combinations of herbs and/or processing of the combined herbs as medicines.

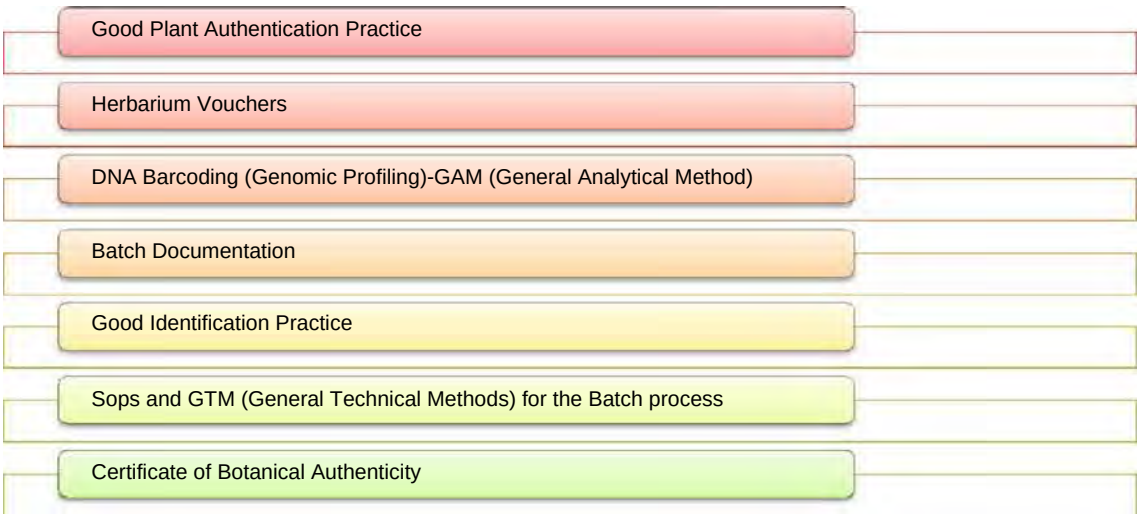

FIG. 19.1 Good identification and authentication practice (GPAIP) for herbs.

19.2 USE OF INDICATIVE SUBSTANCES

The bioactive extract, being a composite mixture of classes and groups of organic compounds in conjunction with many extraneous materials (both organic and inorganic), may be better understood in terms of Indicative Substances. To rationalize the use of natural products, a need-based and novel concept of biomarkers is used in herbal extracts. Unfortunately, the development of this paradigm is not an easy task due to the complex interplay of many variables in this approach. The biomarkers, in combination with other chemical entities via poorly understood mechanisms of synergy or antagonism, are supposedly responsible for the efficacy of the standardized extracts for any particular therapeutic area or disease. However, defining the biomarker has to be very specific and a lot of insight has to go into it before declaring any distinct molecule as a biomarker. Moreover, a good number of biomarkers are desirably required to achieve the elusive goal for the rational use of any given standardized extract. The selection of marker and biomarker compounds from extracts is a tricky business with a lot of subjectivity and experimentation. Details on Marker/Biomarker and related aspects have been described in Chapter 12.

TLC is a simple and reliable aid for supporting the macroscopic and microscopic identification of a plant drug and also for identification of an extract and of any medicinal dosage forms that may be prepared from it. If no tested authentic plant drug or extract material is available, indicative substances are also chromatographed. They can be selected from among the constituent substances of the drug plant, irrespective of whether these are active or accompanying substances. They should, however, be characteristic of the drug plant under investigation and easily demonstrable. Analytical data other than those obtained by TLC can also be used for identification and quality assurance. However, it is not sufficient to demand that identically colored zones or spots from the investigated solution appear in the chromatogram at the level of the indicative substances. Rather, the color and R_t value of the zones that appear, in addition to the indicative substance spots, must be described.

The German Pharmacopeia permits the use of external indicative substances, which are not contained in the drug plant itself. Arnica flowers may be quoted as an example. A comparative solution of rutin in methanol is also applied to the thin-layer plate in addition to the solution under investigation, which is prepared by extraction of the drug powder with methanol. This flavonoid (rutin), which does not occur in Arnica flowers, is used to recognize any possible adulteration by marigold (Calendula) flowers, as it is stated that no zones with a yellowish-to-green color should appear in the chromatogram of the investigated solution (Mukherjee, 2002).

There are various ways in which the quality of an herbal medicine can be identified, one of which is the identification of the Q-marker of the medicine. The concept of the Q-marker came into existence from traditional Chinese medicine. The intrinsic or processing/preparation-resultant chemical substances, which are associated closely with the properties that are shown by the raw materials and products of TCM, and that furthermore can be used as indicators for the quality control of TCM to ensure safety and effectiveness, are known as Q-Markers of TCM. However, this concept does not include the chemicals that are absorbed or newly generated after an in vivo process in need of additional structural elucidation. The basic properties of Q-markers can be defined in four aspects:

a) They are the intrinsic chemical components in TCM materials and products or result from processing/preparation.
b) They are associated with functional properties, with definite chemical structures.
c) They can be qualitatively characterized and quantitatively determined.
d) For the formulae, the preventative substances of the Monarch are firstly considered.

In establishment of Q-markers, special attention should be paid to the components that ensure the authenticity (identity marker), differentiate the quality difference (superiority/inferiority marker), and identify the geo-authenticity (geo-authentic marker) (Yang et al., 2017).

19.2.1 Indicative Substances for Identification

A chemical compound found in plant material that may or may not be chemically active and that characteristically acts as a fingerprint for that plant is known as a marker. Generally, HPTLC and HPLC can visualize and ascertain this compound and its limits. However, biomarkers are the group of compounds found in plants that have biological efficacy (Mukherjee, 2002).

The development of parameters for quality control of Ayurvedic drugs is a big task involving biological evaluation for a particular disease area, chemical profiling of the raw material, and laying down specifications for the finished product. Therefore, the word “standardization” should encompass the entire field of study from the birth of a plant to its clinical application. It is a vast study and involves various disciplines that only interdisciplinary laboratories can undertake. The

industry, however, cannot wait for this to happen and looks forward to easy and quick answers. To solve their problems, the first and foremost task is selection of the right kind of plant material, which is therapeutically efficacious. The difficulty can be elucidated with the example of *Withania somnifera*. This plant is reported to have three chemotypes, depending upon the presence of a class of closely related steroidal lactones, such as Withanolides and Withaferin A. In spite of this classical chemotype distinction, the selection of the right chemotype to which all clinical effects are attributed is still elusive. Therefore, the need arises to lay down standards by which the right material is selected and incorporated into the formulation. The content of Withanolides and Withaferin A and other definite biologically active compounds may be variable, depending upon the geography, genome, and the time of collection of the plant material, among other factors. Therefore, there is a need for establishing limits for the content of these multiple components in clinically acceptable material. Thus, there arises the concept of multiple markers and biomarkers to define the right material.

The assay part of standardization is chemical and biological profiling by which the chemical and biological effects may be assessed and curative values established. Safety for use may also be assessed through this parameter. In biological assays, the drug activity is evaluated through a pharmacological model. For example, insulin, again a natural product, is expressed in IU (international units). These units correlate with the activity of a unit weight of insulin to lower blood glucose levels. Similarly, the efficacy of Digitalis, a cardiotonic drug, can be effectively evaluated by biological assay of its action on cardiac muscles. The force of contraction of these muscles on administration of a known amount is an indicator of its biological activity. However, biological testing is highly subjective in nature due to many variations and, secondly, there are strict regulatory controls on animal testing. Therefore, the development of standardization procedures for quality control through biological assays shall be running against time (Mukherjee 2002).

Chemical profiling, on the other hand, is a versatile technique and can be used in standardization. Fingerprints may be generated with known analytical procedures. Fingerprinting, in essence, is chemoprofiling, which means establishing a characteristic chemical pattern for the plant material or its cut, fraction, or extract. It is important to understand that a plant extract consists of established classes of chemical compounds. These include the primary metabolites, secondary metabolites, and inorganic salts and metals. Primary metabolites are compounds, such as carbohydrates, proteins, and lipids, which are essential for the plant physiology as such, but are formed as by-products in the biochemical pathways. These also include very interesting and useful classes of compounds, such as alkaloids, flavonoids, coumarins, terpenoids, and anthocyanins. Many modern drugs originated from these secondary metabolites, such as morphine from Opium, reserpine from *Rauwolfia serpentina*, and vinblastine and vincristine from *Vinca rosea*. In fact, this is the point of divergence between the modern system of medicine and classical systems of medicine, such as Ayurveda, with the former laying emphasis on pure compounds. These secondary metabolites can be utilized for the identification of plant material because our knowledge of chemistry has advanced sufficiently and sophisticated analytical techniques allowed us to measure these compounds qualitatively and quantitatively. If we can measure the presence of a unique secondary metabolite in a plant material, it is not always sufficient to say with certainty that the plant material is of the desired quality. As an example, take Vasaka. Vasicine is a very characteristic alkaloid found in Vasaka (*Adhathoda vasica*). But merely establishing the presence of this compound and measuring it is not a sufficient indicator of plant quality because vasicine, as a pure entity, has no biological activity attributed to Vasaka. There are no databases for characterizing compounds obtained from specific plant materials.

Marker testing is in no way a substitute for other tests, such as physicochemical, chemical, macroscopic, and microscopic characterizations. Nevertheless, it is an efficient procedure to ensure the identity and purity of herbal drugs (Mukherjee, 2001). There are several constraints in adapting this technique for the regular testing of herbs and herbal formulations in the traditional system of medicine as follows (Mukherjee, 2002):

- Unavailability of library of marker compounds isolated from herbs in their pure form.
- Lack of proper communication and the willingness of quality assurance departments to undertake tedious testing procedures.
- Unavailability of a system in which standard or properly identified herbs are available for the industry.
- High cost involved in procurement of costly instruments and also validation and proper maintenance of them.

19.2.2 Indicative Substances for Content Determination

Only those substances that actually occur in the material being evaluated can be considered in the selection of indicative substances for quantitative evaluation of a drug plant material or of a product prepared from it. An external indicative substance cannot be used.

The choice of the indicative substance is also governed by the problem in question. The quantitative determination of any substance regularly occurring in the drug plant for ensuring the constant action of the drug or of its preparations

is meaningless if this substance cannot be demonstrated to be the active principle. It can, however, be meaningful if it is carried out, for example, on an extract that occurs up to a certain percentage in a tablet preparation, as the extract content in the ready-to-use preparation can be ascertained via determination of the indicative substance. In such cases, the choice of the indicative substances is made on an analytical basis, that is, they must be easy to isolate and to determine exactly in small quantities without great effort and expense (Mukherjee 2002).

19.2.3 Indicative Substances for Stability Testing

The shelf life or the stability limits of plant drugs cannot be determined in cases in which their products containing active substances cannot be detected and determined chemically or biologically. The use of indicative substances as a substitute for the unknown active substance can be regarded as justified in so far as, by definition, no one substance or group of substances, but rather the whole range of extractable constituent substances, is considered to be responsible for the action of phytopharmaceuticals. Difficulties occur in the selection of the indicative substance. The multiplicity of constituents, their qualitative and quantitative differences through the growing period, and the numerous influences (time of harvesting, further processing of the plant drug material, etc.), make the demand that the most labile compound must always be chosen as the indicative substance for stability testing, hardly logical. Furthermore, each type of preparation requires its own choice of indicative substance, as its lability differs greatly under various environmental conditions. A compound susceptible to hydrolysis would not be a suitable indicative substance in dry preparations. The same applies to substances sensitive to oxidation or to light. In this situation, it seems more logical to check phytopharmaceutical preparations and their precursors both organoleptically for taste, smell, and appearance and chemically and physically for any alterations at regular intervals during storage. Determination of the density, refractive index, and alcohol content of liquid preparations and, especially, the comparison of thin-layer chromatograms of fresh material with those prepared upon completion of manufacture of the test preparation, provide definite indications of the stability limits.

Thus, quantitative determination of one or more indicative substances permits no definitive statement on the stability of the activity when the active principle is not known. Care should therefore be taken not to interpret every little alteration as a serious instability (Mukherjee, 2002).

19.2.3.1 Analytical Methods

19.2.3.1.1 Ambient Ionization Techniques

For fast and easy analysis of pharmaceutical products and herbal medicines under ambient and open-air conditions, efforts have been taken since the invention of ambient ionization techniques.

Further, a large field of ambient ionization techniques have been published for analysis of pharmaceutical products and herbal medicines to date, with analytical strategies emphasized.

19.2.3.1.2 Desorption Electrospray Ionization

Desorption electrospray ionization (DESI) is an ambient ionization technique in which charged solvent droplets are made to hit the sample surface with rapid formation of analyte ions after the subject hits the sample surface. The main working of DESI is based on the mechanism of electrospray ionization with an analytical strategy of direct desorption/ionization, and it is extremely suitable for surface analysis and possesses the capability of imaging. Raw herbal materials, such as alkaloids in the seeds, stems, leaves, roots, and flowers of *Conium maculatum*, *Datura stramonium*, and *Atropa belladonna*; diterpene glycosides in Stevia leaves; diterpenoids in *Salvia divinorum* leaves; and camptothecin and 9-methoxy camptothecin in the leaves and stems can be analyzed by DESI. The following strategies have been used for the analysis of pharmaceutical products and herbal medicines with DESI:

- Surface desorption/ionization of analytes
- Reactive DESI
- Surface imaging of analytes
- Coupling thin-layer chromatography (TLC) to DESI–MS for analysis.

19.2.3.1.3 Direct Analysis in Real Time

Direct analysis in real time (DART) is based on the mechanism of atmospheric pressure chemical ionization (APCI), which helps in the analysis of low-molecular-weight volatile/semivolatile compounds in gas, liquid, and solid samples. DART is performed by application of an electrical discharge to a gas (typically nitrogen or helium) for the formation of plasma excited-state species, and the species are carried by the heated gas stream toward the sample to desorb and ionize analyte molecules from the sample for subsequent MS analysis (Yang and Deng, 2016).

19.3 GMP AND HACCP IN TRADITIONAL SYSTEMS OF MEDICINE

Herbal medicines are prepared with material from herbal sources, which are collected from a variety of geographical or commercial sources, unlike conventional pharmaceutical products, which are prepared with the help of synthetic materials and reproducible mechanized techniques and procedures. Therefore, it is difficult to determine the conditions in which they have been grown. Further, their composition and properties may vary. Besides manufacturing and quality control, production techniques of herbal medicines are rather different than for conventional pharmaceutical products. GMP guidelines are formed to provide GMPs for pharmaceutical products. These guidelines cover various herbal medicines developed by several WHO Member States and the European Union. Details on this context are described in Chapter 21.

HACCP is the collection of guidelines created by The National Advisory Committee on Microbiological Criteria for Food (NACMCF) (Buchanan, 1997). It has been strategized that the production of traditional medicinal formulations should be based on thorough analysis of the main conditions during purchase, production, quality control, packaging, and marketing (Buchanan, 1997). Quality assurance is considered to be an integral part of all the traditional systems of medicine (Anonymous, 2011). To ensure food safety from harvest to consumption, several basic principles were established. HACCP covers a methodical approach for identification, prevention, and control of food safety hazards. These approaches are depicted by seven stated principles, as described below (Buchanan, 1997).

19.3.1 Conduct a Hazard Analysis

The main objectives of a hazard analysis are as follows:

- to identify the hazards and associated control measures,
- to assure product safety, the needed modifications should be identified, and
- to provide a basis for determination of critical control points (CPPs).

19.3.2 Determine CPPs

A point, step, or procedure in food processing that can be controlled, and results in the prevention of food safety hazards is known as a CPP. Critical points are the locations in a process that help in the determination of food safety hazards by the application of some portion of control.

19.3.3 Establish Critical Limits

Critical limits (CL) are the parameters that help in identifying the measure of control on CPPs. Therefore, they help to indicate whether a CCP is in control or not.

19.3.4 Establish Monitoring Procedures

Formerly, after setting CLs for each CCP, the establishment of procedures for monitoring the CCPs should be done. To check the control over CCP, monitoring is performed, as it is a well-planned sequence of measurements.

The main objectives of monitoring are as follows:

- To track control of the process.
- To determine when there is a loss of control and a deviation occurs.
- To provide a written document to be used in verification.

19.3.5 Establish Corrective Actions

When a CL fails to meet the optimum level in determination of a CCP, corrective actions must be established. Production of food type and process determines the corrective actions. It consists of the following:

- identifying and eliminating the cause of the deviation,
- ensuring that the CCP is under control after the corrective action is taken,
- ensuring that measures are established to prevent recurrence, and
- ensuring that no product affected by the deviation is shipped.

19.3.6 Establish Record keeping and Documentation Procedures

Proper documentation and recording of the operation of the HACCP system should be kept. Documentation of all measurements and corrective actions should be documented. The HACCP records should contain:

- The date and time of the activity reflected on the record.
- The signature or initials of the employee making the entry.
- The information entered on the record at the time it is being observed.
- Actual observations or data values obtained.

19.3.7 Establish Verification Procedures

A systematic verification of HACCP systems must be done. To guarantee that the HACCP plan is executed as planned, and to confirm the proper monitoring of the CPPs, verification is performed (Anonymous, 2016).

19.4 PHYSICAL QUALITY ASSURANCE

"Quality assurance" is a wide-ranging concept covering all matters that individually or collectively influence the quality of a product. It is the totality of the arrangements made with the object of ensuring that pharmaceutical products are of the quality required for their intended use. Quality assurance therefore incorporates GMP and other factors, including product design and development (WHO, 2007). These are tightly controlled by European and national legislation. Monographs provide legally binding quality assurance procedures for products. The variability in the content and concentrations of constituents of the plant material, together with the range of extraction techniques and processing steps used by different manufacturers, results in a marked variability in the content and quality of all herbal products. To ensure the reproducibility of the pharmacological effect and clinical effectiveness of a botanical material, precise data on the composition of the extract are needed. The quality control of all these steps will greatly improve the quality of the resultant HMPs (Da-Costa-Rocha et al., 2012). Unlike pure pharmaceutical preparations, herbal medicinal products have few generic equivalents. Products from different manufacturers vary considerably, because it is inherently difficult to control all the factors that affect a plant's chemical composition. Environmental conditions, such as sunlight and rainfall, as well as manufacturing processes, such as selecting, drying, purifying, extracting, and storing herbs, can create substantial variability in product quality and in the concentration of plant chemicals within different products (Rotblatt, 1999).

It has been observed that quality assurance of herbal medicines has always been discussed from a chemical and physiological viewpoint. The physical quality has always been neglected even though it plays an important role in the processing and manufacturing of the plant extract.

Without the addition of suitable adjuvant substances, many plant extracts occur in a form that makes further processing considerably more difficult, often even impossible. Hence, extracts of Crataegus fruits, Curcuma extracts, and many others cannot be processed to more manageable dry products either by roller, belt, or spray drying. One particular example is the male fern extract, which is produced as a solvent-free thin extract. In all such cases, the manufacturer cannot handle this without considerable additions of inert adjuvant substances. Before drying, therefore, a proportion of Aerosil, lactose, maltodextrin, glucose syrup, or starch constituting up to 50% of the end-product is added to such plant extracts. As the ratio of active substances to accompanying plant substances remains unaltered here, the manufacturer has only to declare the measures he has taken.

19.5 QUALITY ASSURANCE BY CULTIVATION AND BREEDING

Although medicinal plant cultivation and breeding are not in the province of pharmaceutical technology, but proper cultivation and collection of medicinal plants well have a great influence on the use of phytopharmaceuticals as useful medicines. Different criteria for QA through GAP guidelines have been described in detail in a separate chapter. They have a major role to play in QA based on the following parameters.

- The fact that many medicinal substances of natural origin cannot be synthesized, or can be synthesized only with unacceptably great effort, necessitates creation of the natural starting material, that is, cultivation of the medicinal plant.
- The unreliability in the supply of drug plants gathered from the wild shows the need for their cultivation. The qualities available in the often widely scattered gathering areas are limited. Expert collectors are becoming increasingly difficult to find. All of this results in the increasing occurrence of mistaken identity and adulteration of drug plant materials.

- The increased demands for safety of medicines in general have also led to increasing demands for purity and quality of phytopharmaceuticals and of the plant drug materials from which they are gathered.
- Legal regulations, such as the 1973 Washington protection of species agreement and the more recent 1980 West German nature protection order, will considerably hinder the trade and processing of wild plants.

Medicinal plants must be cultivated with phytochemical aspects in view, as the success of such cultivation depends less on the quantity of plants produced and much more on their quality. The active substance content of a cultivated medicinal plant can be affected by various factors:

- Genetic variation and hereditary transmission of the secondary substances.
- Morpho and ontogenetic variability, that is, differences in the active substance contents in various parts of the plant and during its growth.
- Environmental influences (location, fertilization).

19.6 STABILIZATION AND STABILITY

WHO's Supplementary guidelines for the manufacture of herbal medicines specifically state that some stability data is very much required, so as to fix the expiry date, indicating the shelf life for an herbal material or herbal preparation along with the storage conditions (WHO, 2006). The European Medicines Evaluation Agency (EMEA) also mentioned its importance. The determination of the stability of the constituents is not sufficient but the stability of the product as a whole is required (Bansal et al., 2016). In the herbal substance or formulation, the stability of different substances present should be demonstrated by TLC fingerprinting or other suitable methods so that their proportional content can be measured at comparable concentrations to the initial fingerprint (CPMP, 2011). Stability studies on herbal drugs involve quantitative determination of specific constituent(s) as active and/or analytical marker(s). It should be ensured that any changes made in the composition should not be extrapolated to deviations in its therapeutic activity. The herb in its entirety is considered as the active ingredient (WHO, 2009; CPMP, 2011). Under the influence of heat, humidity, and/or light during manufacture, transportation, and storage, constituents of different chemical nature in an herbal product may undergo varied intramolecular or intermolecular reactions. The stabilization of a phytopharmaceutical preparation consists of measures that guarantee its storage quality or stability. Stability means the unaltered state of the product under customary or precisely defined storage and transport conditions. Alterations that reduce the quality and shelf life can be of a physical, chemical, or microbial nature (Bansal et al., 2016). They can therefore be detected in comparison with the original state:

- Organoleptic (sensory)
- Physically (refractive index, measurement of turbidity, colorimetric, etc.)
- Chemically (determination of one or more of the constituents)
- Biologically and microbiologically (bacterial count determinations, etc.)

Adjusted preparations, the lower and upper limits of the active substance content of which are stated in pharmacopoeias or other published directions, does not necessarily mean that no other alterations have occurred. Stability is not to be understood as an absolute term. Alterations take place gradually under the customary storage conditions or even upon strict adherence to storage instructions until finally a state that could be described as unstable is attained. Here, the active substance content has fallen to such an extent, that the physical state has altered so much, or microbial contamination has assumed such proportions, the professional use and the activity of the product can no longer be guaranteed to be harmless to the patient. The storage quality of products that have been standardized is ascertained by comparing the properties of the fresh product used for the standardization procedure with those found after certain periods of storage.

19.7 STABILIZATION METHODS

19.7.1 Guidelines for Stability Testing

For the purposes of manufacturing and the distribution of stable molecules and products for patient consumption, the regulatory authorities of different countries have constructed provisions in the drug regulations for manufacturers to comply with the stability data.

In order to ensure uniformity in testing, these stability testing guidelines were established to meet requirements for the application dossier and stepwise execution harmonized by the International Conference on Harmonization (ICH). The US

FDA also issued a guidance document entitled, "Expiration dating of solid oral dosage form containing Iron." WHO also published guidelines for stability testing in a global environment.

Later, additional guidelines for active pharmaceutical ingredients, drug products, or formulations and excipients was published. The codes and titles covered under the ICH guideline are outlined in Fig. 19.2.

19.7.2 Real-Time Stability Testing

To allow significant product degradation under the suggested storage conditions for a longer period of time, real-time stability testing is preferred. The stability of the product itself decides the period of the test; it should be of considerable time duration to indicate clearly that there is no measurable degradation to distinguish degradation from interassay variation. Data are collected during the test to show the instability. Interpretation of the data by comparing with a reference material with established stability characteristics will make the testing easier (Bajaj et al., 2012; Bansal et al., 2016). As per ICH guidelines, the long term stability testing condition is 25°C ± 2°C/60% RH ± 5% RH for 12 months and for intermediate stability testing condition is 30°C ± 2°C/60% RH ± 5% RH for 6 months should be performed.

19.7.3 Accelerated Stability Testing

In accelerated stability testing, a product is put through high temperature and heat, which determines the failure of its stability. This is performed to subject the product to conditions that accelerate degradation and used to predict the shelf life or to evaluate the relative stability of alternative formulations. This method helps in early determination of the shelf life of the product, which enables a shortening of the development schedule. The stress conditions apart from temperature and heat are moisture, light, agitation, gravity, pH, and packaging. Due to the short time period of the accelerated stability testing, there is less instability in the measurement system when compared with real-time stability testing. Therefore, the samples are stressed, then refrigerated and assayed simultaneously. As per ICH guidelines, the accelerated stability testing condition is 40°C ± 2°C/75% RH ± 5% RH for 6 months should be monitored.

In addition, the comparison between the unstressed product and the stressed material is made within the same assay and the percent of unstressed sample recovery depicts the stressed sample recovery. When considered statistically, four different stress temperatures are suggested for accelerated stability projections. Although, for components, such as thermolabile and proteinaceous, denaturing stress temperatures are avoided for relatively precise stability projections. The concept of accelerated stability testing is based upon the Arrhenius equation and modified Arrhenius equation. $InK = InA + \frac{\diamond E}{RT}\log\left(\frac{K2}{K1}\right) - \frac{-Ea}{2.303R}\left(\frac{1}{T2} - \frac{1}{T1}\right)$ (Bajaj et al., 2012; Bansal et al., 2016) where $K1$ and $K2$ are the rate constants at temperatures $T1$ & $T2$ (degree kelvins); whereas Ea is the activation energy and R is the gas constant.

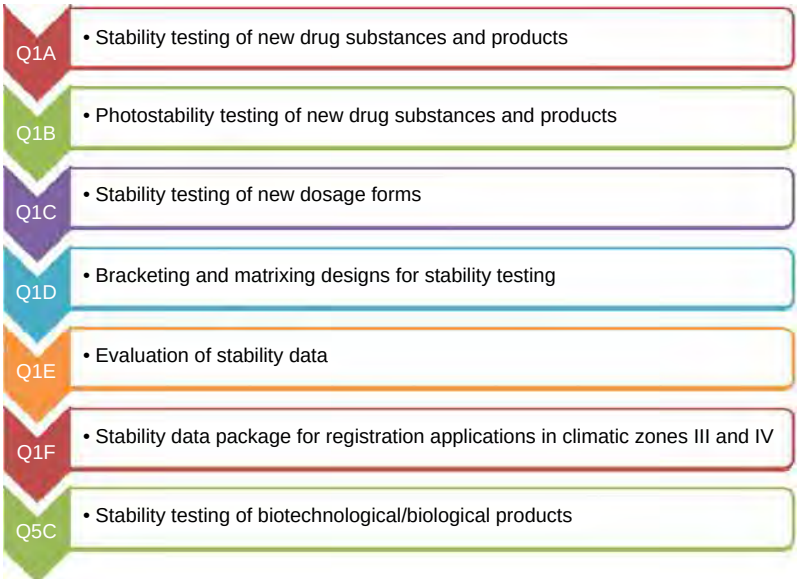

FIG. 19.2 Codes and titles covered under ICH guidelines.

19.7.4 Retained Sample Stability Testing

To determine the stability data for marketed products, retained sample stability testing should be performed. The stability of samples that have been retained and stored for at least one batch in a year are selected. If the number of batches marketed exceeds 50, then stability samples from two batches are recommended. At the time of first introduction of the product in the market, the stability samples of every batch may be taken. The stability of the samples is tested at predetermined intervals, that is, if a product has a shelf life of 5 years, it is conventional to test samples at 3, 6, 9, 12, 18, 24, 36, 48, and 60 months. This conventional method of obtaining stability data on retained storage samples is known as the constant interval method. Stability testing by evaluation of market samples is inherently more realistic because it challenges the product not just in the idealized retained sample storage conditions, but also in the actual marketplace (Bajaj et al., 2012; Bansal et al., 2016).

19.7.5 Cyclic Temperature Stress Testing

In this testing, conditions similar to market place storage are mimicked by cyclic temperature stress for 24 h. The temperature levels for this cyclic stress testing are selected on a product-by-product basis. They are selected considering various factors, such as recommended storage temperatures, and repeated for 20 cycles per product to understand the specific chemical and physical degradation properties of the products (Bajaj et al., 2012; Bansal et al., 2016).

19.7.6 Stability Testing With the Help of Drying

Physical, chemical, and microbial alterations take place in the liquid state, especially in an aqueous medium, more easily than in dry products. It follows from this that the drying of phytopharmaceutical preparations, and especially keeping such preparations dry, is the simplest and best method of protection (WHO, 2017). The pharmacopeias have recognized this for a long time. The residual moisture content in dried extracts is generally limited to a maximum of 5%. As extracts dry to a greater extent, they readily attract water from the surrounding atmosphere. An equilibrium of 6%–7% residual moisture is established in extracts that are not hermetically sealed during storage under the average relative atmosphere humidity prevailing. The storage of dried extracts over blue silica gel is useful only if the partial vapor pressure above the protected extract is greater than that above the drying agent at a given atmospheric humidity, if the drying agent is more hygroscopic than the extract. The rate at which the extract absorbs moisture depends very much on its specific surface area. This means that spray dried and freeze dried (lyophilized) extracts are more sensitive than roller or oven-dried ones. This fact must also be considered when grinding dried extracts, that is, this should not be done in rooms in which there is a high atmospheric humidity. There should also be no noticeable downward temperature difference from the air in the room to the material being milled, as otherwise water vapor will condense on the cold extract (Lauro et al., 2007).

Large quantities of extracts cannot be stored over a drying agent, as uneconomically large quantities of the latter will be required to ensure an adequate absorption capacity. Physical alterations scarcely occur at all under the above-mentioned conditions. Chemical processes, such as enzymatic reactions, which are detectable down to a residual moisture content of about 10%, as well as oxidations, hydrolysis, and others, proceed extremely slowly when storage in a cool place protected from light, as well as adequate drying, are guaranteed. The entry of oxygen must be restricted by the choice of suitable packing materials. In extreme cases, the material must be packed in vacuum or sealed under an inert gas. The impermeability of the packing material to oxygen should be checked if this packing material is a synthetic substance (Mukherjee, 2002).

Microbial alterations, that is, multiplication of the bacteria present in the product to a number that is no longer acceptable, as explained previously, are also dependent on the residual moisture in the product. The microbial growth depends on the so-called AW value (AW = water vapor pressure above the substrate/vapor pressure of pure water).

19.8 FACTORS AFFECTING STABILITY FOR HERBAL FORMULATIONS

Phytopharmaceutical formulations should be stable in different conditions. Alternative processes continue more rapidly in liquid phytopharmaceutical preparations than in dry ones. Several physical, physicochemical, and other parameters for the formulations should be recognizable as follows:

- Physical alterations, such as the formation of sediments, color changes, etc.
- Alterations due to microbial growth, recognizable by the formation of a pellicle of mold, cloudiness, or a sediment that can be easily disturbed.

- Chemical alterations, when these can be detected organoleptically by smell, taste, or appearance.
- Chemical alterations, such as hydrolytic decomposition, racemization, oxidation, etc., can only be detected with difficulty, that is, with analytical apparatus and agents.

The stability testing of a phytoformulation is dependent on several factors and a single measure cannot give an appropriate answer to this. It is obvious that the existing methods are not enough to fulfill the demand at this point and further investigation of the techniques/methodologies in this field is required to further control stabilization and quality assurance (Mukherjee, 2002).

Almost all foods and products derived therefrom, as well as herbs and herbal preparations, including phytopharmaceuticals, contain a group of metabolites. These metabolites are sometime biologically active, while some may not be active but impart colors, flavors, aromas, odors, and protection against diseases. These compounds, including phenolics, thiols, carotenoids, ascorbic acid, tocopherols, sulforaphane, indoles, isothiocyanates, and glucosinolates, may help to protect human cellular systems from oxidative damage through a variety of mechanisms; they therefore lower the risk of chronic disease in human beings. Several phytoceuticals possess unique health benefits and are also used as natural colorants. The phytochemical stability of herbs and herbal products are affected by many variables, including pH, oxygen, temperature, time of processing, light, water activity (AW), enzymes, structure, self-association, concentration, metallic ions, atmospheric composition, copigments, the presence of antioxidants, and storage conditions, suggesting that these molecules are unstable and highly susceptible to degradation and decomposition (Yang et al., 2013) as shown in Fig. 19.3.

19.8.1 Effect of pH

Phytomolecules, such as anthocyanins, are sensitive to pH, and show faded color above a value of 2. However, acylation with hydroxycinnamic acids does not only bring about distinct bathochromic and hyperchromic shifts, but also promotes stability at near neutral pH, which is explained by intramolecular copigmentation due to the stacking of the hydrophobic

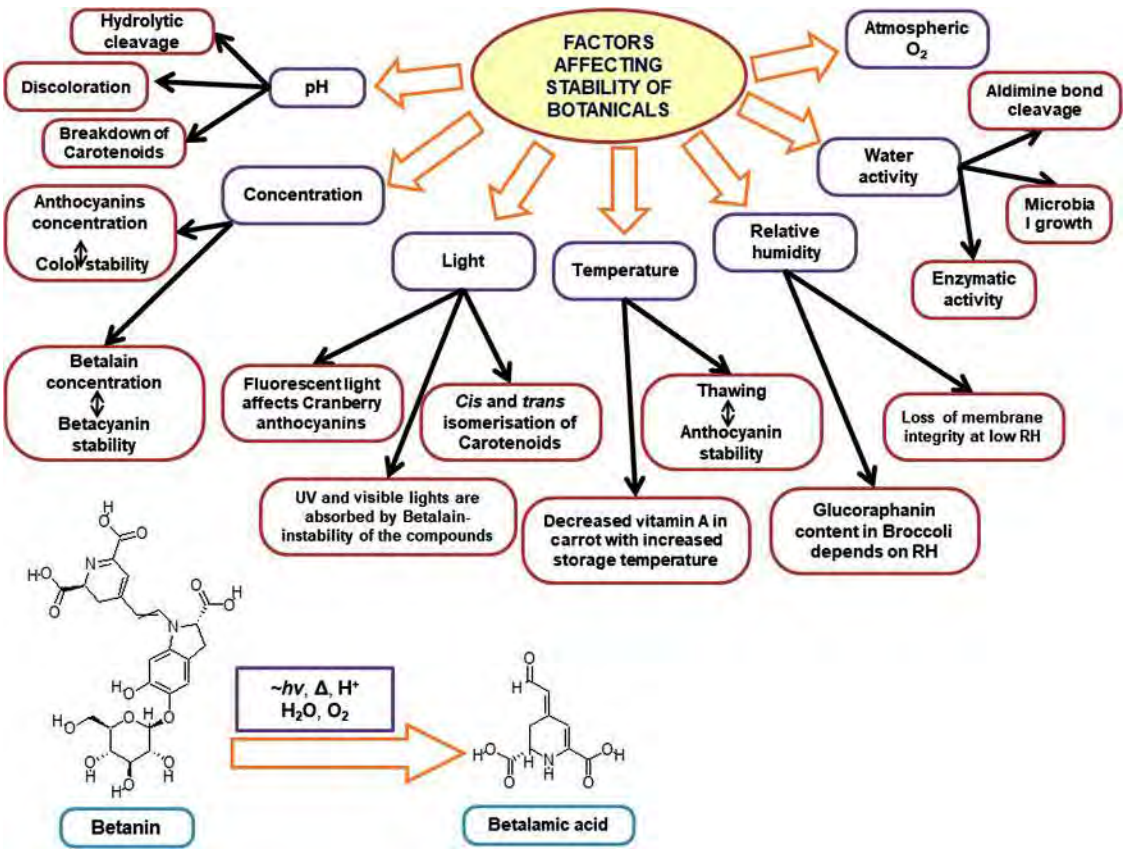

FIG. 19.3 Factors affecting the stability of botanicals.

acyl moiety and the flavylium nucleus, thus reducing anthocyanin hydrolysis (Brouillard and Dangles, 1993). The extent of color loss upon a pH increase is translated into hydration constants, which inversely are used to predict the stability at a given pH (Stintzing et al., 2002). In 2005, Bao et al. characterized anthocyanin and flavonol components from the extracts of four Chinese bayberry varieties, and investigated color stability under different pH values. The study indicated that the anthocyanin was most stable at pH 1.5. The result further showed that all color parameters significantly changed above pH 4, and the peaks above pH 4.0 at 515 nm were also remarkably reduced, suggesting the pigment was highly unstable above pH 4.0 (Bao et al., 2005).

There are a few reports on the stability of carotenoids based on their pH. Acidification of milled carrots to pH 4 or 5 with citric acid could improve color of the juice. β-carotene is stable to pH change and was reported to be stable in foods over the range pH 2–7 (Chen et al. 1995, 1996). During processing of carrot juice, the sterilization temperature has to be raised because carrots are mildly acidic (pH 5.5–6.5). However, this treatment can result in substantial loss of color. To remedy this problem, carrot juices are often acidified before processing so that the sterilization temperature can be lowered. It has been reported that heating carrots in an acetic acid solution can prevent coagulation of the extracted juice during heat sterilization. Luteoxanthin can be formed from violaxanthin under acidic conditions. Neochrome can be attributed to conversion of neoxanthin under acidic conditions too (Chen et al., 2007). Catechins as a mixture are extremely unstable in neutral or alkaline solutions ($pH > 8$), whereas in acidic solutions ($pH < 4$) they are stable (Chen et al., 2001). Their stability is pH dependent (pH 4–8); the lower the pH, the higher the stability. EC is the most stable isomer followed by ECG. EGCG and EGC are equally unstable in alkaline solutions (Su et al., 2003). In alkaline solution, green tea catechins change their color from light brown to dark brown due to degradation. Acid hydrolysis of glucosinolates leads to the corresponding carboxylic acid together with hydroxyl ammonium ion and has been used in the identification of new glucosinolates. Base decomposition of glucosinolates results in the formation of several products. In addition to allyl cyanide and ammonia, thioglucose is obtained from 2-propenylglucosinolate with aqueous sodium hydroxide (Fenwick et al., 1981).

The content of glucoraphanin decreased to less than 0.03 mg/mL when it was kept at a pH value of less than 6 for 9 days, but the content of glucoraphanin still remained at 0.0806 mg/mL when the extraction pH was 6.6. In addition, the degradation of glucoraphanin was accelerated if stored under acidic conditions. Therefore, glucoraphanin should be stored at a neutral pH condition (Wang et al., 2012). Alkali pH values decrease the stability of 4-hydroxybenzyl isothiocyanate by promoting the formation of a proposed quinone that hydrolyzes to thiocyanate (Borek and Morra, 2005). On the basis of experimental data obtained using glucobrassicin (GBS) extracted from kohlrabi leaves, a general scheme in which various indole derivatives (indole-3-carbinol [I3C], indole-3-acetonitrile [IAN], and 3,3′-diindolylmethane [DIM]) is generated, depending on the pH of the reaction (Clarke, 2010). In the enzymatic breakdown of GBS, myrosinase acts at pH 7 and, at room temperature, leads to the complete breakdown of GBS after 1 h regardless of the lighting conditions. The chemical breakdown of GBS was studied using aqueous buffered solutions with pH 2–11. Whatever the pH, no degradation product of GBS was noticed after 2 h. Moreover, in this study, a number of other glucosinolates were found to be stable in the same conditions (Lopez-Berenguer et al., 2007). It was found that pH had a significant effect on sulforaphane nitrile production. A neutral or alkaline pH resulted in predominately sulforaphane production, whereas an acidic pH (3.5, typical of salad dressings) gave rise to more sulforaphane nitrile (Ludikhuyze et al., 2000).

19.8.2 Concentration

The color and pigment stability of strawberry and blackcurrant syrups, which were processed and stored under identical conditions, has been reported (Skrede et al., 1992). The study revealed that color stability was dependent on total anthocyanin level rather than qualitative pigment composition, because anthocyanin pigments of blackcurrant syrup were more stable than those of unfortified strawberry syrup. The color stability of strawberry syrup fortified with equal anthocyanin levels was similar to blackcurrant syrup. At high concentrations, anthocyanins may self-arrange, resulting in reduced hydrolytic attack (Hoshino and Tamura, 1999), which was found to result in color intensification in red raspberry (Melo et al., 2000). The concentration of betalain plays a crucial role in stabilizing betalain during food processing, because betacyanin stability appears to increase with pigment concentration (Mobhammer et al., 2005).

19.8.3 Light

The effect of fluorescent light on the degradation rates of the major cranberry anthocyanins was assayed in model systems in the presence of oxygen in the temperature range 25–55°C (Attoe and von Elbe, 1981). The study revealed that light

degraded most of the anthocyanins at 40°C. Light exhibited a significant effect on anthocyanin degradation in the presence of molecular oxygen. Conversely, light-induced *trans-cis*-isomerization of coumaric acid substituents in anthocyanins offers a way to stabilize color (George et al., 2001). Acerola is a good source of ascorbic acid, carotenoids, as well as cyanidin-3-α-*O*-rhamnoside and pelargonidin-3-α-*O*-rhamnoside. Açai is rich in the anthocyanins cyanidin-3-glucoside and cyanidin-3-rutinoside. The addition of anthocyanic extracts from acerola (*Malpighiae marginata* DC.) and acai (*Euterpe oleracea* Mart.) as a colorant and functional ingredient in isotonic soft drinks and in buffer solution was evaluated. The study revealed that the degradation of anthocyanins from both tropical fruit sources followed first-order kinetics in all the systems under air, either in the presence or absence of light. Light exerted a significantly negative influence on anthocyanin stability in both acai-added systems, isotonic soft drink ($P < 0.001$), and buffer ($P < 0.001$). The degradation rate of açai anthocyanin extract in the buffer system was 7.1 times greater under light than in the dark. Additionally, in the presence of light, the anthocyanin degradation was 1.2 times faster for acerola and 1.6 times faster for açai in soft drink isotonic systems, as compared with their respective buffer solutions. Light was found to affect betalain stability (Cai et al., 1998; Herbach et al., 2006), which can be attributed to betalain absorption of light in the UV and visible range, resulting in the excitation of electrons of the chromophore to a more energetic state, thus bringing about higher reactivity or lowered activation energy of the molecule (Jackman and Smith, 1996).

For lycopene pigments, light effects were more destructive than high-temperature (Nachtigall et al., 2009). The sensitivity of carotenoids to nonsensitized direct light is dependent on the wavelength of irradiation. Under fluorescent light, which means that the involvement of singlet oxygen was ruled out, the higher the unsaturation, the slower the rate of carotenoid autoxidation. This reveals that a higher degree of unsaturation offers a greater protection to β-carotene against autoxidation. Also, it has been reported that the deterioration of the carotene was probably due to absorption of light in the visible region. The photocatalyzed oxidation of β-caroteneis was also more severe in ultraviolet than in visible light (Bonnie and Choo, 1999). Exposure to light, especially direct sunlight or ultraviolet light, induces *trans-cis*-photoisomerization and photo destruction of carotenoids. Thus, work on carotenoids must be performed under subdued light; for example, all the extraction procedures were conducted under dimmed light to avoid isomerization or degradation loss of carotenoids (Chen et al., 2007). As compared with pure carotenoids, carotenoid-arabinogalactan complexes exhibit an enhanced stability toward photodegradation (Polyakov et al., 2010). Open columns and vessels containing carotenoids should be wrapped with aluminum foil, and thin-layer chromatography development tanks should be kept in the dark or covered with dark material or aluminum foil. Polycarbonate shields are available for fluorescent lights, which are notorious for emission of high-energy, short-wavelength radiation (Sajilata et al., 2008).

19.8.4 Temperature

In order to extend the shelf life of herbs and fruits, it is essential to manipulate the metabolism in fruits and vegetables during storage as a postharvest treatment. Ferreres et al. (1997) reported the stability of the anthocyanin pigments of Spanish red onions (cultivar "Morada de Amposta") stored in perforated films for 7 days at 8°C. A small increase in anthocyanins was found after 1 day of storage, followed by a decrease after 7 days of storage, which showed a huge difference in the stability of the individual anthocyanins. The glucosides were more stable than the corresponding arabinosides. The malonated anthocyanins were more stable than the corresponding nonacylated pigments, suggesting that anthocyanin acylation is one of the major structural factors influencing pigment stability (Mazza and Miniati, 1993).

Rodríguez-Saona et al. (1999) have evaluated two acylated pelargonidin-based anthocyanins from red-fleshed potatoes (*Solanum tuberosum*) and red radishes (*Raphanus sativus*) and two extraction methods (C-18 resin and juice processing) during 65 weeks of storage at 25 and 2°C in the dark. It was shown that higher stability was obtained in juices with C-18 purified radish anthocyanins (22-week half-life) and the lowest stability was obtained with potato juice concentrate (10-week half-life). The addition of 10%, 20%, and 40% of sucrose by weight to IQF strawberries prior to freezing displayed a protective effect on the anthocyanin degradation after storage for 3 years (Wrolstad et al., 1990). The effect of cultivars (Chandler, Tudla, and Oso Grande) and storage temperature on the color stability of strawberry jam (Fragaria × ananassa) was investigated (García-Viguera et al., 1999). The effect of storage time, temperature, and light on the degradation of monomeric anthocyanin pigments extracted from skins of grape (*Vitis vinifera* var. Red globe) was evaluated through stepwise regression analysis (Morais et al., 2002). The extracts of pigments dissolved in distilled water containing 0.01% HCl were stored in the air at 24, 32, and 40°C, and analyzed after 1, 3, 6, 8, and 14 days of storage, both in light, using a lamp of 1.5 W, and in the dark. It was concluded that the overall decomposition rate of peonidin-3-glucoside and malvidin-3-glucoside was significantly dependent on storage time and temperature. However, light exerted a negligible impact on the decomposition rate.

A lower storage temperature can extend the shelf life of catechins and for ready-to-drink tea beverages. A low temperature (4°C) and acidic pH (4.0) were found to be the optimal storage conditions for catechin preservation (Bazinet et al., 2010). The addition of butylated hydroxytoluene (BHT) at a level of 0.1% was reported to have a significant effect on the longer stability of catechins, with over 90% EGCG remaining on day 130 stored at 37°C (Demeule et al., 2002). BHT in glycerin was also found to improve the t_{90} (time for 10% degradation to occur) to up to 76 days at 50°C, which offers a potential for glycerin-based vehicles to stabilize EGCG (Proniuk et al., 2002).

19.8.5 Relative Humidity (RH)

To maintain the postharvest quality of herbs, fruits, and vegetables, RH plays a major role. A high RH of 98%–100% is recommended to maintain postharvest quality in broccoli (Rodrigues and Rosa, 1999). The RH appears to be a critical factor in glucosinolate retention when postharvest temperatures rise above approximately 4°C. For example, glucoraphanin content declined by more than 80% in broccoli heads left at low RH and 20°C for 5 days. Similarly, broccoli heads stored in open boxes with low RH at 20°C showed a 50% decrease in glucoraphanin content during the first 3 days of storage, whereas heads stored in plastic bags with a high RH of more than 90% displayed no significant loss at the same temperature. The decrease in glucoraphanin coincided with a marked loss of visual quality (yellowing), indicating a probable loss of membrane integrity and mixing of glucosinolates with myrosinase at low RH (Toivonen and Forney, 2004).

19.8.6 Water Activity (AW)

AW plays a crucial role in the stability profiling of several phytocomponents. Betanin is susceptible to aldimine bond cleavage because of the water-dependent hydrolytic reaction, a reduced mobility of reactants, and limited oxygen solubility. Amaranthus pigment powders showed higher stability than the respective aqueous solutions, this being ascribed to varying AW values (Cai et al., 1998). Additionally, some stabilizers, such as pectin and guar gum, appeared to improve the storage stability of red beet solutions by lowering the AW value. The stability of betacyanins was reported to increase after a reduction of the AW value by spray drying (Cai and Corke, 2000) and by concentration (Castellar et al., 2006).

The study from Lavellia et al. (2007) gives rise to some practical points about the processing and storage conditions required to maintain high carotenoid content in dehydrated carrots. The partial dehydration of carrots to intermediate moisture levels could be proposed instead of removing the water completely, according to the following protocols: (1) reduction of AW values to 0.31–0.54, corresponding to 6%–11% of moisture (on a wet-weight basis)—in this AW range, microbial growth is arrested, enzymatic activity and nonenzymatic browning are at minimum, and our data indicate maximum carotenoid stability and (2) reduction of AW values to 0.54–0.75, corresponding to 11%–22% of moisture—in this AW range, the microbial growth rate and the enzymatic activity are still at a minimum; however, the most effective factors that account for carotenoid stability are still to be investigated. Furthermore, the occurrence of nonenzymatic browning cannot be ruled out. Both criteria should be combined with optimized packaging conditions, which reduce the exposure of the product to air and light during storage.

19.8.7 Atmosphere

In the presence of O_2, both betanidin and betanin were found to be unstable. The stability of betanin was negatively correlated with oxygen concentration (Czapski, 1990), indicating the involvement of O_2 in betanin degradation. Conversely, betanin stability was observed to be improved in an N_2 environment (Drunkler et al., 2006).

19.9 VALIDATION OF ANALYTICAL PROCEDURES

Generally, a validation of the analytical procedure is required by the four most common types of analytical procedures:

- Identification tests
  By comparing the properties of the sample to that of the reference standard, an identification validation test can be performed. These tests are performed to confirm the identity of the analyte in the sample.
- Quantitative tests for the content of impurities
  To ensure the purity of the sample, a test for impurity is performed with the help of a quantitative test or limit test.
- Limit tests for the control of impurities.

- Quantitative tests of the active moiety in samples of the drug substance or drug product or other selected component(s) in the drug product.

However, there are many other analytical procedures, such as dissolution testing and particle size determination, that have to be validated as these are also equally important analytical procedures.

For some considerable time, acronyms, such as good laboratory practice (GLP), good manufacturing practices (GMP), and standard operating procedure, and terms, such as accreditation, auditing, and certification, have introduced a degree of uncertainty into the use of concepts of quality. However, there is no question as far as the analyst is concerned: "The method compiled should do what it is intended that it shall do." It is necessary to document and demonstrate the quality of the working instructions. This involves nothing more or less than determining a range of parameters and demonstrating their reliability by means of statistical methods.

19.9.1 Identity Testing

In order to be certain in, for example, thin-layer chromatography, that a certain band corresponds to a defined reference substance, the migration distances in chromatography are compared with each other. This comparison must be carried out under defined working conditions. The quality of modern HPTLC plates ensures that a positive result probably means that the two substances are identical. It is best to apply the sample and reference substances as bands. If matrix effects occur, identity can be confirmed by overlapping the application of the bands. In addition, there are sufficient microchemical derivatization methods (the use of reagents) and biological–physiological methods for determining the effectivity profile to allow further confirmation of the identity.

19.9.2 Specificity

Specificity can be defined as the ability to reach the desired component explicitly in the presence of other components that are estimated to be present in the sample. Normally, these might include impurities, degradants, matrices, and others (Anonymous, 2005).

A chromatographic method can only determine an active substance specifically if the chromatographic system selectively separates the active substance from impurities, degradation products and excipients. The separation should be confirmed with suitable chromatograms.

A further confirmation is provided by direct spectrometric data yielded from the chromatogram. It is possible to carry out in situ measurements of UV/vis, FTIR, Raman, and mass spectra (FAB, SIMS, LD) (Mukherjee, 2002).

19.9.3 Linearity

The ability of an analytical procedure to acquire test results, which are directly proportional to the concentration (amount) of analyte in the sample, is known as linearity of the analytical procedure (Anonymous, 2005). Ideally, there should be a linear calibration curve that passes through the origin when tested graphically or by regression analysis (test for linearity according to the curve fitting test of Mandel). This ideal is best approached for the measurement of fluorescence when small quantities are applied and in the absence of constant systematic errors. Absorption measurements always yield second-degree functions. It is therefore necessary to check that a limitation of the measurement range allows the two-standard method to yield exact results (Mukherjee, 2002).

19.9.4 Precision

The precision of an analytical procedure depicts the nearness of the degree of scatter between the series of measurements. Under prescribed conditions, these measurements are obtained from multiple sampling of the same homogeneous sample (Q2-R1 guideline). The coefficient of variation of the method shows the precision of an analytical method. The measurement of the repeatability of a determination method is depicted by precision. There should be discrimination between the precision on replication (=repeatability) and the precision on comparison (=reproducibility). Generally, precision is reflected at three levels:

a) Repeatability

When the same individual repeats the analysis several times in immediate succession using same apparatus and sample, then it is identified as replication of the method.

b) Intermediate precision

Intermediate precision is observed when the same sample is analyzed in the same laboratory but on different days using a different apparatus.

c) Reproducibility

When the analysis is performed on the same sample, but in different laboratories with a different apparatus, then it is known as reproducibility of the method. The better the different results are clustered together, the higher is the precision and the narrower is the confidence range, which can be calculated using the Student's *t*-test (Mukherjee, 2002).

19.9.5 Trueness

The systematic errors involved in a method determine the trueness of the method. The agreement between the measured analysis value with the "actual" value (=*x* actual) defines trueness. Naturally, the recovery must be expected to be excessive as well as deficient. In order to quantify this, it is necessary to determine the recovery rate (REC) with different substance concentrations added (*x* added).

$$\mathrm{REC} = X\,\text{found} / X\,\text{added}\,100\,(\%)$$

This is a criterion for the evaluation of each analytical method. If a recovery rate of 100% is found on checking the individual steps of the analysis, the method is free from constant and proportional systematic errors. The slope of the recovery function is then 1.

$$X\,\text{found} = af + bf\ x\,\text{added}$$

If there is interference from the matrix components, these can be independent of the concentration of the component being analyzed. This results in a parallel displacement of the calibration curve. One cause for this displacement could be the inclusion of a matrix component. In such a case, the analytical method is not accurate enough. A blank matrix must be available in order to be able to test this. If such a matrix is not available, it is not possible to determine constant systematic errors. A warning to this effect should be entered in the analytical report. If proportional systematic errors are found, then the size of the error increases in proportion to the concentration of the components to be analyzed. These deviations can result from errors of extraction, interference by the fruits, and absorption to glass vessel walls, among others; in such cases the recovery rate is obtained from the slope bf, of the recovery function, where.

$$\mathrm{REC} = bf\,100\,(\%)$$

Hence, the interferences that occur must be recognized and eliminated. If this is not possible, it will be necessary to carry out the analysis in the future by standard addition. The spiking of the solution is best carried out in equal steps with a reference solution that is as concentrated as possible to avoid dilution errors and alterations to the matrix.

19.9.6 Detection and Determination Limits

The lowest amount of analyte present in a sample that can be detected but cannot be quantitated as an extract value is known as the detection limit of an individual analytical procedure (Anonymous, 2005).

The detection limit of an analytical method is of particular interest if substances are to be determined in the trace or ultra-trace range. Here, it is not only necessary to consider the detection limit of the basic analytical method (=ideal detection limit), but also the influence of the matrix. This latter often means that the actual detection limit is 5–10-fold higher. The determination limit (XB) is the lowest concentration of a substance that can be determined with a given analytical precision—reported as the permissible relative confidence range of the analysis result. It is calculated from.

$$\mathrm{CR\,rel.perm.} = \mathrm{CR}\,(\mathrm{XB}) / \mathrm{XB}\,100\,(1\%)$$

where

$$\mathrm{CR\,rel.perm.} = \text{maximum permissible relative confidence range}$$

$$\mathrm{CR}\,(\mathrm{XB}) = \text{confidence range of the concentration } x = \mathrm{XB}$$

$$\mathrm{CR}(\mathrm{XB}) = S\,\mathrm{XB}t$$

Solved for XB, this yields:

$$\mathrm{XB} = \mathrm{CR}(\mathrm{XB}) / \mathrm{CR\ rel.perm.}100(1\%)$$

Hence, the value of the determination limit depends on the largest random error that can be tolerated in reporting the result. Example: when CR rel. perm. = 10%, XB becomes 10. CR (XB), crudely estimated XB = 10 S xo t (Mukherjee, 2002).

19.9.7 Sensitivity

The sensitivity describes the ability of an analysis method to react to changes in the substances (e.g., concentrations). It is a measure of the extent to which the signal (=measured value) changes as a function of concentration and is thus the slope b of the calibration curve.

$$Y = a + bx$$

The steeper the calibration curve, the smaller the random errors. Hence, the precision and the sensitivity of the measure are large.

19.9.8 Ruggedness of the Method

Along with the above-mentioned factors, the analytical method should also give details concerning:

- Whether the sample and reference solutions are stable or whether only freshly prepared solutions should be used.
- Role of temperature in chromatography.
- The negative effect of changes in atmospheric humidity on the chromatographic separation (resolution).
- Whether positioning errors on application of sample/reference solutions make themselves evident in the evaluation. If measurements are made after a certain degree of spot optimization or with aliquot evaluation of chromatogram bands with slit-shaped measurement areas, these should not play any role.
- Whether the chromatographed substances in the chromatogram remain stable with time. Here the requirement is whether it is necessary to evaluate immediately or after a certain latency time.

All of this information that characterizes the ruggedness of the analytical method should be checked regularly. The availability of such information is of great value for routine investigations. The discussion above reveals that it is not possible to validate a chromatographic method without statistical analysis. Hence, a large number of individual data points are necessary and these can be collected rapidly, reliably, and economically. Validation of the method provides the necessary information. The process removes uncertainties that have been attributed, for example, to the effects of a plant protection agent or of a pharmaceutically active substance. Hence, quality control and validation serve to increase the reliability of analysis, which is what the analyst is aiming for and what the law correctly demands (Mukherjee, 2002).

Stability testing is an integral part of the drug formulation process and it is employed at every step of manufacturing and development. Accelerated stability testing (at relatively high temperatures and/or humidity) is performed primarily in the early stages, which helps in determination of the effect of long-term storage on product degradation. To determine shelf life and expiration dates of products with long-term shelf storage, less stringent conditions are followed at slightly elevated temperatures. Particularly for herbal drugs, the content uniformity and stability are key concerns as they mostly contain multiple ingredients together with other adjuvants.

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Chapter 20

Phyto-Pharmaceuticals, Nutraceuticals and Their Evaluation

Chapter Outline

20.1 NUTRACEUTICAL AND DIETARY SUPPLEMENTS FROM BOTANICALS

The Father of modern medicine Hippocrates (460–370 BC) said, "Let food be your medicine and medicine be your food." The term "nutraceutical" was introduced during the 20th century; it was coined to illustrate a bridge between nutrition and medicine in which both of them played a role as major contributors to human health and wellbeing (Haller, 2010). Nutraceuticals may be outlined as diet supplements that contain bioactive agents from a food in a concentrated, nonfood matrix and utilized in dosages with the aim of enhancing human health and wellness (Zeisel, 1999). From early times, natural products have been used as distinguished sources of therapeutic agents for the cure and mitigation of diseases in humans and animals (Mukherjee et al., 2015a). There is growing research interest in biologically active compounds exhibiting promising applications in food and nutritional products across different fields, therefore, creating good business and public health development. Nutraceuticals and functional foods have similarities in nature (Mukherjee et al., 2017a,b). However, there is some distinction in the patterns by which they are consumed. Nutraceuticals are consumed like prescription drugs (capsules, pills, tablets, etc.), whereas functional foods are consumed as foods and have many potential health advantages (Coppens et al., 2006).

Nutraceuticals, as defined by the European Nutraceutical Association, are nutritional products that have effects that are relevant to health. However, unlike prescription drugs, they are not artificial substances or chemical compounds developed for specific indications. They are products that contain nutrients (partly in concentrated form) and are designated under a food classification. They are sold over the counter in pharmacies, supermarkets, specialist outlets, and the web. The European Food Safety Authority (EFSA) defines botanicals as food supplements, however, not strictly as nutraceuticals because of their nonfood origin. As outlined by the American Nutraceutical Association, a nutraceutical is any substance that

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is a food or food part that provides medical or health advantages and prevention and treatment of ailments. Such products may be within the classification of isolated nutrients, dietary supplements, specific diets, herbal products, and processed foods, such as cereals, soups, and beverages, to genetically modified designer foods (Gonzalez-Sarrias et al., 2013). Dietary supplements are outlined within the US Drug Supplement Health and Education Act of 1994 as products (other than tobacco) that feature one or more dietary ingredients, such as vitamins, minerals, herbs, amino acids, or any other substance to supplement food by enhancing total dietary intake, metabolite, constituent, extract, or a combination of any of these (Mukherjee et al., 2015a; Proctor and Murphy, 2001).

Products from botanical sources are the mainstay of the food market and numerous types of product have been developed and promoted. They embrace whole foods with health advantages other than basic nutrition, such as grains, beans, pulses, cereals, fruits, spices, vegetables, tea, and coffee. Typical products in this class are rosemary, tea leaf, and garlic extracts (Mukherjee et al., 2017a,b). Many dietary supplements are marketed in several pharmaceutical dosage forms, such as tablets, capsules, powders, and liquids, that are derived from plants and used as primary food sources (Franz et al., 2011). Botanicals have been historically used for the promotion and management of human health problems, for instance, ginkgo, garlic, shrub, coffee tree, wild carrot, *Dillenia indica, Emblica officinalis*, *Moringa oleifera*, and many botanic preparations (raw materials and finished products) have a history of human use either as foods or as different sources (Mukherjee et al., 2015a). Thus, valuation of a botanical preparation should exploit all existing knowledge and should be scientifically validated (Kwak and Jukes, 2001).

Several aspects need to be considered for development and promotion of nutraceutical and dietary supplements. Wild fruits and berries have been consumed as a source of vitamins, minerals, and nutrient supplements and are used in the preparations of energy drinks, antioxidants, multivitamin pills, natural flavors, wine, and color and food ingredients. The market of natural antioxidants is increasing exponentially every year and demand in the Asia Pacific region is much higher compared with other regions. This excessive use of natural antioxidants might be due to the fact that they are clinically proven, effective against cancer, cardiovascular, neurodegenerative and aging-related oxidative problems, and that their possible mechanisms of action have been explored in a number of studies. Wild fruits and berries from Himalaya have been widely consumed and explored for the presence of vitamins, minerals, natural antioxidants, secondary metabolites, and others, and thus are considered a nutraceutically important resource. Among others, the berries of Berberis species has been investigated for polyphenolics, alkaloids, vitamins, minerals, and other secondary metabolites that show nutritional and antioxidant potential. The dried fruit juice of Berberis has been clinically tested and found to be effective against inflamed acne lesions, to activate the immune system, and to help in prevention of scurvy. The fruit extract has been reported for its beneficial effect on the cardiovascular and nervous system and can be used for the treatment of hypertension, tachycardia, epilepsy, and convulsions. Various food products, such as juices, jams, pickles, syrups, and candy, have been prepared and thus have the potential to be used as nutraceutical products. The formulation and development of products based on plants requires deep knowledge of the tissue type and process variables to meet the correct quality and quantity of the products.

20.2 PLANT-MADE PHARMACEUTICALS

Plant made pharmaceuticals (PMPs) are the result of a breakthrough application of biotechnology to plants to enable them to produce therapeutic proteins that could ultimately be used by the medical community to combat different illnesses (Mukherjee et al., 2008). In this process, plants themselves become "factories" that manufacture therapeutic components. These metabolites are then extracted, refined, and used in pharmaceutical production (Mukherjee, 2003). Globally, herbal medicine has been considered as an important alternative to modern allopathic medicine. Although the herbal medicines are very popular in society, only a few medicinal herbs have been scientifically evaluated for their potential in medical treatment. In most countries, herbal drugs are poorly regulated and are often neither registered nor controlled by the health authorities.

Plant-made pharmaceuticals are strictly regulated by the regulatory agencies in the United States and differ from traditional agricultural products. Plant-made pharmaceutical research does not represent a new wave of value-added commodity agriculture. When a pharmaceutical company or sponsor already holds an approved application and wants to substitute a plant-based pharmaceutical ingredient in the drug or biologic, it must file a supplemental application reconfirming the safety and efficacy of the final drug/biologic product. However, as the application is being reviewed and evaluated, the agency must reinspect the facility and production line where the new drug or biologic will be manufactured to ensure that it will be made according to good manufacturing practices (GMPs) (Mukherjee et al., 2008). The entire manufacturing process, from seed banks, field preparation, planting, cultivating, harvesting, storage, transportation, and extraction, to the purification of ingredients to be used in the finished product, must also comply with GMPs. The sponsor also needs to demonstrate that the pharmaceutical ingredient contains no allergens, antinutrients, toxic compounds, heavy metals, or other

materials from the host plant that could adversely affect the safety or effectiveness of the pharmaceuticals. Under NEPA, FDA must consider the potential environmental impact of its decisions. Most drugs and biologics are eligible for exclusion from this rule because their ingredients are manufactured in enclosed facilities. Many plant-made pharmaceuticals likely would be field produced and not necessarily excluded; however, it is likely that FDA would defer to APHIS for environmental reviews of pharmaceutical plants grown outdoors. FDA reviews for NEPA would probably focus on the potential environmental impacts posed by uses after harvest that are not subject to APHIS oversight (Mukherjee et al., 2008).

Phytochemicals refer to various chemical constituents that are bioactive ingredients or phytochemicals identified from food plants and animal sources. Some of these vegetables and fruits are associated with the prevention of certain diseases. Researchers have examined numerous phytochemicals, which, in general, have been manufactured by plants against insects or have other biological functions, antioxidant, or hormone-like activity in plants; there are various beneficial health effects in humans who eat the plants (McGhie et al., 2012). Examples of phytochemicals include phenolics (e.g., flavonoids, phenolic acids, and phenols), nitrogen-containing compounds, alkaloids, terpenoids, and many others (Wang and Song, 2014).

20.3 HEALTH BENEFITS OF NUTRACEUTICALS AND FUNCTIONAL FOODS

Nutraceuticals are biologically active molecules found in foods that may not be essential for maintaining normal human functions, but may enhance human health and wellbeing by inhibiting certain diseases or improving human performance. Numerous classes of nutraceuticals are found in both natural and processed foods, including carotenoids, flavonoids, curcuminoids, phytosterols, and certain fatty acids. Many of these nutraceuticals have the potential to act as therapeutic agents, and may therefore be suitable for incorporation into functional or medical foods as a means of preventing or treating certain types of cancer. Nutraceuticals vary considerably in their chemical structures, physiochemical properties, and biological effects. For example, nutraceuticals vary in their molar mass, structure, polarity, charge, and functional groups, which influence their chemical reactivity, physical state, solubility characteristics, and biological fate and functions. Some nutraceuticals are naturally present in whole foods, such as fruits, vegetables, and cereals, and are therefore often consumed in this form. Conversely, other nutraceuticals are isolated from their natural states and converted into additives that can be incorporated into functional foods, dietary supplements, or pharmaceuticals. Several parameters for the evaluation of nutraceuticals are further explained in Fig. 20.1.

The consumption of food for medication, or "dietetic medical care," has long been a strong belief in Chinese culture over thousands of years. However, the term "functional foods" was introduced in Japan in the 1980s (Ozen et al., 2012), Japanese interest in this field has also brought awareness of the necessity of using foods to Europe and also the United States. As the originator of functional foods, Japan is the sole country that acknowledges functional foods as a definite class in its food regulatory system. Also, the Japanese functional foods business is currently among the most advanced in the world. In Japan, functional foods are referred to as foods with specific uses for health. They are composed of useful ingredients that have benefits, for example, to maintain or regulate specific health conditions, such as gastrointestinal health, cardiovascular health, immunity, and blood cholesterol levels (Arai et al., 2002). In different countries, there is no

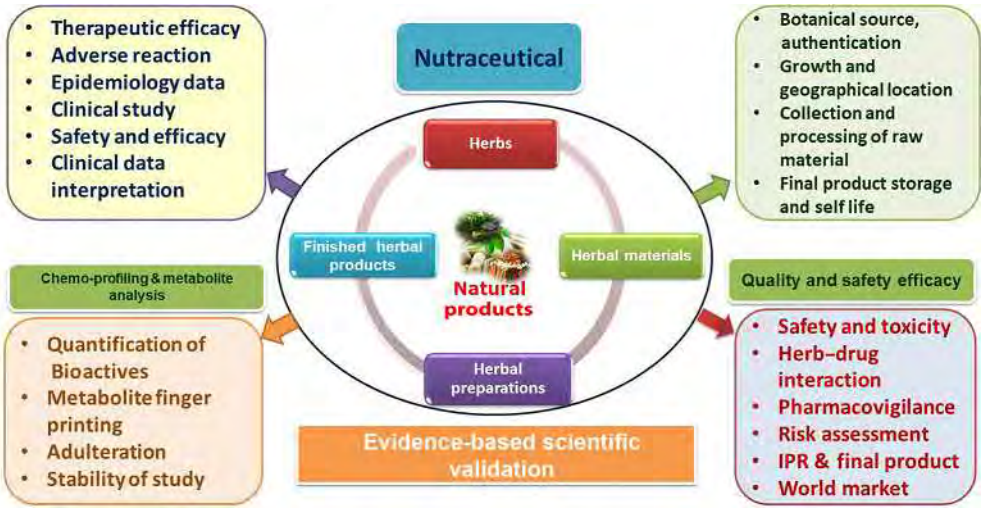

FIG. 20.1 Evaluation of nutraceuticals.

clear boundary between standard foods and functional foods. One argument is that any food is useful and provides nutrients that incorporate a physiological impact. Therefore, functional food is more of a promotional term for a food that involves false claims in its health advantages and also it affects the perception of the product (Mukherjee et al., 2017a,b; Wang and Song, 2014).

Academic institutions and national authorities have also tried to define functional foods. The simplest definition is "foods that provide health benefits beyond basic nutrition" (Ozen et al., 2012), which was approved by the International Food Information Council (IFIC). These substances provide essential nutrients often beyond quantities necessary for normal maintenance, growth and development, and/or other biologically active components that impart health benefits or desirable physiological effects (Hurley et al., 2011). The International Life Sciences Institute (ILSI) Europe and the European Commission's Concerted Action on Functional Food Science in Europe (FuFoSE) defined functional foods as follows: "a food product can only be considered functional if, together with the basic nutritional impact, it has beneficial effects on one or more function of the human organism thus either improving the general physical condition or/and decreasing the risk of the evolution of diseases." FuFoSE also developed a working definition of functional foods as foods that are "satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutrition effects, in a way that is relevant to either an improved state of health and wellbeing and/or reduction of risk of disease" (Ozen et al., 2012). In 1989, Dr. Stephen coined the term "nutraceuticals," which has both key contributions to human wellness from "nutrition" and "pharmaceutical" (Kalra, 2003). Foods and drugs have no absolute distinction as "a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease" (DeFelice, 1993). The simplest definition of nutraceutical is set forth by the Nutritional Improvement Law Enforcement Regulations of Japan as "foods for specified health use (FOSHU)." Nutraceuticals are also defined as naturally occurring bioactive compounds with health benefits (Wang and Song, 2014). Health Canada officially defined nutraceuticals as "a product isolated or purified from food, generally sold in medicinal forms not usually associated with food and demonstrated to have a physiological benefit or provide protection against chronic disease" (Hasler and Brown, 2009). There are various well-known bioactive compounds and minerals that are ideal examples to illustrate the potential health benefits of phytochemicals; they are listed in Table 20.1. However, some are widely used and are discussed in preceding sections.

The array of nutraceuticals was expanded from food plants to incorporate bioactive compounds from nonfood sources. Nutraceuticals may be given in one kind or in a combination of many forms, which is the most evident distinction between nutraceuticals and prescribed drugs that are used as medication or synthesized or refined pure chemicals. A universally accepted definition of functional foods and nutraceuticals is lacking. Some specialists insist that functional foods and nutraceuticals connect totally different ideas; however, these terms cannot be distinguished in most cases. A functional food for one will act as a nutraceutical for another (Kalra, 2003). Nutraceuticals are natural foods or foods that are changed to have a useful influence on the health and wellbeing of the consumer through the addition, removal, or modification of specific parts. The terms functional foods and nutraceuticals should not imply that there are wholesome foods or unhealthy foods but that all should be included in a healthy diet; however, safety is the major concern of functional foods (Wang and Song, 2014).

20.4 DEVELOPMENTAL FACTORS OF BOTANICALS AS NUTRACEUTICALS

There are many factors involved in the development of nutraceuticals and dietary supplements. There are many vital aspects that ought to be considered for their international acceptance, as well as efficaciousness, safety and stability (McAlindon, 2006). Some issues connected to the assembly and consumption of nutraceuticals are that the composition and contents of active constituents in natural plants vary based on the season, climate, temperature, humidity, soil, and several other factors. Therefore, the assortment, identification and maintenance of uniform quality, standardization, substance identification, efficacy, safety, bioavailability, stability, and restrictive problems are important factors to be considered for greater understanding of the health-promoting effects of nutraceuticals (Proctor and Murphy, 2001). Nutraceuticals should initially be present in functional foods at a sufficiently high level to have a beneficial physiological effect (McClements and Xiao, 2017).

- The nutraceuticals should remain stable within functional foods during manufacturing, storage, and utilization, otherwise they may lose their beneficial health effects.
- The nutraceuticals should not have an adverse effect on the color, taste, or shelf life of a food product.
- After ingestion, the nutraceuticals should be released from functional foods and delivered to the appropriate site-of-action within the human body.

The safety of these nutraceuticals is a major concern. In the United States, the Food and Drug Administration (FDA) has estimated that over 50,000 adverse events are caused by botanical and other dietary supplements. In addition, for most herbal drugs, the efficacy is not proved and the quality is not assured. The Traditional Medicine (TM) Strategy

TABLE 20.1 Bioactive Components Used as Nutraceuticals

Amino acids, peptides, proteins and their derivatives	
L-Carnitine	Weight loss, Alzheimer's disease prevention and treatment, improvement in blood cell count
Taurine	Betterment of cardiac and cognitive function
Casein phosphopeptides	Enhanced immunomodulatory activity, mineral solubility and absorption
Collagen	Pain relief and improvement of joint function in patients with osteoarthritis, rheumatoid arthritis, promotion of skin health
Lactoferrin	Immune system augmentation
Lipids and fatty acids	
Lecithin	Cognitive impairment and dementia prevention, cholesterol is decreased
Phosphatidylserine	Improvement in age-related cognitive impairment, Alzheimer's disease
DHA	Normal brain function, Alzheimer's disease age-related macular degeneration
CLA	Colorectal cancer and obesity prevention
α-Linolenic acid	Blood cholesterol levels are maintained
γ-Linolenic acid	Cardiovascular disease prevention
Carbohydrates, fiber, prebiotics	
Many types of dietary fiber	Prevention, obesity, prevention of cardiovascular disease, diabetes, colorectal cancer prevention, reduction in Blood cholesterol
Fructooligosaccharide	Improvement in gastrointestinal function
Pectins	Improvement in gastrointestinal function, reduction in blood cholesterol blood glucose after meals, prostate cancer prevention
Lactulose	Enhancement in intestinal health
L-Arabinose	Absorption of sucrose Inhibition, improvement in gastrointestinal function to prevent obesity and diabetes
β-Glucan	Contribution to the maintenance of normal cholesterol levels, immune function enhancement

2014–2023 of the World Health Organization (WHO) focuses on promoting the safety, efficacy, and quality of TM by expanding the knowledge base and providing guidance on regulatory and quality assurance standards. Herbal medicine products include herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants, other plant materials, or combinations thereof as active ingredients. Herbs include crude plant material, for example, leaves, flowers, fruits, seeds, and stems. Herbal materials include, in addition to herbs, fresh juices, gums, fixed oils, essential oils, resins, and dry powders of herbs. Herbal preparations are the basis for finished herbal products and may include comminuted or powdered herbal materials, or extracts, tinctures, and fatty oils of herbal materials. Finished herbal products consist of herbal preparations made from one or more herbs. The regulatory scenario regarding herbal preparations varies from country to country (World Health Organization, 2013). Globally, several diverse regulatory approaches are there, such as:

- Same regulatory requirements for all products, with certain types of evidence not required for herbal medicines;
- Exemption from all regulatory requirements for herbal medicines concerning registration or marketing authorization;
- Herbal medicines subject to regulatory requirements concerning registration or marketing authorization.

In Europe, for marketing approval, herbal preparations are classified into three categories as follows:

- Provisions for traditional medicinal use (traditional use) are accepted on the basis of sufficient safety data and plausible efficacy

- Well-established medicinal use provisions (well-established use) demonstrated with the provision of scientific literature establishing that the active substances of the medicinal products have been in well-established medicinal use within the European Union for at least 10 years, with recognized efficacy and an acceptable level of safety.
- Safety and efficacy data from the company's own development (stand alone) or a combination of own studies and bibliographic data (mixed application).

FDA Botanical Drug Development Guidance describes proper development strategies for botanical drugs to be approved in new drug applications (NDAs) and specific endorsements on submitting investigational new drug applications (INDs). The term botanical means products that include plant materials, algae, macroscopic fungi, and combinations thereof. FDA guidance recommends that INDs must contain sufficient information to demonstrate (FDA, 2007). The issues related to the appropriateness of conventional biomedical and clinical models for evaluating the efficacy of traditional medicines remain very critical. A holistic approach based on systems biology should be suited to study the therapeutic efficacy and pharmacodynamics of traditional medicine-based drug development (Verpoorte, 2005). It is also argued that instead of the randomized controlled trials normally used as the gold standard in routine biomedical research, strategies of pragmatic or management clinical trials may be better suited for traditional medicine–inspired reverse pharmacology approaches (Fønnebø et al., 2007).

20.5 CLASSIFICATION OF NUTRACEUTICALS

Nutraceuticals are classified in various ways. Because nutraceuticals target specific health fields or populations, nutraceuticals are often categorized based on the targeted population or their health benefits (disease prevention). Nutraceuticals can also be categorized based on the contents or food types:

(i) *Herbal or edible plant substances*: herbal extracts and/or concentrates.
(ii) *Nutrients*: Includes substances with certain physiological functions, such as vitamins, minerals, fatty acids, amino acids, and certain ingredients from plant-based substances (Wang and Song, 2014).
(iii) *Functional diet*: The ingredients may contain vitamins, minerals, amino acids, enzymes, botanicals, or other dietary supplements.

All functional foods or nutraceuticals can be supplied to consumers in different dosage forms (e.g., powders, tablets, liquids, capsules, extracts, and concentrates). However, there are several other classifications of nutraceuticals, which are shown in Table 20.2 (Ghosh et al., 2015).

20.5.1 Bioactive Compounds and Phytochemicals

Several scientific studies, including several projects funded by scientific authorities, have led to the identification and understanding of the mechanisms of biologically active components in foods, which may improve health and possibly reduce the risk of disease while enhancing overall wellbeing. Almost all ingredients, ranging from macronutrients (e.g., proteins, fats, and carbohydrates) to micronutrients (e.g., vitamins and minerals) have specific physiological functions, and some are consumed as functional foods and nutraceuticals. The most commonly studied health benefits of phytochemicals are the prevention of cardiovascular disease and cancer as well as immunological effects, which are associated with their high antioxidant activity. However, some specific phytochemicals may have other physiological functions beyond their antioxidant activity.

20.5.1.1 Curcumin

Curcumin is an active biomarker of turmeric (*Curcuma longa* Fam: Zingiberaceae). Its endogenous antioxidant defense mechanisms have been investigated for many years, and it has been suggested to have an antiinflammatory activity. Appropriate intake of curcumin has a potential role in decreasing the incidence of obesity and its associated risk factors (Shehzad et al., 2011). The ability of curcumin to delay the onset of cancer has also been the topic of extensive research for many years, especially for colorectal and skin cancers. Curcumin has also been shown to facilitate diabetes prevention through glycemic control, which further supports its role in cardiovascular disorders. Biochemical analyses and clinical studies have demonstrated that curcumin promotes human health in various ways, including regulation of lipid metabolism, as well as through its antiinflammatory and antioxidant properties. Additionally, it has been shown that oral curcumin can relieve symptoms of dyspepsia, osteoarthritis, Alzheimer's disease (AD), and rheumatoid arthritis.

TABLE 20.2 Classification of Nutraceuticals and Their Health Benefits

Health Fields	Examples of Nutraceuticals Used
Child growth/early development and growth	*Cognition, sensory: PUFAs, iron, zinc, iodine. Growth and body composition: essential amino acids, unsaturated fatty acids; Skeletal development: calcium, vitamins D and K2; Gastrointestinal health: prebiotics, probiotics; Immune function: vitamins A and D, antioxidant vitamins, trace elements, L-arginine, nucleotides, probiotics, prebiotics, neutral and acidic oligosaccharides*
Pregnancy	*PUFAs, certain amino acids, folic acid, iron, zinc, iodine*
Gastrointestinal health	*Synbiotics, (mixtures of pre- and probiotics), other non- and poorly digestible carbohydrates*
Mental health and performance	*B vitamins, n-3 PUFAs, S-adenosylmethionine, phytochemicals, plant extracts, Glucose and sugar-derived products*
Physical performance	*Caffeine, specific amino acids, creatine, and carnitine*
Cancer prevention	*Colon cancer: prebiotics, dietary fiber, calcium, selenium, folate, low-fat dairy, phytochemicals* (e.g., *carotenoids, curcumin, polyphenolic compounds*), *some plant extracts; Breast cancer: folate, vitamin D, isoflavones, α-linolenic acid, phytochemicals* (e.g., *lignan, resveratrol*), *some plant extracts* (e.g., *green tea, pomegranate*); *Prostate cancer: folate, selenium, isoflavones, some plant extracts* (e.g., *green and black tea*)
Alzheimer's disease prevention	*Antioxidant vitamins, phytochemicals, DHA*
Energy balance/body weight management/obesity prevention	*Conjugated linoleic acid, dietary fiber, polyols, medium-chain triglycerides and other poorly digestible carbohydrates, chitosan, diglycerides some plant extracts, replacements, green tea, phytochemicals, caffeine, calcium, capsaicin foods with low glycemic index or glycemic response fat and sugar*
Diabetes prevention	*Lipoic acid, soluble dietary fiber, chromium*
Cardiovascular disease prevention	*Vitamins, minerals, anthocyanins, proanthocyanidins, PUFAs, dietary fiber*
Bone protection/musculoskeletal disease prevention	*Soy protein, conjugated linoleic acid, glucosamine, S-adenosylmethionine, chondroitin, collagen hydrolysate, methylsulfonylmethane, soybean unsaponifiables, fructooligosaccharides, inulin, vitamins* (*D, K, and C*), *minerals* (e.g., *calcium, manganese, copper, zinc*)

Based on Contents

Category	Examples of Nutraceuticals
Herbs/botanical extracts	Strawberry, grape, mushroom, tomato, chia, cinnamon, Ginseng, garlic, onion, echinacea, ginger, licorice, *Ginkgo biloba*, pomegranate, broccoli, valerian, chamomile, St John's wort
Functional diet	Functional eggs, minimally refined grains red wine, functional dairy foods, functional drinks
Nutrients	Peptides, dietary fibers, functional carbohydrates, proteins, amino acids, vitamins, minerals, fatty acids, phytochemicals extracted from plants

20.5.1.2 Anthocyanins

Anthocyanins are flavonoids that exist in various fruits and vegetables. Anthocyanins are widely known as nutraceuticals and are a group of soluble vacuolar pigments, which are red, purple, or blue in color based on the pH of the micro environment. Over 600 anthocyanins are known to be present in natural foods. Human consumption of anthocyanins is among the very highest of all flavonoids. The toxicity of dietary anthocyanins is low. Anthocyanins possess medication and anticarcinogenic activity and are ameliorative in CVD and neurodegenerative disorders. They are also useful in weight management and polygenic disorders. All of these effects are more-or-less related to the antioxidant properties of anthocyanins; enzyme inhibition and different pathways might also be relevant. For the prevention of metabolic disorders, mechanistic studies support the helpful effects of anthocyanins on the established biomarkers of CVD risk. Anthocyanins could reduce glucose levels by improved insulin resistance, protective β cells, increasing the secretion of insulin, and reducing the digestion of sugars within the intestine. The metabolism, absorption, and bioavailability of anthocyanins as nutraceuticals have been examined over the past decade; but, more study is needed to work out the anthocyanins that are needed to realize "optimal" human health (Wang and Song, 2014).

20.6 NUTRACEUTICAL AND DIETARY SUPPLEMENTS FOR OBESITY MANAGEMENT

Obesity management through diet could also be accomplished by the bioactive constituents of food supplements, which might modulate the molecular pathways and gene/protein expressions related to the metabolism of an obese individual along with diet management and physical activity. The most acclaimed ways for weight management by functional food ingredients are: inhibition of food intake (by inhibiting anorectic signals or enhancing anorectic signals), limiting the bioavailability of nutrients (by suppressing the organic process enzymes and/or interacting with them to physically stop their absorption), stimulation of energy expenditure (thermogenesis), and modifying the composition of the gut microbiota. The precise roles of gut microbiota are in modulating metabolic energy storage by increasing the capability to reap energy from the diet and in modulating the plasma lipopolysaccharide levels that activate the inflammatory pathways and, therefore, the onset of obesity and type 2 diabetes. The best and most useful option for overweight and obese individuals is calorie restriction and exercise. Most research on food has shown its ability to modulate some specific physiological functions in the organism through food intake. Different natural products have significant weight-reducing effects (Mukherjee et al., 2015b).

20.6.1 Berries

The word berry is employed for several sorts of tiny fruits that bear many seeds and may be used as food. Some examples are raspberry, blueberry, and lingonberry. There are totally different species of berries that contains different types of ingredients, most often polyphenols. Acai berries (*Euterpe oleracea* Mart.) are referred to as "super food" with antiaging and weight-loss properties. This fruit is small and purple in color. The fruit pulp is full of antioxidant. It reduces glucose level, hypoglycemic agent levels, total sterol, and LDL-cholesterol in healthy overweight adults (Udani et al., 2011; Mukherjee et al., 2015b).

20.6.2 Blackberry (Family: Rosaceae)

The blackberry is an edible berry of several species within the Rubus genus within the family Rosaceae. It contains an anthocyanin compound (C3G) that has powerful antioxidative and antiinflammatory activity. A literature review shows that consumption of C3G-rich blackberries is effective in reducing weight gain and inflammation related to ovariectomy-iatrogenic menopause in a rat model. A treatment consisting of a diet containing 100% blackberry (w/w) for 100 days normalized NF-kB and Cox expression levels in vivo (Kaume et al., 2012; Mukherjee et al., 2015b).

20.6.3 Indian Gooseberry (Family: Phyllanthaceae)

Indian gooseberry (amla) has been historically employed in Ayurvedic herbal preparations or rejuvenating medications. In the HFD mice model, it considerably suppressed weight gain. Amla normalized the fatty RNA expression of nuclear transcription factor, PPARg. Its liquid extract inhibited lipoid accumulation in 3T3-L1 mouse adipocytes treated throughout differentiation (Mukherjee et al., 2015b).

Peng and coauthors have investigated the potential of mulberry in fat management. Mulberry water extracts (MWE) contain polyphenolic elements, such as acid, chlorogenic acid, rutin, and anthocyanins, which, by reducing humor triacylglycerol, sterol, free carboxylic acid, and LDL/HDL level, might be responsible for the hypolipidemic action in 6-week-old male hamsters. MWE protects livers from impairment by decreasing internal organ lipids through regulation of lipogenesis and lipolysis (Mukherjee et al., 2015b).

20.6.4 *Rubus idaeus* (Family: Rosaceae)

Rubus idaeus are berries that are effective in obesity management. The main compound that decreases the internal organ triacylglycerol content in HFD-induced mice is 4-(4-hydroxyphenyl) butan 2-one (RK). It translocates lipase from the cytoplasm to lipoid droplets in rat epididymal fat cells, thereby considerably increasing norepinephrine-induced lipolysis. Specifically, RK alters the lipoid metabolism and will increase norepinephrine-induced lipolysis in white adipocytes. In these ways, RK prevents elevations in HFD-induced weight and therefore the weights of the liver and visceral fatty tissues (Morimoto et al., 2005; Mukherjee et al., 2015b).

20.6.5 *Solanum lycopersicum* (Family: Solanaceae)

The *Solanum lycopersicum* is an edible red fruit/berry from the Solanaceae family. An investigation of high-fat-diet-induced C57BL/6 mice showed that its extract attenuates fat, which can be related to activation of the AMPK pathway (Choi et al., 2014).

In another investigation, the result of phytochemicals containing tomato vinegar (TV) has been evaluated in vitro and in vivo. In HFD-induced rats, TV suppressed adipocyte differentiation of 3T3-L1 preadipocyte and lipid accumulation and differentiation (Lee et al., 2013; Mukherjee et al., 2015b). Supplementation with tomato vinegar markedly weakened visceral fat weights, triglycerides, and other sterol levels. Moreover, plasma LDL-cholesterol and the atherogenic index was reduced. It conjointly elevated HDL-cholesterol to total sterol ratio. Thus, this study found that TV may be used as an antiobesity agent (Lee et al., 2013; Mukherjee et al., 2015b).

20.6.6 Capsicum

Capsicum is the fruit of various species of capsicum plants. *Capsicum lycopersicum plant* is additionally referred to as red pepper or chili pepper or bell pepper. Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the commonest capsaicinoid molecule. Others, such as capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin, are also present. The antiobesity effects of water extracts of *Capsicum annuum* L. were examined through the analysis of lipoprotein lipase (LPL) RNA expression level in mouse preadipocytes. In another study of the antiadipogenic effects of *C. annuum* L., seeds in 3T3-L1 adipocytes cells were examined (Udani et al., 2011). From the experimental outcome, a decrease in the expression of LPL RNA level and adipogenic transcription factors C/EBPb, C/EBPa, and PPARg was discovered; this might be a potential mechanism of the antiobesity activity of the capsicum pepper plant. Many studies indicate capsicum as a possible antiobesity food (Mukherjee et al., 2015b).The antiobesity effects of water extracts of *C. annuum* L. were examined through an analysis of compound protein enzyme (LPL) RNA expression level in mouse preadipocytes. In another study of antiadipogenic result of *C. annuum* L. seeds in 3T3-L1 adipocytes cells were examined (Udani et al., 2011). From the experimental outcome, it was observed that there was a decrease in adipogenic transcription factors C/EBPb, C/EBPa, and PPARg. Red chili pepper consumption may cause increased satiation, thus reducing energy and fat intake (Westerterp-Plantenga et al., 2005). Another potential mechanism of the antiobesity effects of capsaicin is thermogenesis caused by primary sensory neurons of the "pain" pathway to stimulate the transient receptor potential of the vanilloid receptor (Caterina et al., 2000). Capsaicin can even increase endocrine (epinephrine, catecholamine (NE), and dopamine) secretion from sympathetic systems and, as a result, will increase metabolism and thermogenesis (Watanabe et al., 1988). Clinical studies have shown that diet-elicited thermogenesis has been amplified by capsaicin in the diet and has been shown to extend energy expenditure by negative energy balance and increases fat oxidization (Janssens et al., 2013; Mukherjee et al., 2015b).

20.7 NUTRACEUTICAL AND DIETARY SUPPLEMENTS FOR GERIATRICS

Geriatric medicines deal with the prevention and treatment of diseases and disabilities in older adults. The development of effective dietary interventions for promoting healthy aging is a difficult area of research. Medicine may be a practice that addresses the complicated requirements of older patients and emphasizes maintaining practical independence even in the presence of chronic disease. The treatment of geriatric patients requires a unique strategy and it is extremely complicated, with the risk of chronic disease, disability, and death. An aging population is a worldwide development. The most widespread conditions affecting older people are cardiovascular disease, the symptoms of a heart condition, dementia, pathology, respiration issues, cataracts, and polygenic diseases, to name some. Decreased immunity is also partly responsible for the increased morbidity and mortality resulting from infectious agents in the aged. Biological process condition is one of the chief variables that explains variations in the incidence and pathology of infection. Aged people are at increased risk for substance deficiencies thanks to a range of things, together with social, physical, economic, and emotional obstacles to consumption. Therefore, there is a secondary imperative to shift priorities to extend our attention to ways in which we can stop chronic sicknesses related to aging. Separately, people should place increased efforts into establishing healthy habits, together with consuming a more healthful diet.

20.7.1 Antioxidants

A distinguished theory of aging and chronic illness has been that it is the extended accumulation of cellular injury as a result of free radicals that leads to an increased risk of illness. In living cells, a pair of enzymes are major defense systems against free radical injury. The first line of defense includes antioxidant enzymes (such as SOD, catalase, and glutathione peroxidase) and so the second line includes mass protein antioxidants (thioredoxin, glutathione, vitamins A, C, E, lycopene, lutein, polyphenols, quercetin, etc.). It is thought, therefore, that diets rich in antioxidants, and lots of bioactive polyphenol compounds found in fruits and vegetables, will combat free radical injury and improve health. This theory has a durable association with better health outcomes, and can have positive effects on cancer, metabolic disorder, and neurodegenerative diseases (Gupta and Prakash, 2015).

20.7.1.1 Plant Polyphenols and Catechins

Polyphenols are secondary metabolites of plants and are usually involved in the defense against ultraviolet light or aggression by pathogens. They are found in fruits, vegetables, legumes, cereals, and beverages. Legumes and chocolate conjointly contribute to polyphenolic intake. The main sources of dietary polyphenols are cereals, legumes (barley, corn, nuts, oats, rice, sorghum, wheat, beans, and pulses), oilseeds (rapeseed, canola, flaxseed, and olive seeds), fruits, vegetables, and beverages (fruit juices, tea, coffee, cocoa, beer, and wine). Fruits, such as apple, grape, pear, cherry, and numerous berries, contain up to 200–300 mg/100 g polyphenols. Tea leaf polyphenols have antiinflammatory and anticancer advantages due to the presence of epigallocatechin-3-gallate (EGCG). Grape seed extract (GSE) may be a targeted supply of polyphenols. Though clinical analysis on GSE for inflammation and cancer is not as advanced as that for the curcumins and tea leaf catechins, there is plentiful animal and in vitro evidence suggesting that GSE also has effectiveness in applications geared toward protection against oxidative stress aiding circulation, in addition to its general antiinflammatory and antitumor effects.

20.7.1.2 Carotenoids: Xanthophyll, Zeaxanthin, and Carotenoid

A human diet supplemented with carotenoids is useful in reducing the chronic conditions associated with coronary heart diseases. β-Carotene and carotenoid have been shown to be reciprocally associated with the danger of CVD diseases and cancers, whereas xanthophyll and carotenoid are associated with disorders associated with the mind. Xanthophylls protect against female internal reproductive organ, prostate, breast, colorectal, and respiratory organ cancers. They conjointly defend against the risk of cancer. Dietary carotenoids are present in pigmented fruits and vegetables having yellow, orange, and red colors.

20.7.2 Plant Sterols (Phytosterols)

Phytosterols are added to functional foods to boost their cholesterol-lowering ability. These compounds help in the inhibition of cholesterol absorption. They are currently widely used as food supplements to assist in lowering blood cholesterol and managing cardiovascular disorders in older patients with dyslipidemia (Gupta and Prakash, 2015). Phytosterols are mostly found in vegetables, nuts, fruits, and seeds. Sitosterol, campesterol, and stigmasterol are most extensive in nature. The first phytosterols in the diet are sitosterol, stigmasterol, and campesterol and typical consumption of plant sterols is roughly 160–400 mg/day.

20.7.3 Vitamin-B

The importance of the vitamins B, folic acid, vitamin B-12, and vitamin B-6 lies in wellbeing and a healthy brain. The B-group vitamins are usually inadequate in the aged and this is associated with the loss of psychological wellness, such as Alzheimer's. The inadequacies may produce impairment of methylation reactions that are crucial to the health of brain tissue. Additionally, these inadequacies may end in hyperhomocysteinemia. Vitamin B6, cyanocobalamin, and folacin are three necessary B-vitamins that are involved in metabolic cycles that supply the body with alkyl groups (1-carbon metabolites) that are necessary for several functions within the body, including homocysteine metabolism, a possible risk factor for cardiovascular disease.

20.7.4 Calcium and Vitamin D

The role of calcium and vitamin D has been related to its necessary function in bone metabolism and also the prevention of pathology. A study found that vitamin D and calcium will cut back the speed of mortality in seniors, thereby providing an increased lifespan. High-calcium diets have been shown to have some efficacy in reducing the danger of carcinoma and also the return of colonic polyps, whereas vitamin D has been involved in a variety of diseases together with polygenic disease and numerous cancers (Gupta and Prakash, 2015).

20.7.5 Omega-3-Fatty Acid

Omega-3 fatty acids are found in animal oils and in some plants, such as oilseed. N-3 fatty acids are renowned for having antiinflammatory drug impacts and for lowering blood triglycerides and have also been found to have a positive effect in patients suffering from cardiac illness (heart attack) or heart conditions (Gupta and Prakash, 2015).

20.7.6 Glucosamine, Chondroitin, Collagen

Glucosamine and chondroitin are a part of animal tissue. Animal tissue acts as a cushion between the bones in a joint. Animal tissue consists of a sort of scleroprotein. Oral consumption of these building blocks of animal tissue is believed to be useful in reducing pain and protecting bone animal tissue. It is found in several forms, together with glucosamine coordination compound, *N*-acetyl-glucosamine, and glucosamine salt, which may be a combination of glucosamine and mineral salt (Gupta and Prakash, 2015).

20.7.7 Dietary Fiber

Dietary fiber is classified into either soluble fiber or insoluble fiber. These types of fiber have completely different effects metabolically due to their different chemical properties. Dietary fiber intake is very important from a metabolic viewpoint (lipid and glucose metabolism), acting as prebiotics on microbiota health, in preventing colonic cancer, in treating intestinal diseases and symptoms, and on mineral absorption. Insoluble dietary fiber is not water soluble and is comparatively flatulent, tending to extend the dry matter content of the stool and aiding in the prevention of constipation (Gupta and Prakash, 2015).

20.7.8 Prebiotics and Probiotics

Prebiotics and probiotics have useful effects with relation to immunity, intestinal barrier function, and enhanced resistance to illness. The gut membrane and microbiota are intimately coupled in the maintenance of a health interface between the host and external threats. The combined effect of prebiotics and probiotics (synbiotics) has a synergistic effect in enhancing immunity and facilitating intestinal barrier function (Gupta and Prakash, 2015). They are also helpful in malnutrition, lactose intolerance, calcium intake, and dysbiosis by overuse of antibiotics.

20.7.9 Whey Protein

Whey is a crucial protein constituent of milk that has health promoting properties. Whey proteins are considered to possess the very highest biological process values of all food proteins. They contain all the amino acids needed by humans, and in the right proportions. The health and biological benefits of whey include: biological supply of amino acids; antimicrobial action; growth improvement of useful gut microflora (e.g., immunoboosting properties); bifidobacteria; and the management of different diseases (Gupta and Prakash, 2015).

20.7.10 Zinc

Zinc is a vital chemical element that is biochemically involved in a wide range of reactions and has important effects on DNA synthesis, cell proliferation, and differentiation. The metallic element zinc is important for the system, and zinc deficiency affects multiple aspects of innate and adaptive immunity. Immunity is compromised in zinc deficiency disease and its supplementation in conjunction with antioxidants is prescribed in elderly patients. In elderly patients, zinc supplementation is done to maintain blood zinc concentrations and to facilitate reducing respiratory illness and associated morbidity. Consequently, oral zinc supplementation demonstrates the potential to enhance immunity and reduce chronic inflammatory responses (Gupta and Prakash, 2015).

20.7.11 Rasayana Therapy in Ayurveda

Rasayana therapy is one of the most important components in Ayurveda, which is primarily promotive, and is essentially meant to rejuvenate the body and mind to impart longevity and immunity against disease. The herbs and foods mentioned in this context are perceived as having adaptogenic, antiaging, and antistress properties, as well as being immunomodulators and memory enhancers. In Ayurveda, Rasayana refers to acquisition, movement, or circulation of the nutrition needed for body tissues (known as Dhatus in Ayurveda) (Debnath et al., 2015). Foods, such as Shatavari (*Asparagus racemosa*), milk, and ghee, help in this process. The second approach is to improve the metabolic process (referred as "Agnivyapara"), which increases the anabolic effect, thereby improving the overall health of the human body. Bhallataka (*Semecarpus anacardium*) is an example that acts at the metabolomics level to promote health.

Another way of attaining rejuvenation is to boost the circulation by promoting competent flow of nutrients through the channels (known as "Srotas") of the body. This may help in better bioavailability of the nutrients all over the body by improving health and desired benefits. There is a general idea that Rasayana is for geriatrics, but it is actually a science of

nutrition that encompasses applications for all ages to augment vitality. Ayurveda lists a separate class of immune-modulatory botanicals named Rasayanas. Several botanicals from these texts have been studied for their immune-modulatory properties and have the potential to provide new scaffolds for safer, synergistic, cocktail-immune drugs. Due to their favorable pharmacokinetic properties, they are considered as adjuvants and are also therapeutically superior to other drugs; although an Ayurvedic Rasayana drug shows less CYP450 inhibition, yet it is highly recommended in Kapha disorders (asthma, respiratory problems) (Mukherjee et al., 2017a,b). Different Rasayana herbs and food supplements are used nowadays, for example, Chyawanprasha and Brahmi Rasayana are quite effective for all age groups (Debnath et al., 2015).

20.8 NUTRACEUTICALS USED FOR CARDIOVASCULAR DISEASES

Although most of the cardiovascular disorders typically affect older adults, the process begins in adolescence. Hence, primary prevention has to be compelled to be started right from childhood. Under such circumstances, considering the high risk of morbidity and mortality, CVD dietary supplements and nutraceuticals could play a major role in CVD (Maulik and Banerjee, 2015). There are several Indian medicative plants and their phyto-compounds that are shown to possess helpful effects against CVD disorders.

20.8.1 *Terminalia arjuna*

Out of all Indian medicinal plants, *Terminalia arjuna* showed potential for development as a medicine for CVD disorders. *T. arjuna* is sometimes called Arjuna or Arjun tree in India. The Arjuna bark has been extensively studied over the last century in various animal models to demonstrate its cardioprotective properties, such as positive inotropic, hypolipidemic, and coronary vasodilatory properties. The stem bark can attenuate cardiac muscle pathology and oxidative stress evoked by chronic receptor stimulation. *T. arjuna* treatment significantly attenuated internal organ dysfunction and cardiac muscle injury in rats with chronic heart disease (Maulik and Banerjee, 2015).

20.8.2 Garlic

Garlic (*Allium sativum*) is another potential herb to be employed in fighting CVD disorder. Garlic has been shown to protect against myocardial infarction, doxorubicin-induced cardiotoxicity, viscus arrhythmias, internal organ hypertrophy, and ischemia–reperfusion injury. The induction of internal organ endogenous antioxidants and so the reduction of lipid peroxidation by garlic have been noted previously. Totally different mechanisms, such as regulating ion channels, modulating Akt signaling pathways, protein deacetylase inhibition, and hemeprotein P450 inhibition, can even be responsible for the cardioprotective results of garlic (Maulik and Banerjee, 2015).

20.8.3 *Tinospora cordifolia*

Tinospora cordifolia is a very well-known immune-modulatory plant used in Ayurveda. It has been investigated for its activity in rats. The PQRST waves were normalized, and atrial, as well as bodily structure, fibrillation was controlled in rats treated with *T. cordifolia*. This study indicated that *T. cordifolia* is going to be utilized in clinical settings and is helpful in heart disease and flutter, and may be useful in tachyarrhythmias. There was a dose-dependent reduction in infarct size and lipid peroxide levels in infarct heart tissue with the previous treatment of *T. cordifolia*.

20.8.4 *Emblica officinalis*

Emblica officinalis, commonly called amla in India, is a very important healthful plant known for its dietary and therapeutic uses in ancient systems of medicine. A study showed the cardioprotective potential of *E. officinalis*. Amla contains a property to adapt tissues against anemia injury by enhancing endogenous antioxidants (Maulik and Banerjee, 2015).

20.9 VALIDATION OF BOTANICALS AS NUTRACEUTICALS

The nutraceuticals derived from botanicals and their products should be properly characterized and standardized through analytical techniques. New reports are published almost daily on the role of existing food ingredients and advances in identifying bioactive compounds and their health benefits. However, more research is needed to translate basic research into consumer-relevant products. Different processes are available for the discovery, design, development, and marketing

of a new nutraceutical, and these processes can be divided into two parts: research and marketing. The acceptance of the prospective health benefits of functional foods and nutraceuticals has been tarnished by numerous published reports that failed to demonstrate the claimed effects. To solve this problem, well-designed scientific research (research process) and effective consumer education programs (marketing process) are necessary (Schilter et al., 2003; Ghosh et al., 2015). A phytopharmaceutical, being a composite mixture of phytoconstituents present in an herb or a mixture of herbs, should be validated from cultivation in the farm to manufacturing in the industry (Mukherjee et al., 2017a,b; Kroes and Walker, 2004).

20.9.1 Safety of Nutraceuticals

The safety of nutraceuticals is of the utmost necessity because it is obligatory in countries. The key challenges in researching functional foods and nutraceuticals involve deciding their performance and health benefits. From a clinical perspective, the direct assessment of enhancements in health and wellbeing and/or the reduction of disease risk are typically tough to match to the end result of a cure by medicine, that is, the state of health and wellbeing does not continuously lend itself to quantitative assessment. The "omics" era has ushered in nutrigenomics, proteomics, and metabolomics as the disciplines that may contribute to the fast development of nutraceuticals; what is more, bioinformatics can integrate all knowledge from multiple sources and cases. It is attainable to know the results of nutrients and foods at the molecular level within the body and therefore the variable effects of dietary elements on every individual. A synergism between developments in functional food and nutraceutical science and "omics" might, in the future, lead to a scenario in which it is attainable for people to create actually informed selections regarding the foods that offer the simplest opportunities for health, wellbeing, and reduced risk of diseases (Ghosh et al., 2015). The details of quality-related issues of the safety of botanicals are discussed in Chapter 18.

20.9.2 Regulatory Aspects of Phyto-Pharmaceuticals, Dietary Supplements, and Nutraceuticals

In order to establish scientific evidence, documentation, and economical values for nutraceuticals and dietary supplements, several common principles and responsibilities have been imposed by various countries through different regulatory bodies. The objective of these regulatory affairs mainly deals with the improvement of quality to increase the interest of consumers to provide safe and effective products. Restrictive frameworks vary from region to region as elaborated in Chapter 21.

In Europe, the Committee on Herbal Medicinal Products (HMPC) established several aspects of the scientific analysis of herbal medicines, which are harmonic and accepted through science-based standards, to confirm public health (Mukherjee et al., 2017a,b; Knoss, 2015).

Some parameters for understanding the event of herbal drug regulation in any nation are the general policy structure, drug registration system, development of collection procedures, national monographs, inclusion in essential medication lists, and drug kind (OTC or prescription). Many countries have herbal drug regulation and registration systems. Korea, Indonesia, India, Myanmar, Sri Lanka, Thailand, China, Malaysia, and Vietnam have National Monographs for herbal medicine (Fan et al., 2012). In Canada, natural health products (NHPs) are subjected to the Natural Health Products rules in which all producers of NHPs have to be compelled to apply for licenses before merchandising them (Fan et al., 2012; Mukherjee et al., 2017a,b).

In India, the Ministry of AYUSH controls the herbal medicine and traditional medicine regulations. The Drugs and Cosmetics Act of 1940 lays down rules for production and promotion of herbal products. Schedule T of the Drugs and Cosmetics Act, 1940 specifically deals with the GMP for AYUSH medicine (Mukherjee et al., 2016). The new legislation on development of phytopharmaceuticals permits the systems of extraction, fractionation, and potentiating. Once an NDA endorsement comes from CDSCO, the new phytopharmaceutical would be the same as that of a drug. The new direction for phytopharmaceuticals is in accordance with controls in the United States and China, together with logical assessment and data age (Narayana and Katiyar, 2013a,b; Mukherjee et al., 2017a,b).

In China, Chinese herbal products are regulated by the State Food and Drug Administration (SFDA) and may be registered as functional foods or medicine. The Department of Food License controls the regulatory approval of functional foods, whereas that of Chinese herbal medicine is controlled by the division of Traditional Chinese Medicines (TCMs) & Ethno-Medicines underneath the Department of Drug Registration, which is quite strict as synthetic drugs. Some specific tips on food supplements have been developed by the Codex Alimentarius. The World Health Organization (WHO) defines provisions for risk assessment, labeling necessities, a negative list of ingredients, provisions for nutrition and health claims, and standards GMP for food supplements (Mukherjee et al., 2017a,b).

In the United States, approval from the Food and Drug Administration (FDA) is not required by dietary supplements before marketing. Companies that manufacture or distribute dietary supplements containing "new dietary ingredients" are

required to submit premarket safety notifications. Phytopharmaceuticals are generally the same as botanical medications in the United States. The administrative situation in regards to botanical arrangements fluctuates from nation to nation. In Europe, herbal arrangements are ordered into three classes: customary use, settled use, and independent/blended application. The FDA's Botanical Medication Advancement Direction portrays suitable improvements and anticipates organic medications to be submitted in NDAs and particular proposals for submitting INDs. The administrative situation with respect to herbal drug evaluation differs from nation to nation.

In Indian regulations, the major classes of Ayurveda, Siddha, or Unani (ASU) drugs are included in the Drugs and Cosmetic Act. Classical ASU drugs, as mentioned in the authoritative books of ASU system drugs, are manufactured and named in accordance with the formulations described in the authoritative texts. For this category, the issue of license to manufacture is based on citation in authoritative books and published literature, unless the drug is meant for a new indication when proof of effectiveness is required. Patent or proprietary medicine makes use of ingredients referred to in the formulations of authoritative texts, but with intellectual intervention, innovation, or invention to manufacture products different from the classical medicine. For this category, the issue of a license to manufacture requires proof of effectiveness, based on a pilot study as per relevant protocol for ASU drugs.

In 2015, regulatory requirements for phytopharmaceuticals were under the purview of the Central Drugs Standards Control Organization (CDSCO). A gazette notification of the Government of India defines regulatory provisions for phytopharmaceuticals and regulatory submission requirements for scientific data on quality, safety, and efficacy to evaluate and permit marketing for an herbal drug on similar lines to synthetic, chemical moieties (Government of India, draft gazette notification, GSR 702(E), dated 24th October 2013). In Schedule Y, the newly added Appendix I B describes data to be submitted along with the application to conduct a clinical trial or import or manufacture a phytopharmaceutical drug in the country. The regulatory requirements for NDA for the phytopharmaceutical drug include standard requirements for new drug-safety and pharmacological information, human studies, and confirmatory clinical trials. For a phytopharmaceutical drug, there are several aspects to be considered, which includes available information on the plant, formulation and route of administration, dosages, therapeutic class for which it is indicated and the claims to be made for the phytopharmaceutical, and supportive information from published literature on safety and efficacy and human or clinical pharmacology information.

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Chapter 21

Regulatory Harmonization and Good Quality Practices for the Development of Herbal Medicine

Chapter Outline

21.1 REGULATORY HARMONIZATION IN HERBAL DRUG DEVELOPMENT

Global harmonization of herbal health claims is inevitable and desirable from both an economic and medical standpoint. The free movement of safe and effective herbal medicines throughout the world will bring better standards of living by enhancing capacity building in developing countries, improving healthcare, and promoting WHO's goal of "health for all." Harmonization of herbal health claims is achievable only for those herbal medicines having sufficient scientific data to support the claims of safety and therapeutic efficacy. However, for others, more scientific information will be required in the form of well-designed, controlled, clinical trials, and basic scientific research. What is needed is a coordinated, forthright, and determined initiative to unite the expertise of healthcare professionals and scientists from industry and academia from around the world, with the financial resources to do the job.

The draft rules from the US FDA on "Guidelines for The Botanical Drug Industry" are used to prescribe several parameters for the development of herbal medicine. German investigators have published 410 monographs on 324 different plants and plant substances used for their medicinal properties. They represent a rational system of historical ancient use with a

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scientific approach. These monographs are used to prescribe several parameters on the standards in herbal medicine, which come from different organizations and associations, such as EMEA, ESCOP, and AESGP. They are available at websites and this can undoubtedly facilitate the formation of principles and rules for herbals in various countries (Mukherjee, 2003).

In India, the Ministry of AYUSH and the Drug Controller General of India (DCGI) control the principles and rules for herbals, together with the Drugs and Cosmetics Act, 1940 and Rules. This act incorporates principles for the implementation of GMP in herbals, which can facilitate the standardization of herbal products to safeguard efficacy and safety.

There have been several amendments to the Drugs and Cosmetics Act, and Rules (D&C Act and Principles). They make regulatory provisions for characterizing phytopharmaceuticals (plant-based medicines) and a schedule, which gives the requirements for scientific information on quality, safety, and efficacy to assess the approval for a plant-based drug as a medicine in light of comparative lines to synthetic and chemical moieties. In India, through the regulatory guidelines on phytopharmaceuticals, a new domain for herbal drugs has been opened; which have been affirmed and differentiated as drugs by the Central Drugs Standards Control Organization (Mahady, 2001; Narayana & Katiyar, 2013). The regulatory status of herbal medicines in developed and developing economies is explained in Fig. 21.1.

21.2 REGULATORY STATUS OF HERBAL DRUGS IN INDIA

In India, herbal drugs are regulated under the Drug and Cosmetic Act (D and C) 1940 and Rules 1945 made thereunder, in which regulatory provisions for Ayurveda, Unani, and Siddha medicine are clearly laid down. The Department of AYUSH is the regulatory authority and the department mandates that any manufacture or marketing of herbal drugs can only be done after obtaining a manufacturing license, as applicable. The D and C Act extends control over licensing, formulation composition, manufacture, labeling, packing, quality, and export. Schedule "T" of the act lays down the good manufacturing practice (GMP) requirements to be practiced for the manufacture of herbal medicines. Official pharmacopeias and formularies are available for the quality standards of the medicines. The first schedule of the D and C Act lists authorized texts, which have to be followed for licensing any herbal product. The Government of India has made the Ministry of AYUSH which consider various needs to maintain the quality and safety of ASU drugs. The Drug and Cosmetic Act (D and C) 1940 and Rules 1945 clearly state, under schedule T, the GMP requirements of Ayurveda, Siddha, Unani (ASU) drugs. The various rules are mentioned in Fig. 21.2. Various quality control and standardization profiles of Ayurvedic drugs are further explained in the Ayurvedic Pharmacopeia and Formularies. Beside these, the quality control guidelines, as prescribed by WHO, are practiced by most of the manufacturers throughout the country. In the following sections, the same relating to these systems is described, as mentioned in different sections of the drugs act (Mukherjee, 2002).

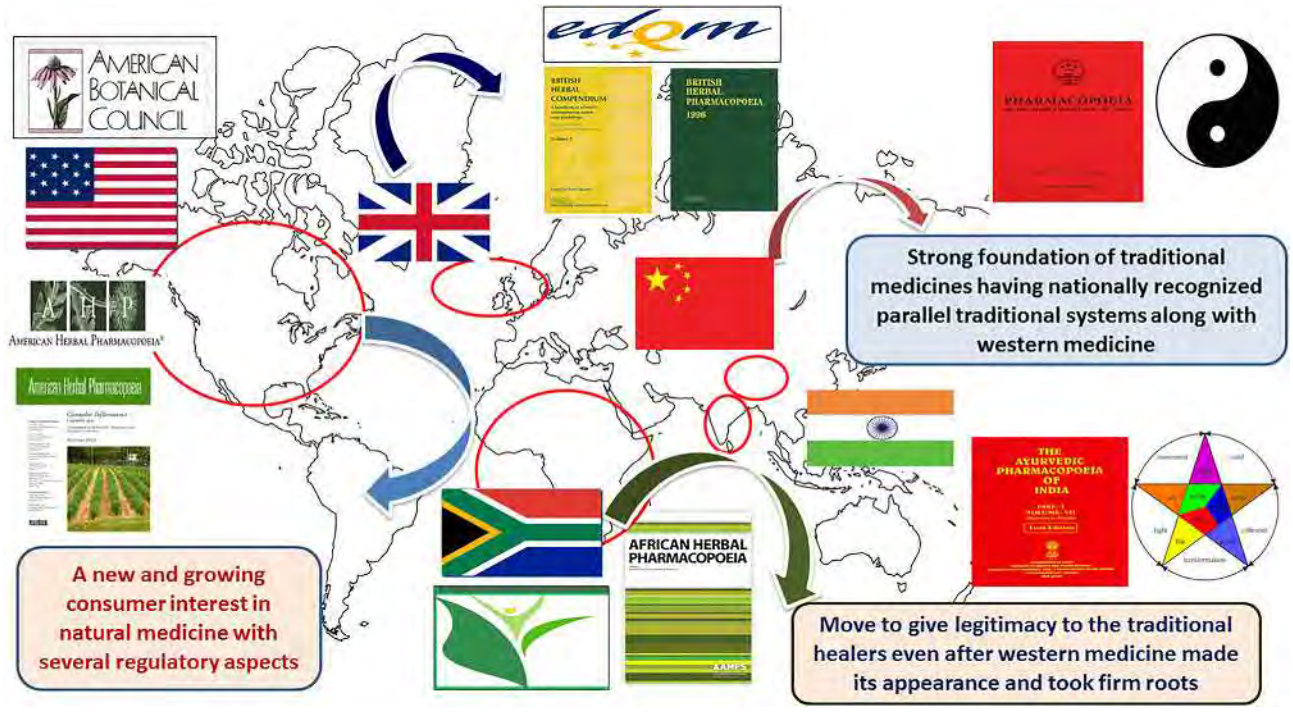

FIG. 21.1 Regulatory status of herbal medicines in different parts of the world.

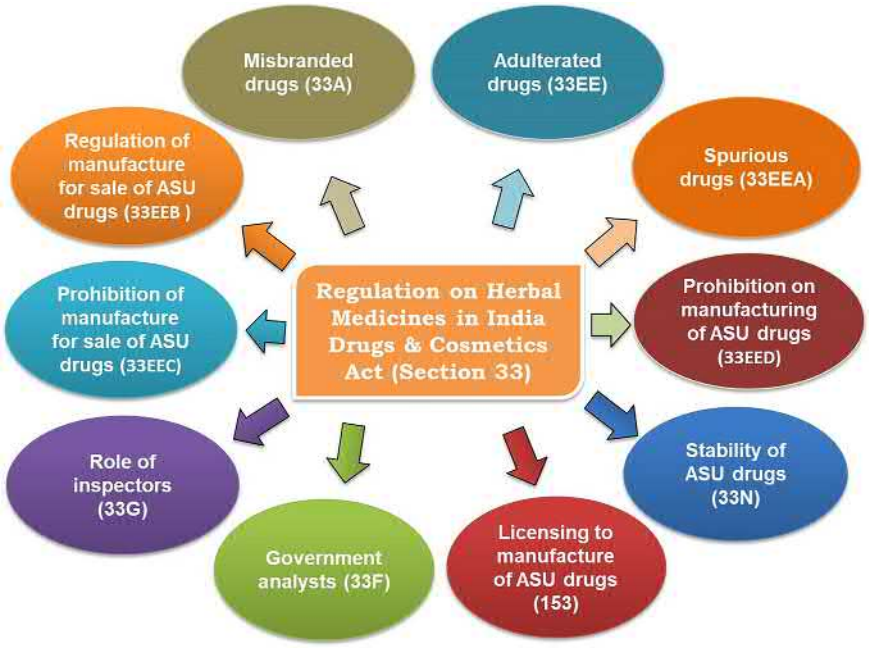

FIG. 21.2 Several aspects of the regulation of herbal medicines in India.

21.2.1 Ayurvedic, Siddha, and Unani Drugs Technical Advisory Board (ASUDTAB)

As mentioned in Section 33-C of the Drug and Cosmetic Act (D and C) 1940 and Rules 1945, the Central Government constitutes the Ayurvedic, Siddha, and Unani Drug Technical Advisory board (ASUDTAB) to advise the Central Government and also the State Governments on technical matters. The Board comprises individuals from varied fields and would regulate the use and production of ASU medicine. The Central Government shall appoint a member as the Chairman of the Board. The Board could, by approval of the Central Government, legislate by-laws fixing control of its own procedures and conducting all business to be transacted by it (Mukherjee, 2003).

21.2.2 Ayurvedic, Siddha, and Unani Drugs Consultative Committee (ASUDCC)

Section 33-D, of the D&C act states that the Central Government could form an Advisory Committee to be known as the Ayurvedic, Siddha, and Unani medicine advisory Committee to advise the Central Government, the State Governments, and also the Ayurvedic, Siddha, and Unani medicine technical board on any matter for the aim of securing uniformity throughout India within the administration of this act because it relates to Ayurvedic, Siddha, and Unani medicine. The traditional constitution and performance of the committee shall encompass two persons to be appointed by the Central Government as representatives of that government and not more than one representative of every state to be appointed by the government involved (Mukherjee, 2003).

21.2.3 Misbranded Drugs

Section 33-E of the Drug and Cosmetic Act (D and C) 1940 and Rules 1945, describe misbranded drugs. An Ayurvedic, Siddha, or Unani drug shall be deemed to be illegal if it is colored, coated, pulverized, or polished in a way that is harmful, or if it is promoted as of higher or larger therapeutic worth than it is in reality; or it is not labeled in the prescribed manner; or its label bears any statement, style, or device that makes any false claim for the drug or that is fake or deceptive in any specific terms (Mukherjee, 2003).

21.2.4 Adulterated Drugs

In Section 33-EE, It has been clearly stated that an Ayurvedic, Siddha, or Unani drug shall be deemed to be adulterate if it consists, in whole or in part, of any filthy, putrid, or rotten substance; or if it has been manufactured, packed, or stored in unsanitary conditions whereby it may be contaminated, or if it consists, in whole or in part, of any toxic substance that

may render the contents injurious to health; or if it bears or contains, for functions of coloring only, a color aside from the one that is prescribed; or if it contains any harmful or poisonous substance that can render it injurious to health; or if any substance has been mixed with it that reduces its quality or strength. If the drug consists, in whole or in part, of any degraded substance only by reason of the fact that such substance is the result of any natural decomposition of the drug, or such decomposition is not because of any negligence on the part of the manufacturer of the drug or the dealer and it does not render the drug injurious to health (Mukherjee, 2003).

21.2.5 Spurious Drugs

As prescribed in Section 33-EEA, an Ayurvedic, Siddha, or Unani drug shall be deemed to be spurious once one or all of the following criteria apply: if it is overprescribed, or offered or exhibited for sale, under a reputation that belongs to a different drug; or if it is an imitation of, or could be a substitute for one more drugs or resembles another drug in a manner possible to deceive, or upon its label carries the name of another drug, unless it is plainly and prominently marked (Mukherjee, 2003).

21.2.6 Rules of Manufacture for Ayurvedic, Siddha, and Unani (ASU) Drugs

Section 33-EEB states the rules for the manufacture and sale of Ayurvedic, Siddha, and Unani drugs. No one shall manufacture, sell, or distribute any Ayurvedic, Siddha, and Unani drug except in accordance with such standards, if any, as could also be prescribed in respect to that drug. Section 33-EEC prescribes some criteria for prohibitions on the manufacture of ASU. No person, either by himself or by another person on his behalf, shall manufacture or distribute any illegal, adulterate, or spurious Ayurvedic, Siddha, and Unani drug. The power of Central Government to ban the manufacture, etc., of Ayurvedic, Siddha, and Unani drugs in the public interest is represented in Section 33-EED. Misbranded, adulterated, and spurious drugs are outlined under Section 33-EED. The Central Government has been authorized to ban the manufacture, sale and, distribution of Ayurvedic, Siddha, and Unani drugs that are irrational or are not safe. Sections 33-I and 33-J are substituted by new Sections 33-I and 33-J to fix the penalty for irrational drugs. Sections-33-EEB and 33-EEC correspond to Sections 33-D and 33-E of the unamended chapter IV A. Section 33-I prescribes the penalty for manufacture, sale, etc., of ASU drugs in violation of this Act (Malik, 2016). Various aspects of the regulation of herbal medicines in India are described further in Fig. 21.2.

21.2.7 Licensing Authorities

The rules related to licensing authorities are stated under Rule 152 of the Drugs and Cosmetics Rules, Government of India. It states that the State Government shall appoint licensing authorities for applications for a license to manufacture Ayurvedic (including Siddha) or Unani drugs. According to Rule 153, an application for the grant or renewal of a license to manufacture Ayurvedic (including Siddha) or Unani medicine should be made in form 24-D to the licensing authority. The type of license to manufacture Ayurvedic (including Siddha) or Unani medicine, subject to the conditions of Rule 157, shall be issued in form 25-D as per Rule 154. A license shall be granted by the Licensing Authority in consultation with an expert in the Ayurvedic (including Siddha) or Unani system of drugs.

The licensing authority shall satisfy himself that the manufacturing unit has adequate instrumentation, staff, and capability for manufacture and facilities for testing, to undertake the manufacture on behalf of the person for a loan license before the grant of a loan license. The certificate of renewal of a license in Form 25-D shall be issued in Form 26-D under Rule 155. The certificate of renewal of a loan license in Form 25-E shall be issued in Form 26-E based on Rule 155-A (Mukherjee, 2003). Rule 156 states that a license in Form 25-D or a revived license in Form 26-D, unless suspended or canceled, shall be valid for 5 years from the date of its issue (Malik, 2016).

Rule 157 of the Indian Drugs and Cosmetics Act has prescribed conditions for the grant or renewal of a license complying with the conditions of the manufacturing unit. The manufacturing conditions for Ayurveda, Siddha, Unani (ASU) drugs have been stated in Rule 157-1; the area and hygienic conditions have been specified in Schedule T. It has been stated that the manufacturing of ASU drugs should be made under the direction and supervision of a competent technical staff with prescribed qualifications as specified in Rule 157-2. The importance of maintaining records related to raw materials used has been specified under Rule 157-A (Malik, 2016).

The conditions under which a license for ASU drugs will be issued is stated in Rule 158 and the specifications for issuing a loan license are stated in Rule 158-A. Several guidelines with respect to ASU drugs are stated in rule 158-B. Provisions for free sale certificates and nonconviction certificates for ASU drugs are given under Rule 158-C through Forms 26-E2-1, 26-E2-2, and 26-E3.

Rule 159 states conditions and provisions regarding cancelation and suspension of a license. Rule 160 states that the raw materials should be identified and tested. Rule 160-A to J deals with laws related to the approval of institutions for carrying out tests for ASU drugs and raw materials used in their manufacture on behalf of licensees for the manufacture for sale of ASU drugs. Rule 161 deals with labeling, packing, and the limit of alcohol in ASU drugs. Rule 161-A deals with labeling and packing of ASU drugs for export. Rule 161-B deals with the shelf-life and expiration date of ASU drugs. Rule 162 deals with the duties of inspectors specially authorized to inspect the manufacturing of ASU drugs. Rule 163 deals with having a pharmacopoeial lab for Indian medicine function as a Central Drug Laboratory for the purpose of testing or analysis of ASU drugs. Rule 164 mentions different test methods to be employed in relation to ASU drugs. Rule 165 to 167 specifies the qualifications and duties of a government analyst. Rule 168 deals with standards to be complied with in manufacture, sale, or distribution. In the cases of Asavas and Aristas, the upper limit of self-generated alcohol should not exceed 12% (v/v). Rule 169 deals with permitted excipients (Malik, 2016).

21.3 GOOD HARVESTING PRACTICES

The starting materials for all phytomedicines are crude plant materials, mostly parts or plant organs of medicinally used species and usually in a dried form. According to WHO, there are 21,000 plant species listed as being medicinally used as plant drugs. Between 70% and 90% of these are commercially obtained by collecting the drugs in the natural habitat. Among these, only about 50–100 species are cultured by plant cell culture techniques (Mukherjee, 2002).

21.3.1 Collection of Plant Materials

Collection practice is, interestingly, not always coincident with the area of the main occurrence of the species. Collection is found mostly in regions with people with low wages. The reasons for the continuing practice of collection are manifold. Some of these arguments include:

- some of the plant species grow slowly;
- many of the plant species are not amenable to agriculture;
- in-culturing of the natural species causes difficulty;
- the tonnage needed for the particular herbal medicine is not always specific; and
- collection of the herbal medicine is more important than harvesting.

Sometimes the amount needed is small, and, therefore, uninteresting from the monetary point of view. Collecting is, in such a case, a more economical alternative. For *Baptisia australis,* for example, the present demand is a grand total of 4–5 tons per year, of which 95% is processed by a single manufacturer (Mukherjee, 2002).

21.3.2 Problems Encountered in the Collection of Medicinal Plants

It has to be noted that there are some dangers inherent in the collection practice of the medicinal plants, which include:

- overharvesting of endemic species;
- reduction and/or elimination of local populations, with a resulting decrease of genetic variety; and
- unnecessary destruction of plants during harvest.

These dictate two main problems, namely, extinction and elimination of genetic variety. Overharvesting of natural resources can lead to extinction of a particular plant species in an entire region (Mukherjee, 2002).

21.3.3 Good Collection Practices

In spite of these problems, the collection of plant material in the native habitat will represent, for a long time to come, the method of gathering starting material for phytomedicines. There are specific aspects of quality and concomitant analytic approaches, which are important for collecting plant drugs, which can be explained through several points:

- correct identity of the plant drugs being collected;
- admixture of the collected original varieties with others; and
- presence of foreign matter.

Collected plant drugs, especially those used under their vernacular name, are very prone to be mislabeled, so that the analytical determination of identity becomes important. The importance of pharmacognostic analysis is of utmost important for the correct identification of the harvested plants (Mukherjee, 2002).

A related problem is admixture. The above example would not have happened, if a proper identification protocol had been followed. But, because it happened in the nonmedicinal OTC trade, none was required and most health food companies do not invest in such enterprises. A third aspect, which should be considered with emphasis, is foreign matter. Collected drugs tend to contain a higher percentage of sand, grass, and nondrug parts of the species than allowed by the general notices of the pharmacopeia. Therefore, specific care should be taken in performing those tests described for this purpose. Generally, heavy metals, unusual residues, and pesticide levels are of little or minor importance. They are rather more frequent in crops from fertilized agricultural fields.

Collecting practices cannot be considered in isolation. They always go together with the concomitant processing, that is, drying and packing for transport. This has several important features that need to be considered. The first is a purely hygienic one. In order to prevent microbiological infestation with pathogen bacteria and molds, care has to be taken to avoid handling by persons carrying internal or external infections and to avoid access of the processed material to domestic animals, pets, rodents, and insects. A second consideration has to do with the avoidance of alterations of the desired ingredient spectrum by direct sunlight, high humidity, and restricted ventilation.

In order to oblige GMP requirements, a manufacturer has to have records of the origin of the raw materials and also of the performance of the proper steps for processing and handling. Thus, documentation by a responsible person should be carried out from collecting to manufacturing for each batch of collected drugs. The training of the collectors and handlers is a major requirement, so that in the future collected material will be available in sufficiently documented quality to be sold and traded. The demand for such material will grow and provide a better income base for the populace involved in this trade.

The above considerations have to be incorporated and are the key to success for the proper development of quality of herbal products. For the commercial collection of medicinal plants from the natural environment, various guidelines have been incorporated as described in the subsequent sections. This constitutes a first proposal, introducing points gathered from practical experience and probably requires amendment (Mukherjee, 2002).

21.4 GOOD AGRICULTURAL PRACTICES

The farming and agricultural processes of all plants listed and utilized for medicinal use should be based on the guidelines for the good agricultural practices (GAP) of medicinal and aromatic plants (MAPs). Hence, it applies to the assembly of all materials utilized in the food, medicinal, plants, and fragrance industries. It conjointly applies to all or any means of production together with organic production in accordance with the European Union rules, as WHO imposes these standards. Strict management should be maintained. Farmers concerned within the production of herbs should make sure that they avoid harm to the ecology and boost the biodiversity of their farms. The current GAP guidelines apply extra standards for the assembly and processing of raw materials and specialize in essential production steps (measures) that are required to ensure good quality.

The main aim of GAP is to make sure that the plant meets the demands of the buyer while maintaining standards of the very best quality. The vital aspects that are required are that they are produced hygienically, so as to scale back microbiological load to a minimum, so that throughout cultivation, process, and storage any negative impacts are avoided.

As within the course of the agricultural processes or MAPs and their product are exposed to an outsized range of each microbiological and different contaminants, the main aim is to guide the producers to scale back raw material contamination to the greatest extent.

21.4.1 SOPs for the Controlled Cultivation of Medicinal Plants

The Standard Operating Procedure (SOP) contains the guidelines that are supposed to contour the cultivation of medicinal plants in keeping with well-regulated strategies and to identify and consistently lay down these steps within the cultivation method that are vital for the production of plant material of good quality. These guidelines are also considered as an aid to mapping potential contamination of plant material by microorganisms or different harmful substances (herbicides and pesticides, heavy metals, aflatoxins, radiation, and fumigants) at an early stage. The SOP for the cultivation of medicinal plants can be described by the following guidelines:

- The cultivation of medicinal plants should be carried out in keeping with standard procedures as far as possible. Protocols should be necessitated for various actions throughout cultivation.
- The principles of good crop care must be observed, including an appropriate crop rotation.

- Generally speaking, disturbance of the environment resulting from cultivation should be avoided.
- Other guidelines should be involved for the biological cultivation of plants (Mukherjee, 2002).

21.4.2 Principles and Guidelines for GAP

21.4.2.1 Seeds and Propagation Material

Botanically identified seeding materials indicating plant variety, cultivar, chemotype, and origin are to be used. The material used ought to be 100% traceable. An equivalent applies to vegetative propagated starting material. The starting material employed in organic production should be certified organic. The strain, variety, chemotype, and origin should be indicated. These specifications can change, for example: (1) species: *Chamomilla recutita* Rauschert; cultivar Bodegold; chemotype bisabololoxide A/chamazulene; seed company, 1996, charge number 4711 or (2) species: *Chamomilla recutita* Rauschert; seed collected on 26-6-1991 by the company, Pleasant Herbs, California, and afterward propagated. It should be possible to trace the origin of the seed fully. A similar standard applies for the material used for vegetative propagation. The starting material ought to meet the standards for purity and germination. The starting materials should be free from pests and diseases to guarantee healthy plant growth. The prevalence of species/variety-identical plants and parts of plants ought to be controlled along the course of the total production technique (cultivation, harvest, drying, and packaging). Plant material or seeds derived from or comprising genetically modified organisms ought to be in accordance with national and European rules. Checks should be applied for the prevalence of plants or elements of plants throughout the complete cultivation and harvesting. Such contamination should be removed instantly (Mukherjee, 2002).

21.4.2.2 Cultivation

SOPs for cultivation depend on the mode of cultivation, for example, conventional or organic. In general, care should be taken to avoid environmental disturbances. The principles of good crop husbandry must be practiced, including an appropriate rotation of crops.

21.4.3 Harvesting of Cultivated Medicinal Plants

The quality assurance (QA) of plant drugs is affected by harvesting cultivated medicinal plants and also the collection of these plants in the wild plays an important role. Various parameters have been developed in this respect in order to guarantee optimum quality, both regarding the content of active ingredients and purity (the absence of contamination) of the harvested plant material to facilitate simple checks of these aspects. Guidelines for plant material collected in the wild have also been included, regarding measures that must be taken to limit any negative effects on the environment.

21.4.4 Collection of Medicinal Plants in the Wild

This is an important process for the QA of herbal drugs in general. The general guidelines to be followed for this purpose can be summarized as:

- Generally speaking, disturbances to the environment resulting from the collection of plant material in the wild must be avoided as far as possible.
- Endangered plant species may not be collected in the wild. Neither should the endangered plant species be damaged in any way during the collection of other plants in the wild.
- The area in which plants are collected must be defined precisely. In addition to the exact location, as much data as possible concerning soil conditions (including potential contamination) and climate must be provided.
- The guidelines for the harvesting of cultivated plant material described above also apply to the collection of plant material in the wild.

21.4.5 Primary Processing of Herbal Products

The steps of processing, such as washing, freezing, distilling, and drying, are the primary guidelines. The guidelines are applicable to the primary processing of freshly harvested material. They are supposed to indicate the processing steps and whether the procedures used are of a sufficiently high standard to forestall loss of quality within the material. The initial processing of freshly harvested plant material consists of washing, freezing, drying, and comminuting. Fresh plant materials are often frozen at a temperature below −18°C (or below −20°C in the case of long-term storage) if this is not damaging

to the quality of the material. If plant material is dried in the open air, it should be spread out in a very thin layer. In order to attain good air circulation, the drying racks must be situated at a sufficient distance from the ground.

21.4.6 Packaging of Collected Materials

The regulation of the packaging, storage, and transport of medicinal plants in an adequate manner is one of the major aspects of GAP in order to get good-quality plant material and to enable the testing efficacy of the procedures used. After the recurrent management and ultimate elimination of low-quality materials and foreign bodies, the merchandise ought to be prepacked in clean and dry, ideally new sacks, baggage, or cases. The label should be clear, and made of nontoxic material. The packaging materials ought to be held in a very clean and dry place, which is free from pests and inaccessible for stock and livestock. It should be ensured that no contamination of the merchandise takes place by the packaging material, especially in the case of fiber baggage. Reusable packaging materials ought to be clean and dried before use. It should be ensured that no contamination takes place by reusing the baggage.

21.4.7 Storage and Transport

Herbal drugs should be stored in optimum conditions and transportation should occur under optimum conditions to ensure the quality of the end-product. The essential oils need to be stored in a very dry, well-aerated building, during which the daily temperature fluctuations are restricted and with optimum aeration. Most products (except Basil) need to be stored between 1 and 5°C, whereas frozen merchandise ought to be stored below −18°C (or below −20°C for longer-term storage). The storage of essential oil should be carried out at acceptable chemical storage standards. Once frozen storage or saturated steam is employed for pest control, the wetness of the material should be controlled after treatment (Mukherjee, 2002).

21.4.8 Equipment and Machinery

The equipment and machinery to qualify the optimum quality of herbal materials being used for the formulation of dosage forms with therapeutic potentials can be prescribed. The equipment used for the cultivation, harvesting, and initial processing steps must be easy to clean in order to prevent contamination. The components of the machines that come into direct contact with the plant material must be cleaned regularly and kept free from oil and other contamination. All machinery should be mounted in an easily accessible way. It must be well serviced and regularly cleaned. Fertilizer and pesticide application machinery must be regularly calibrated. Implements used in cultivation, harvesting, and processing must be in perfect technical condition. The equipment used for the application of fertilizers and plant-protection products must be calibrated regularly. The blades of the harvesting machines must be adjusted such that as few soil particles as possible are picked up with the plant material. Preferably, nonwooden equipment should be used unless tradition demands wooden material. Once wooden equipment (such as pallets, hoppers, etc.) is used, it should not come into direct contact with chemically and contaminated/infected materials, so that infection will be prevented.

21.4.9 Requirements for the Staff

Specific guidelines exist for the staff involved in the cultivation, harvesting, and primary processing of medicinal plants, as well as the equipment and buildings used. They set requirements for the levels of training and the personal hygiene of the employees and the maintenance of the machines and buildings so that the optimum quality of the plant material can be guaranteed.

21.4.10 Requirements for Buildings and Related Items

Requirements for the buildings and related items for the cultivation up to the primary processing of the plant medication are necessary to make sure of the standard of the plant materials so that the optimum quality is warranted for formulations of therapeutic importance. Buildings within which the processing and storage of the plant material takes place should be clean and well vented and may not be used for keeping of bovine or other animals. It is recommended to use rooms with floors and walls of concrete or some other material that is simple to wash. The buildings should be built in such a way that they offer protection against insects, rodents, birds, cows, and other domestic animals. Sufficient measures against vermin should be taken in each area that is used for the processing and storage of plant materials. The door openings and windows should, for instance, be fitted with netting. The treatment of rooms against vermin should be carried out by qualified workers (Mukherjee, 2002).

21.5 GOOD LABORATORY PRACTICES

Good Laboratory Practice (GLP) are collection of principles meant to assure the quality and integrity of nonclinical laboratory studies that are intended to support the analysis or sale permits for products regulated by government agencies. The term GLP is most ordinarily associated with the pharmaceutical trade and, thus, the desired nonclinical animal testing that has to be performed before approval of a drug. However, GLP applies to many different nonpharmaceutical agents, such as color additives, food additives, food contamination limits, food packaging, and medical devices. The actual laws from the US FDA are found in 21CFR 58 and for the European Union via the Organization for Economic Cooperation and Development (OECD). These principles of GLP ought to be applied to nonclinical safety testing (OECD, No. 1). GLP does not apply to clinical studies, but only applies to nonclinical studies and testing. This will be important because clinical studies are dominated by Good Clinical Practice (GCP), and completely different laws intended to safeguard human participant safety. Moreover, a good deal of the GLP structure depends on the roles and responsibilities of the Study Director, who is in control of the oversight and execution of all aspects of the nonclinical study. First, GLP may be a quality management system, not a scientific management system. Or, in other words, GLP defines a collection of quality standards for study conduct, data assortment, and results. GLP does not outline scientific standards. If a study practices GLP, then one can be relatively sure that the results were collected as made public within the study protocol; but, one cannot be sure that the study truly addresses the scientific hypothesis.

The GLP system of quality standards also targets the idea of a QA unit. This QA unit is supposed to be a freelance group or person that monitors the whole study conduct, analysis, and news. The aim of the QA unit is to verify that all written procedures are followed throughout the study. Harmonization of the procedures adopted to observe GLP compliance, as well as equivalence of their quality and rigor, are essential in order to facilitate the mutual acceptance of test data generated for submission to the regulatory authorities of OECD Member countries. The aim of this document is to provide elaborate practical guidance to OECD member countries on the structure, mechanisms, and procedures they must adopt once establishing national GLP compliance monitoring programs in order that these programs may be internationally acceptable. It is recognized that member countries can adopt GLP principles and establish compliance monitoring procedures consistent with national legal and administrative practices, and in line with priorities they provide to, for example, the scope of initial and consequent coverage regarding classes of chemicals and kinds of testing (WHO, 2009).

21.5.1 Principles of GLP

The definitions of terms in the "OECD Principles of good Laboratory Practice" are applicable. The periodic review of test facilities and/or auditing of studies for the purpose of validating adherence to GLP principles is necessary. Overviews of GLP inspections are circulated to members of the OECD panel on GLP and also the OECD Secretariat annually before the end of March. The subsequent minimum set of data ought to enable harmonization of the overviews that have changed among national GLP observance authorities:

- *Identification of the facility inspected*: Appropriate information ought to be enclosed to allow the identification of the facility unequivocally, that is, the name of the test facility, the city, and country within which it is located, together with inspections abroad.
- *Dates of reviews and decisions*: Month and year of inspection, and, if acceptable, date of final call on GLP compliance status.
- *Nature of scrutiny*: A transparent indication ought to be given of whether or not a full GLP inspection or solely a study audit was carried out, also as whether the examination was routine or not and any other authorities that were involved.
- *Areas of experience of the facility inspected*: Because GLP compliance is related to the tests performed by a facility, the area(s) of expertise of the test facilities inspected ought to be included within the annual overviews, using the following broad categories.

(1) Physico–chemical testing, (2) toxicity studies, (3) mutagenicity studies, (4) environmental toxicity studies on aquatic and terrestrial organisms, (5) studies on behavior in water, soil, and air; bioaccumulation, (6) residue studies, (7) studies on effects on mesocosms and natural ecosystems, (8) analytical and clinical chemistry testing, and (9) alternative studies. It is emphasized that these classes are to be employed in a versatile manner.

GLP, based on scientific principles and practices, is indispensable for providing scientific confidence in studies conducted for chemical safety determinations. There are obvious reasons for government agencies worldwide to have GLP compliance, and reasons why it is entirely acceptable for greater weight to be given to GLP studies than to non-GLP studies that are solely available as articles in scientific journals. Noncompliance with GLP must not be used as the sole criterion

for excluding studies from thought in restrictive decision-making. Each study, GLP and non-GLP, ought to be evaluated and weighed in accordance with elementary scientific principles. Regulatory agencies (Food and Drug Administration [FDA] and US Environmental Protection Agency [EPA]) and also the National Toxicology Program (NTP) require studies to be conducted in accordance with GLP (FDA, 2004) and, therefore, the Organization for Economic Cooperation and Development (OECD) GLP principles (OECD, 1998) apply to any or all OECD member countries.

21.6 GOOD MANUFACTURING PRACTICE

The manufacturing process is one of the key steps whenever quality control is needed to confirm the quality of medicative products, as well as herbal medicines. Good Manufacturing Practices (GMP) is one of the foremost vital tools for this purpose. The core needs for GMP for herbal medicines are common to GMP for pharmaceutical products. There is little doubt that GMP may be a key step in ensuring the safety and efficacy of herbal medicines. However, meeting GMP requirements from manufacturers could be particularly troublesome for developing countries. Investment in GMP could increase production prices, resulting in a higher value of the ultimate product. This can impact the affordability of the medicines. Therefore, relevant national health authorities ought to take this impact into account and take suitable measures to encourage and motivate the manufacturers to improve their GMP.

The maintenance of GMP is important in herbal medicine for manufacturing high-quality herbal products. GMP for pharmaceutical products ensures that products are systematically produced based on the guidelines. Thus, GMP takes into consideration all aspects of the development of pharmaceutical substances, as well as herbal products. It includes specifications for all the operational procedures:

(a) All processes of manufacturing are clearly outlined, consistently reviewed under expertise, and shown to be capable of systematically manufacturing pharmaceutical products of the desired quality that accommodate the specifications. Important steps of manufacturing processes and any vital changes manufactured to the processes are valid.
(b) All necessary facilities are provided, including qualified and trained personnel, adequate premises and area, appropriate instrumentation and services, correct materials, containers, and labels, approved procedures and directions, and appropriate storage and transport.
(c) Adequate personnel, laboratories, and instrumentations for in-process controls.
(d) Instructions and procedures are written in clear and unambiguous language, and are applicable to the particular facilities.
(e) Operators are trained to carry out procedures properly.
(f) Records are unbroken (manually and/or by recording instruments) throughout manufacture to indicate that all the steps required by the outlined procedures and directions have been performed and that the amount and quality of the product are as expected; important deviations are totally recorded and investigated.
(g) Records covering manufacture and distribution that modify the whole history of a batch to be derived are preserved in a comprehensible and accessible form.
(h) The correct storage and distribution of the product minimizes any risk to its quality.
(i) A system is available to recall any batch of product from sale or provision.
(j) Complaints regarding marketed products are examined, the causes of quality defects are investigated, and applicable measures are taken concerning defective products and to prevent the return of defective product.

Botanicals have been used for a long time and have an extended heritage. GMP is critical for botanicals so as to rationalize the risky procedures by which they are manufactured, from cultivation to production:

- To guarantee an associated applicable infrastructure or "quality system" encompassing the structure, procedures, processes, and resources.
- To offer systematic action necessary to confirm with adequate confidence that the botanicals satisfy the prescribed demands for quality.
- To reduce the danger inherent in any botanical preparation that cannot be completely prevented by testing the final product.

According to some GMPS, such as the EU-GMP, WHO-GMP, or PIC/S, herbal products are considered to be herbal medicinal products, whereas the United States considers those products to be dietary supplements that do not have any claims of medical specialty actions. Meticulously, in keeping with federal agency guidelines, dietary supplement regulation and drug regulation are separate branches. GMP parameters, testing and QA techniques, all are designed to confirm that a product is safe for human consumption. A number of the common GMP parameters are as follows:

(a) *Hygiene*: The manufacturing facility should maintain a clean and sanitary manufacturing space.
(b) *Method controls*: Manufacturing processes should be clearly outlined and controlled. All of the important processes are confirmed to achieve consistency and compliance with specifications.
(c) *Validation*: Manufacturing changes that have an effect on the standard of the product are validated as necessary.
(d) *Procedures*: Directions and procedures are written in clear and unambiguous language. Personnel are trained on storage and document procedures.
(e) *Records*: Manufacturing records are produced, manually or by instruments, throughout the manufacturing process, which demonstrate that the steps required by the outlined procedures and directions were indeed taken and indicating the amount and quality of the product are as expected. Deviations are investigated and documented.
(f) *Distribution records*: Distribution records are specified on the whole history of a batch to be derived and are preserved in a comprehensible and accessible form.
(g) *Recall systems*: A system is available for recalling any batch of product from sale or provision.
(h) *Records of complaints*: Complaints regarding marketed products are examined, the causes of quality defects are investigated, and applicable measures are taken in respect of the defective product to forestall recurrence.

GMP rules are not prescriptive directions to manufacture products. They are instead a series of general principles that have to be determined throughout manufacturing. Once a manufacturer fixes its quality control program and manufacturing methodology, there may even be some ways in which to meet GMP requirements. It is the company's responsibility to work out the most effective and economical manufacturing methods to meet the requirements of the regulatory agencies.

Thus, GMP will guarantee the consistent quality of herbal products that are applicable to their intended use as needed by the promoting authority. GMPs for botanicals include all aspects from cultivation to manufacturing and internal control. There has been a trend toward the development of GMP for botanicals in several countries. India, being a significant producer of herbal medicine for its completely different systems of medicines, needs GMPs to be enforced to the fullest extent for herbal medicine. The objectives of GMP for herbal medicine are usually expressed in terms of what is to be achieved in many areas of manufacture and management, as well as cultivation and assortment. During this era of worldwide herbal drug revolution, there is a desire to implement GMP within the production of herbal medicine (Mukherjee, 2002).

21.7 GOOD CLINICAL PRACTICE

The history of Good Clinical Practices (GCP) traces back to one of the oldest enduring traditions in the history of medicine: The Hippocratic Oath. The complexities of modern medicine research necessitate more elaborate guidelines to address a Physician's ethical and scientific responsibilities in biomedical research, such as obtaining informed consent or disclosing risk. GCP is a set of guidelines for biomedical studies, which encompass the design, conduct, termination, audit, analysis, reporting, and documentation of studies involving human subjects. The fundamental tenet of GCP is that in research on humans, the interest of science and society should never take precedence over considerations related to the health of the study subject. It aims to ensure that the studies are scientifically and ethically sound and that the clinical properties of the pharmaceutical substances under investigation are properly documented (Vijayananthan and Nawawi, 2008).

21.7.1 GCP for Indian Systems of Medicine: Ayurveda, Siddha, and Unani

Ayurveda has placed great stress on ethical guidelines when treating a patient through medical/surgical interventions. The utmost priority has been accorded to ethical issues and prior consent of the patient was prompted within the Ayurvedic texts. As the guiding system of rules, it is primarily known for its edict to undertake and do no harm to the patient. However, the complexities of ASU drug analysis necessitate many elaborate sets of guidelines that address a physician's moral and scientific responsibilities, such as getting consent or revealing the risk concerned in ASU drugs. Standard, randomized, and controlled clinical trials are considered to be the gold standard. However, when applying them to evaluate herbal and ancient drugs, particularly Ayurvedic medicines, their limitations return to the forefront. A careful study of a holistic approach to treatment practiced in traditional medicine suggests that this technique of conducting clinical trials has numerous limitations in evaluating the proof of affectivity of Ayurvedic or ancient drugs. Many limiting factors make the task of conducting clinical trials on ancient drugs in a standard approach difficult. An associated integrated/holistic approach is important when undertaking a trial with an Ayurveda drug and also the factors need to be considered as individual variables and may be accommodated when planning the trial protocols for Ayurvedic/herbal medicines (GCP, AYUSH).

The ASU-GCP guidelines seek to establish two cardinal principles, namely, the protection of the rights of human subjects and the credibility of ASU drugs. They must be followed for all ASU drug research in the Republic of India in the least stages of drug development, whether before or after product registration in the Republic of India.

The guidelines are addressed to researchers and all those who have an interest in conducting clinical trials on ASU drugs. The main targets are to highlight scientific validation and for promoting evidence-based use of ASU treatments and they are meant for voluntary use. The target is to encourage clinical studies in ASU systems undertaken in accordance with moral and scientific standards, safety aspects, and with the rights of participants protected.

With the introduction of the Drugs and Cosmetics Rule 158-B in August 2010, the requirement of proof of effectiveness for licensing of patent or proprietary ASU drugs has necessitated the adoption of GCP. It provides assurance of the safety and efficacy of contemporary and traditional ASU formulations. These standards of clinical tests need to be followed. They describe the roles and responsibilities of clinical trial sponsors, clinical analysis investigators, monitors, and others. The target is to infuse the culture of conducting ASU intervention-based clinical studies in accordance with requisite scientific standards and rationally designed methodologies. The results and findings of clinical trials should be properly recorded, analyzed, and reported. Sincere adherence to these guidelines will facilitate the acceptance of clinical data by the national and global scientific community. The foundations of the basic principles written by the CDSCO for taking part in GCP with necessary modifications have been made to suit the ASU principles and treatment methodologies (Vijayananthan and Nawawi, 2008).

21.8 QUALITY REPORTING OF HERBAL CLINICAL TRIALS

The integration of herbal drugs into evidence-based clinical practice and research also rests on the acceptance of its scientific evidence by the traditional medical community, together with medical practitioners, pharmacists, nurses, and different healthcare workers. Studies in other complimentary alternative medicine (CAM) modalities, such as acupuncture, have been designed with specific details of the experiment (e.g., reasonable needle used, location of the points, depth of needle insertion, and techniques for rotating the needles) and the nature of the control methodology after considering a placebo effect (Sherman et al., 2002). If possible, proof generated for herbal drugs ought to be derived from the most powerful technique of testing the impact of treatment intervention, the randomized controlled trials (RCT). With a plausible biological basis, herbal products may be evaluated through double-blind, placebo-controlled, multicenter trials. Reflecting this, the World Health Organization (WHO) has published a number of guidelines for the clinical analysis of herbal and TMs (WHO, 2013).

Trials with poor outcome measurements will exaggerate the estimates of treatment effects (Schulz et al., 1995). Thus, in the EBM paradigm, RCTs should be reported in accordance with the 22-item Consolidated Standards of Reportage Trials (CONSORT) listing (available at www.consort-statement.org). This includes a detailed description of patient eligibility criteria, sample size calculation, specific objectives and hypotheses, implementation of the trial, and statistical methods, regardless of whether or not the intervention is conventional or herbal (Gagnier et al., 2006).

In parallel with other methodologies necessary to the design of the trials, outcome measurement is central to the development of CAM, together with herbal drugs (Long, 2002). Thus, item six (outcomes) of the CONSORT checklist was suggested to reflect the intervention and indications tested while considering their underlying theories and ideas when reporting RCTs on herbal drug intervention (Gagnier et al., 2006). At the request of the agency (FDA), the Institute of Medicine (IOM) convened an operating committee in 2005 and made a report entitled Complementary and Alternative Medicine within the United States. In this report, the core recommendation was that "the same principles and standards of proof of treatment effectiveness apply to any or all treatments, whether or not presently labelled as typical drugs or CAM." Maybe one obstacle is the holistic idea and approach being emphasized by the distinctive philosophy of herbal medicine. For this reason, some subjective measurements, including the proportion of patients perceiving benefits and also the variety of patients "recovering" from the condition, were commonly reported in TCM trials (Ernst, 2006). The development of the quality of life parameters for herbal medicine trials by using an EBM approach has received a great deal of attention (Leung et al., 2005; Wu et al., 2009).

21.9 INTELLECTUAL PROPERTY RIGHTS AND PATENT ISSUES WITH HERBAL MEDICINES

Intellectual property comprises legal rights concerning industrial, scientific, literary, and artistic accomplishments by an applicant. It can be either a scientific or literary creation. Intellectual property rights help in protecting a person's rights to an innovation, product design, an artistic or literary work. Thus, the person is protected from his competitors. A few examples of registrable or nonregistrable rights are as follows:

- Patents are of two types, namely, product patents and process patents.
- As the products or processes patented are for commercialization purposes, they come with provisions for trademarks or copyright symbols, which guarantee the originality of the product.
- The appearance of the industrial product is protected by industrial designs for proper product identification.
- Trade secrets, such as basic formulas, methodologies, and information, need to be protected from industrial espionage.
- Therapeutic products, medical devices, and veterinary products having exclusive rights for marketing are given by regulatory authorities.
- Third-party access to commercially sensitive data, experimental knowledge, or biological materials, such as proteinous materials, ought to be handled with care. They must never be allowed to be accessed by everyone.
- In the case of a phytotherapeutic product, the supply for patenting is not quite simple.

For maintaining a marketable advantage, trade secrets and trademarks are quite useful. Trade secrets are also used, consistent with the formulation and preparation of the phytopharmaceuticals and the prescription of phytotherapies by practitioners. For complete awareness and consumer acceptance, emblems are essential. The packaging of the merchandise conjointly plays a crucial role in the protection of property rights. It conjointly is a trademark or style registration. Even for a famed phytomedicine, patent protection may also be achieved, depending on the sort of innovation. The patent serves more necessary roles than trade secrets. If a brand new phytopharmaceutical product is being promoted for approval, then the restrictive exclusivity might offer information science protection. Within the final stages of promotion, patents, trademarks, style registrations—all of these things play crucial roles for promoting a valuable product (Sahoo et al., 2011).

21.9.1 Patents of Herbal Drugs

To obtain a patent, one needs to go through some legal proceedings. First, a patent application is filed. Then, the patent authority goes through the application, in order to examine whether the applicant meets all the legal requirements. Also, whether there is any infringement—that also needs to be verified through the review process. If a patent is granted, then the patent applicant gets an exclusive right to manufacture and market the particular innovation for a particular period of time. In general, the patent is granted for a fixed period of time. After completion of the specific period, the patent liquidates automatically. In certain cases, due to delay in the patent reviewing and granting procedure, the applicant is awarded an extension. On the other hand, some patents are relatively shorter in duration, in comparison to the general term (Ramzan, 2015).

21.9.2 Patentability Requirements

In the current patent system, an invention has to satisfy the examiner in many respects, including its novelty, inventive step, industrial applicability, and enablement. According to TIPO Guidelines for Patent Examination (the Guidelines), an invention refers to any creation of technical concepts by utilizing the rules of nature. According to the guidelines, inventions that are mere discovery, against the laws of nature, not using the laws of nature, or nontechnical in character are not considered as inventions.

21.9.3 Inventive Step and Nonobviousness

A patent examiner who acts as a determines the inventive step or nonobviousness. Failure to satisfy the nonobviousness requirement bars the patentability of the invention. Besides chemical modification, if isolated and purified compounds can prove unexpected results, then it defines the inventiveness in the claimed invention. A similar position is adopted in Japan. The Japanese Examination Guidelines for Pharmaceutical Inventions also state that an invention may not involve an inventive step if the medical uses of the invention and the prior art can be correlated with each other in view of the mechanism of the biological effect. However, if unexpected advantageous effects can be recognized, the claims may involve an inventive step. This also applies to a new combination of known drugs and optimizes the dosing schedule/dosage amount to reduce toxicity and improve efficacy (Hsiao, 2007).

21.10 INTERNATIONAL REGULATORY STATUS OF HERBAL MEDICINE

The use of extracts of natural origin in therapy, as well as their validation as medicines or health promoting agents, are the main aspects of phytotherapy. Phytotherapy is mainly based on the concept of synergy, instead of isolating a single constituent, which is supposed to be the active component obtained from a particular plant. Thus, phytotherapy aims to

preserve the complexity of the plant under study with relatively less processing. Several countries have developed regulatory profiles specifically regulating health products to control the manufacture and sale of phytotherapeutic agents and the ingredients used therein, which are intended to protect the public from harm and also the traditional medicines that have been practiced for several years. Several regulatory bodies promoting different regulatory provisions throughout the world are listed in Table 21.1.

TABLE 21.1 Regulatory Status of Herbal Medicine Across Different Countries

Country	Regulatory Body
Argentina	Ministry of Health, Committee on Harmonization of Vegetable Drugs Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (National Administration of Drugs, Foods and Medical Devices—ANMAT)
Australia	Therapeutic Goods Act (TGA) Australian Register of Therapeutic Goods (ARTG) Australian Regulatory Guidelines for Complementary Medicines (ARGCM)
Bhutan	Institute of Traditional Medicine Sciences, Ministry of Health, Bhutan
Brazil	Sistema Único de Salud (Unified Health System—SUS)
Canada	Natural Health product Regulation (NHPR) Natural Health Products Directorate (NHPD) Health Products and Food Branch Inspectorate (HPFBI)
Chile	Unidad de Medicina Tradicional y Otras Practicas Médicas Alternativas (Unit for Traditional Medicine and Other Alternative Medical Practices)
China	Administrative Department of Public Health under the State Council
Democratic People's Republic of Korea	Department of Traditional Koryo Medicine of the Ministry of Public Health
Egypt	National Applied Research Centre for Medicinal Plants, National Organization for Drug Control and Research (NODCAR)
European Union	European Economic Community (EC)
Iceland	Icelandic Medicines Control Agency
India	Ministry of AYUSH, Govt of India
Indonesia	National Agency of Drug and Food Control
Islamic Republic of Iran	Department of Pharmaceutical Affairs
Kuwait	Kuwait Drug and Food Control Administration
Malaysia	Drug Control Authority (DCA)
Mexico	Dirección de Medicina Tradicional (Traditional Medicine Directorate)
Mongolia	Traditional Medical Science Technology and Production Corporation
Myanmar	Department of Traditional Medicine, Union of Myanmar
Nepal	Department of Ayurveda
Philippines	Philippine Institute of Traditional and Alternative Care
Republic of Korea	Bureau of Oriental Medicine
Singapore	TM/CAM National Office under Ministry of Health
Sri Lanka	Department of Ayurveda in the Ministry of Health
Switzerland	Swiss Agency for Therapeutic Products (Swissmedic)
Thailand	Department for Development of Thai Traditional and Alternative Medicine, Ministry of Public Health

TABLE 21.1 Regulatory Status of Herbal Medicine Across Different Countries—cont'd

Country	Regulatory Body
UAE	Committee for Evaluation of Qualifications of Doctors and Specialists in Complementary and Alternative Medicine
Ukraine	Committee on National and Nontraditional Medicine
United Kingdom of Great Britain and Northern Ireland	Medicines and Healthcare Products Regulatory Agency and the Department of Health in England
United States of America	DSHEA Dietary Supplement Health and Education Act under USFDA
Vietnam	Department of Traditional Medicine

21.10.1 Australia

Phytotherapies and health products in Australia are known as "complementary medicines." The ingredients of these medicines are herbs, vitamins, minerals, organic health supplements, homeopathic, and aromatherapy preparations; they are mentioned as and are regulated as medicines under the Therapeutic Goods Act 1989. Any therapeutic substance consisting of one or more active herbal ingredients is defined as a complimentary medicine. Schedule 14 of the law mentions that clearly established efficacy and traditional use is required. The traditional medicines, such as Ayurvedic medicines, Traditional Chinese medicines, nutri-supplements, and aroma therapy oils, are also included in this. Australia encompasses a risk-based approach with a two-layer system for the regulation of natural health products and complementary medicines, which are classified as high and low risk, and are included in the Australian Register of Therapeutic Goods (ARTG). Some complementary medicines are exempt from the requirement to be included on the ARTG, such as preparations of homeopathic medicines. The Australian Regulatory Guidelines for Complementary Medicines (ARGCM) provides details on the regulation of complementary medicines and assists sponsors to satisfy their legislative obligations. TGA postmarket surveillance activities relate to the observation of the continued safety, quality, and efficaciousness of listed, registered, and included therapeutic products once they are on the market. Information on the approach of the TGA to managing compliance risk is obtainable at the TGA web site. The TGA conjointly, undertakes listed medicine compliance reviews. Generally, medicines, together with complementary medicines, have sudden and undesirable effects. The TGA has a robust review program, that involves the assessment of adverse events that are reported to the TGA by customers, health professionals, the pharmaceutical business, international medicine regulators, or by the medical and scientific consultants on TGA consultative committees. Sponsors of medicines are required to report back to the TGA suspected adverse reactions for their medicines. Under the TGA, therapeutic products, including complementary medicines, should be promoted and advertised for the standard use of the merchandise and in a way that is socially accountable, and does not mislead or deceive the buyer. The advertising of therapeutic merchandise in Australia is subject to the advertising requirements of the Therapeutic merchandise Act, that adopts the Therapeutic Goods Advertising Code (TGAC) and the supporting laws, the Trade Practices Act 1975, and different relevant laws (Ramzan, 2015).

21.10.2 Canada

In Canada, complementary/alternative and traditional medicines are called natural health products (NHPs) and are subjected to food and drug laws. Natural health products embrace herbal medicines; ancient Chinese, Ayurvedic, and native North American medicines; medical care preparations; and food and mineral supplements. NHPs are products that are used and marketed for a variety of health functions, such as for the prevention or treatment of any disorder or condition, the reduction of health risks, or the maintenance of health. As outlined in Canada, an NHP could be a substance or a mixture of drugs represented in Schedule-1 of the Natural Health Product Regulation (NHPR), a medical care drug, or a conventional drug, which is meant to have a medicinal activity or other direct impact in the identification, treatment, mitigation, or prevention of a sickness, disorder, or abnormal physiological condition or its symptoms in humans in a fashion that maintains or promotes health. Also, some consumer products, such as toothpastes, antiperspirants, shampoos, facial products, and mouthwashes, are classified as NHPs in Canada due to the presence of natural ingredients and because of their uses.

In Canada, the industrial sale of natural health products is subjected to the licensing provisions of the Natural Health product laws that came into force in 2004. The aim of the laws is to assure that Canadians have access to NHPs that are

safe, effective, and of top quality. To be lawfully sold in Canada, all NHPs should have a Product License and therefore the Canadian sites that manufacture, package, label, and import these products should have website licenses. There are specific labeling and packaging requirements and good manufacturing standards and proof norms that have to be met so as to get product and website licenses. The licensing necessities of the Natural Health product laws to be applied to a person or company that manufactures, packages, labels, and/or imports NHPs for industrial sale in Canada. The licensing requirements do not apply to health care practitioners who compound products on a private basis for their patients or to retailers of NHPs. Canadian physicians choosing to supply various treatments should adjust to guidelines set by the faculty of Physicians and Surgeons of the relevant province.

Most of Canada's legislation, such as the Canada Health Act, focuses on traditional medical practitioners. However, the regulation of medical professionals is a provincial matter and a few Canadian provinces have become tolerant of nontraditional aid suppliers. The Natural Health product laws are administered by Health Canada's Natural Health Product Program, which is comprised of three directorates, each one with its own specific roles and responsibilities. They are the Natural Health Product Directorate, Marketed Health Product Directorate, and Health Product and Food Branch Inspectorate. The Natural Health Product Directorate (NHPD) is the lead board of directors for the Natural Health Product Program and is the controlling authority for the industrial sale of NHPs in Canada. The NHPD is responsible for the assessment and supply of product and website licenses.

The Marketed Health Product Directorate (MHPD) provides a uniform approach to postapproval safety, assessment of signals, safety trends, and risk communications regarding all regulated marketed health products, including NHPs. The management of adverse reactions involving NHPs is also the responsibility of the MHPD. People having an adverse event are encouraged to report it to the MHPD straightaway. The Health Products and Food Branch Inspectorate (HPFBI) are accountable for the social control of the laws and carries out any needed compliance action, together with product recalls and investigations. Health Canada conjointly consults often with two external consultative committees: the Management Advisory Committee (MAC) and the Expert Advisory Committee (EAC) comprised of industrial and consumer representatives. Members of skilled and scientific communities compose the EAC, which provides knowledgeable recommendations to Health Canada on problems regarding the security, efficacy, and quality of NHPs (Ramzan, 2015).

21.10.3 China

The administrative department of public health under the State Council is responsible for the management and control of the protection of varieties of Traditional Chinese Medicine (TCM) throughout the country. The State Administrative departments for the manufacture and commercialism of Traditional Chinese medicines (TCM) shall assist the executive department with the management of the protection of the varieties of Traditional Chinese Medicine (TCM) throughout the country. Regulations in China are developed for the upliftment of the standard of ancient Chinese drugs, protecting the legitimate rights and interests of TCM manufacturing enterprises, and promoting TCM. The laws apply to varieties of TCM drugs in China, together with patented ancient Chinese medicines, extracts, and preparations from natural healthy materials, as well as Traditional Chinese Medicines.

Research and development of traditional Chinese medicine with respect to clinical effects and practices has set up a classification protection system for different types of traditional Chinese medicine to ensure quality and efficacy. In the national pharmaceutical standards, the types of traditional Chinese medicine have been classified under this regulations system. The classification of traditional Chinese medicine according to the standards of provinces, autonomous regions, and municipalities are controlled by the department of Public Health under the State Council or under the Central Government. They are mainly two types, first-class protection and second-class protection. For new drug approval, the manufacturer directly applies to the state or central authority in a class they find suitable according to the rules and regulation of the central government. At first, the approval is taken from the local administrative department for the production and trading of traditional Chinese medicine followed by the department of public health (Ramzan, 2015).

21.10.4 European Union

Herbal medicines are manufactured following GMPs to maintain the quality of the finished product and to ensure safety. For herbal medicinal products, it must be demonstrated that the herbal medicine has been in use for a minimum of 15 years within the EU and 30 years outside the EU. The European traditional herbal product directive has established a restrictive approval technique for herbal medicines in the Economic Community (EC). This Directive desires each member state to have a general herbal registration scheme for manufactured traditional herbal medicines that is applicable to be used not as medical management. The EU medicinal product Directive 2001/83/EC requires that applications for authorization to

position a health supplements on the market to have a dossier containing particulars and documents relating especially to the results of physicochemical, biological, or microbiological tests, as well as pharmacological and toxicologic tests and clinical trials allotted on the product and so proving its quality, safety, and effectiveness. However, the applicant can demonstrate by elaborate references based on scientific literature that the constituent or the constituents of the health product will have a well-established health use with recognized efficacy and an acceptable level of safety within the meaning of Directive 2001/83/EC with no need of preclinical or clinical trial reports (Ramzan, 2015).

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Quality Control and Evaluation of Herbal Drugs

Evaluating Natural Products and Traditional Medicine

Prof. Pulok K. Mukherjee

Quality Control and Evaluation of Herbal Drugs brings together current thinking and practice in the area of quality control and standardization of herbal drugs. The use of herbal medicine in therapeutics is on the rise in both developed and developing countries, and this book facilitates the necessary development of quality standards for these medicines.

A leader in the field, Pulok K. Mukherjee describes and discusses the various methods, techniques, and approaches for evaluating the purity, quality, safety, and efficacy of herbal drugs. Particular attention is paid to methods that assess these drugs' activity, the compounds responsible, and their underlying mechanisms of action. The book describes the quality control parameters followed in India and other countries, including Japan, China, Bangladesh, and other Asian countries, as well as the regulatory profiles of the European Union and North America. This book is also useful as a bioprospecting tool for traditional-medicine-inspired drug discovery and development.

Key Features

- Provides new information on the research and development of natural remedies - essential reading on the study and use of natural resources for preventive or healing purposes.
- Brings together current thinking and practices in quality control of herbal drugs, highlighting several approaches for plant metabolomics, chemo-profiling, marker analysis and standardization.
- Aids in developing knowledge on various techniques - macroscopy, microscopy, HPTLC, HPLC, LC-MS/MS, GC-MS etc for quality evaluation of herbal drugs.
- Assessment of herbal drugs through bio-analytical techniques, bioassay guided isolation, enzyme inhibition, pharmacological, microbiological, antiviral assays and safety related quality issues.
- Highlights on the development of integrated methods for evaluation of botanicals used in traditional medicine.
- References global organizations WHO, USFDA, CDSCO, TCM, and others to serve as a comprehensive document for the enforcement agencies, NGOs, and regulatory authorities concerned with herbal medicine at large.

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