Evidence-Based Validation of Herbal Medicine

Edited by **Pulok K. Mukherjee**

EVIDENCE-BASED VALIDATION OF HERBAL MEDICINE

This page intentionally left blank

EVIDENCE-BASED VALIDATION OF HERBAL MEDICINE

Edited by

PULOK K. MUKHERJEE
School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

ELSEVIER

AMSTERDAM BOSTON HEIDELBERG LONDON NEW YORK OXFORD
PARIS SAN DIEGO SAN FRANCISCO SINGAPORE SYDNEY TOKYO

Elsevier
Radarweg 29, PO Box 211, 1000 AE Amsterdam, Netherlands
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK
225 Wyman Street, Waltham, MA 02451, USA

Notices
Knowledge and best practice in this field are constantly changing. As new research and experience broaden our understanding, changes in research methods, professional practices, or medical treatment may become necessary.

Practitioners and researchers must always rely on their own experience and knowledge in evaluating and using any information, methods, compounds, or experiments described herein. In using such information or methods they should be mindful of their own safety and the safety of others, including parties for whom they have a professional responsibility.

To the fullest extent of the law, neither the Publisher nor the authors, contributors, or editors, assume any liability for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions, or ideas contained in the material herein.

ISBN: 978-0-12-800874-4

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

Contents

This page intentionally left blank

Contributors

Aijaz Ahmad Department of Pharmaceutical Sciences, Tshwane University of Technology, Pretoria, South Africa

Shiv Bahadur School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Sanjay K. Banerjee Drug Discovery Research Center, Translational Health Science and Technology Institute (THSTI), Faridabad, Haryana, India

Subhadip Banerjee Bengal Institute of Pharmaceutical Sciences, Kalyani, West Bengal, India

Santanu Bhadra School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Sauvik Bhattacharyya School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Rajarshi Biswas School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Anthony Booker Centre for Pharmacognosy and Phytotherapy/Research Cluster Biodiversity and Medicines, UCL School of Pharmacy, London, UK

Rainer W. Bussmann William L. Brown Center, Missouri Botanical Garden, St. Louis, MO, USA

Joydeb Chanda School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Debprasad Chattopadhyay ICMR Virus Unit, I.D. & B.G. Hospital, Beliaghata, Kolkata, India

Sushil K. Chaudhary School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Søren Brøgger Christensen Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, Denmark

Geoffrey A. Cordell Natural Products Inc., Evanston, IL, USA

Manoj K. Dalai School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Pratip K. Debnath Gananath Sen Institute of Ayurvdiya and Research, Kolkata, India

Parikshit Debnath S.D.M College of Ayurveda and Hospital, Hassan, India

Fabio Firenzuoli Center for Integrative Medicine, Careggi University Hospital of Florence, Florence, Italy; Referring Center for Phytotherapy, Tuscany Region, School of Medicine, University of Florence, Florence, Italy

Luigi Gori Referring Center for Phytotherapy, Tuscany Region, School of Medicine, University of Florence, Florence, Italy

Debayan Goswami ICMR Virus Unit, I.D. & B.G. Hospital, Beliaghata, Kolkata, India; School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Ranjit K. Harwansh School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Michael Heinrich Centre for Pharmacognosy and Phytotherapy/Research Cluster Biodiversity and Medicines, UCL School of Pharmacy, London, UK

Christian Huck Institute of Analytical Chemistry and Radiochemistry, CCB – Center for Chemistry and Biomedicine, Leopold-Franzens University, Innsbruck, Austria

Deborah Johnston Department of Economics, School of Oriental and African Studies, University of London, London, UK

Amit Kar School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Sanmoy Karmakar Department of Pharmaceutical Technology, School of Natural Product Studies, Jadavpur University, Kolkata, West Bengal, India

Deepak Kasote Department of Pharmaceutical Sciences, Tshwane University of Technology, Pretoria, South Africa

Mahnaz Kazemipoor Department of Science & Technology Studies, University of Malaya, Kuala Lumpur, Malaysia

Werner Knöss Federal Institute for Drugs and Medical Devices, Bonn, Germany

Gail B. Mahady Department of Pharmacy Practice, College of Pharmacy, PAHO/WHO Collaborating Centre for Traditional Medicine, University of Illinois at Chicago, Chicago, IL USA

Subir K. Maulik Department of Pharmacology, All India Institute of Medical Sciences, New Delhi, India

José L. Medina-Franco Facultad de Química, Departamento de Farmacia, Universidad Nacional Autónoma de México, Mexico City, Mexico

Achintya Mitra National Research Institute of Ayurvedic Drug Development, Kolkata, India

Supriya Mondal ICMR Virus Unit, I.D. & B.G. Hospital, Beliaghata, Kolkata, India

Kakali Mukherjee School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Pulok K. Mukherjee School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Lutfun Nahar Medicinal Chemistry and Natural Products Research Group, School of Pharmacy and Biomolecular Sciences, Faculty of Science, Liverpool John Moores University, Liverpool, United Kingdom

Neelesh K. Nema School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

Durbadal Ojha ICMR Virus Unit, I.D. & B.G. Hospital, Beliaghata, Kolkata, India

Bhushan Patwardhan Interdisciplinary School of Health Sciences, Savitribai Phule Pune University, Maharashtra, India

Mukhlesur Rahman Medicine Research Group, School of Health, Sports and Bioscience, University of East London, Stratford Campus, London, UK

Roy Upton R.H. American Herbal Pharmacopoeia, Scotts Valley, CA, USA

Indra Neil Sarkar University of Vermont, Burlington, VT, USA

Ratul Sarkar Department of Pharmaceutical Technology, School of Natural Product Studies, Jadavpur University, Kolkata, West Bengal, India

Satyajit D. Sarker Medicinal Chemistry and Natural Products Research Group, School of Pharmacy and Biomolecular Sciences, Faculty of Science, Liverpool John Moores University, Liverpool, United Kingdom

Tuhinadri Sen Department of Pharmaceutical Technology, School of Natural Product Studies, Jadavpur University, Kolkata, West Bengal, India

Alvaro Viljoen Department of Pharmaceutical Sciences, Tshwane University of Technology, Pretoria, South Africa

Foreword

EVIDENCE-BASED MEDICINE, A NEED FOR PARADIGM SHIFT!

We are living in a rapidly changing world, with new economic realities, and new challenges for the production of food and for health care. In the past 60 years a number of novel medicines have been introduced which are used to treat various diseases, though only a few, mainly the antibiotics, do cure patients. Now for the most important ailments medicines are available. But to develop better ones, or to develop medicines for minor diseases or diseases of the poor, the costs are far too high to bring a novel drug to the market for the patients concerned. Estimations are around 1 billion € for a single novel drug, moreover drug development is a time-consuming process, with 8−12 years from finding a hit to the clinical application. Pharmaceutical industry has no economic incentives in developing novel drugs for small markets and as a result the pipeline of novel small molecule drugs is stalling, with every year less real novel small molecule drugs. At the same time globally still up to 80% of the people are using traditional, mostly herbal, medicines. Some 40,000−70,000 plant species have one or more medical applications in various systems of traditional medicine. In general they are cheap and locally readily available.

Already since many years a number of governments and international organizations have advocated the use of such local traditional medicine in primary health care. But to achieve this, one should have at least evidence for nontoxicity and preferably even know the active compound(s) and their mode of action. With other words there is an urgent need for evidence-based traditional medicines to be able to meet the needs of the poor. At the same time, it may, open the way for novel ideas to develop small molecule drug by finding new leads, targets, or even concepts, like the use of synergy between compounds. Moreover, such information is needed for an adequate level of quality control. Natural products research has the important challenge to deliver the information needed for evidence-based use of traditional medicines.

There are, however, a number of hurdles to overcome. First of all in the present system in the Western world organizations like EMA and FDA make the rules for registration of novel medicines. These rules are based on a single target, single compound paradigm. In traditional medicine often mixtures of plants are used, in which each ingredient does have a certain meaning, and the ingredients and amounts given are part of a personalized medication. Personalized medicine in the Western world is only used in case of very toxic medicines, e.g., in cancer therapy, but otherwise more or less all people get exactly the same dose of a medicine. The formulations are made with very high precision containing the active compound in a certain amount with not more than 1% standard deviation. Obviously such a system is not at all ready to deal with the problem of every person receiving a different mixture of plant extracts, using raw materials in which the variability in the content of active ingredient(s) with no doubt is more than such a 1% standard deviation.

New approaches are thus required to deal with these problems. Particularly in Asia much of the traditional knowledge has already been recorded in books thousands of years ago and still plays a very important role in health care. Therefore this is the place for developing novel approaches supported by their rapidly growing economies: a unique momentum for local pharmaceutical companies.

This book is an effort to bring together the views, expertise and experience of leaders in the field of medicinal plant research and development with the aim to show what is expected and thus needs to be done to come to evidence-based medicinal plants. Eventually this should lead to approaches that generate all the necessary information to register medicinal plants. Evidence that also convinces the regulatory authorities that now advocate the single target, single compound paradigm. There is a major task for us in the natural product research field. A task which requires the close collaboration between pharmacology, toxicology, natural products chemistry, and bioinformatics. New approaches as systems biology to study single patients treated with personalized traditional medicines, instead of large-scale clinical trials, using the various omics technologies need to be developed and used in conjunction with reductionist approaches to confirm activity of compounds identified with activity. It requires more in depth studies and not the more of the same as we see too much happening now, like in vitro screening of many plants at single dose for one single activity. We need first of all to show pharmacological activity and safety of the traditional medicines, based on that one may see if there are possibilities to develop novel leads from these plants.

To encourage the study of traditional medicine there is also an urgent need for protecting the rights of the development of an evidence-based traditional medicine, just as for any novel single compound medicine. The present patent laws will not accept the development of an example, traditional antidiabetic medicine as an innovation, consequently when that evidence has been obtained, anyone may use that information and produce and market the medicine. With other words there is a need for an economic incentive for developing traditional medicine.

This book should thus make a major contribution to the global discussion how to explore and exploit the ancient knowledge to the benefit of mankind. To again make discoveries like morphine, atropine, and artemisinin, evidence-based medicine needs a paradigm shift to get access to the heritage of our ancestors!

Prof. Dr. Rob Verpoorte
Natural Products Laboratory, IBL, Leiden University
The Netherlands
E-mail: Verpoort@Chem.leidenuniv.nl.

Preface

EVIDENCE-BASED VALIDATION OF HERBAL MEDICINE

In today's world, the role of herbal medicine has increased manifold. The use of herbal medicine in therapeutics is becoming more popular. In the era of revolution in herbal medicines, the need of the day is the development of an evidence base for validation for production, evaluation, regulation, safety, and allied aspects of natural products. Herbal medicines are evaluated, validated, and regulated in various countries according to their own system. Globalization in the context of modern drug development will increase the practice and use of natural products worldwide, and herbal medicine is still open to fascinating realms of research. Development of secondary metabolites and natural leads by high-throughput screening offers exciting frontiers of future research. This book highlights several aspects of natural products for validating the quality, safety, and efficacy of herbal medicine, particularly methods to assess their activity and underlying mechanisms of action with a view to improve standards used in different systems of medicine. It will provide a current cutting-edge scientific research on natural remedies, and therefore, reading of this edited volume will be essential for everyone whose professional life impinges on the use of natural resources.

Development of natural products requires the confluence of modern techniques and integrated approaches in various fields of science and technology. This book provides state-of-the-art reviews from researchers around the world on various aspects for evaluation of herbal medicine and will help researchers to know about their validation to exploit traditional medicines (TMs) for drug discovery and development. It will be a very useful publication, which will not only serve as a handy tool for students and researchers in this area but will also provide the most recent methodologies developed for evidence-based validation, phytochemical and pharmacological evaluation of herbal drugs in all aspects from field to bed side. With the emerging interest, this book will encourage the continuing efforts to understand TM-inspired drug development as well as the roles of TM in the global health care at large. The main aim of this book is to improve the level of understanding of various aspects on evaluation of natural products to provide a comprehensive validation of herbal medicine, so that they can be used with greater confidence, because of improved quality and raising a scientifically sound evidence base. It will also be an imperative essential reference for those involved in the fields of herbal medicine, traditional remedies, pharmaceutical sciences, and natural product research. I am sure it will be meant for a global readership and to provide a structured approach to the evidence-based evaluation of herbal medicinal products.

Pulok K. Mukherjee, PhD, FRSC
Editor,
School of Natural Product Studies,
Department of Pharmaceutical Technology,
Jadavpur University, Kolkata, India

This page intentionally left blank

Acknowledgments

EVIDENCE-BASED VALIDATION OF HERBAL MEDICINE

The enormous growth of herbal medicinal products worldwide has been one of the most interesting aspects of healthcare. Harmonization on the different facets of development of herbal medicine, including their quality, safety, efficacy, validation, and regulation, is best possible through international coordination.

The intention of this book is to describe and assess various approaches for evidence-based validation of herbal medicine, which has been described in different chapters of this edited volume by eminent scientists and technologists from different countries. I would like to express my gratitude to all of them for their valuable contributions.

I would like to express my heartfelt thanks to Prof. Robert Verpoorte from Leiden, Netherlands, for his support and encouragement for my works and particularly for writing the Foreword for this book.

It would not be possible to complete this work without the active help from my research group.

I gratefully acknowledge the help and support rendered by my research scholars particularly Mr Sushil K. Chaudhary, Mr Ranjit K. Harwansh, and Dr. Neelesh K. Nema for their active cooperation and assistance. I also thank Dr. Santanu Bhadra, Mr Amit Kar, Mr Rajarshi Biswas, Mr Shiv Bahadur, Mr Joydeb Chanda, Sk Milan Ahmmed and Mr Sankarshan Saha for their active help.

I extremely appreciate the interest taken by Elsevier, USA, for producing this edited volume. I gratefully acknowledge the cooperation and support received from Jill Cetel, Katey Birtcher, Beth Campbell, and Sharmila Vadivelan from Elsevier in bringing out this book.

My father, Shri Harihar Mukherjee inspired me a lot in all of my works. Unfortunately, he passed away a few months before this book was written. A man I intensely admire, he continues to live in my heart.

I am thankful to my family, my mother, my wife Dr. (Mrs) Kakali Mukherjee, daughter Maria and son Manish, for all the support and love they give to me always, you are the reason behind my every successful step.

Pulok K. Mukherjee PhD, FRSC
Editor,
School of Natural Product Studies,
Department of Pharmaceutical Technology,
Jadavpur University, Kolkata, India

This page intentionally left blank

CHAPTER

1

Quality Related Safety Issue-Evidence-Based Validation of Herbal Medicine Farm to Pharma

Pulok K. Mukherjee, Shiv Bahadur, Sushil K. Chaudhary, Amit Kar, Kakali Mukherjee

School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

OUTLINE

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00001-5

1.1 HEALTH CARE THROUGH HERBAL MEDICINE

Herbal medicines attract the interest of both patients and scientists, in all aspects of drug development from natural products and also for validation of traditional medicine (TM). Several developing countries rely on TM because of their accessibility and affordability, and scientists all over the world consider medicinal plants as a source of new chemical entities and use them to isolate compounds such as digoxin, morphine, taxol, atropine, and vinblastine [1]. Herbal medicines have an important position in health care systems worldwide; their current assessment and quality control are a major bottleneck. Many adverse events of herbal medicines can be attributed to the poor quality of the raw materials or the finished products. Quality issues of herbal medicines can be classified into two categories, external and internal. External issues include toxic metals, pesticides residues, microbes, adulteration, and misidentification of medicinal plants. The internal issues affecting the quality of herbal medicines are complexity and nonuniformity of the ingredients. Through the use of modern analytical methods and pharmaceutical techniques, previously unsolved internal issues have become solvable [2]. The increasing search for therapeutic agents derived from plant species is justified by the emergence of diseases. Medicinal plants serve as the most valuable source for curing many diseases. Herbal medicines include herbal extracts, herbal drug preparations, and herbal drugs. Herbal drugs are unprocessed parts of plants or whole plants [3]. Herbs include crude plant material such as leaves, flowers, fruit, seed, stems, wood, bark, roots, rhizomes, or other plant parts, which may be entire, fragmented, or powdered. Herbal preparations include comminuted or powdered materials or extracts, tinctures, and fatty oils of herbal materials, which may be produced by extraction, fractionation, purification, concentration, or other physical or biological processes [4].

Modern allopathic medicine has developed from ancient medicine, and it is likely that many important new remedies were discovered and commercialized following the leads provided by traditional knowledge and experiences. The study of these traditions not only provides an insight into how the field has developed but it is also a fascinating example of our ability to develop a diversity of cultural practices [5]. The administering of a pure chemical or a plant extract containing the same chemical entity is essentially different. The difference is mainly due to the complexity of a plant extract that introduces many variables to conventional phytomedicinal research, which could possibly contribute to chemical complexity and bioactivity. On administration of plant material of *Artemisia annua* versus the pure drug, for example, artemisinin, showed that the bioavailability from the leaves was 45 times more than that of the pure drug [6]. Thus, the complexity of the plant extract could have contributed to the increased bioavailability and thus the bioactivity. A genuine interest on various traditional practices now exists among practitioners of modern medicine and a number of practitioners of traditional, indigenous, or alternative systems are beginning to accept and use some of the modern technologies. Proper methodologies for the research and development, manufacturing, and quality control of the formulations in TM and investigations of the therapeutic potentials of plants used in those systems with support of scientific methods may help to use them with maximum possible efficacy [7].

1.2 INTEGRATED APPROACHES FOR DEVELOPMENT OF HERBAL MEDICINE

The international trade in herbal medicine has attracted most of the pharmaceutical companies, including the multinationals. Until a few years ago, only small companies had interest in the marketing of herbal medicines. Currently, several large multinational companies are interested in commercializing herbal drugs [8]. The world market for herbal medicine, including herbal products and raw materials, has been estimated to have an annual growth rate upto 15%. Several integrated approaches in herbal research for promotion and development of natural products are shown in Figure 1.1.

1.2.1 Opportunities and Challenges in Herbal Medicine

With the global increase in the demand for medicinal plant or plant-derived medicines, there is a call for ensuring the quality and safety of herbal drugs using several modern analytical techniques. Chemical constituents in herbal medicine may vary depending on harvest seasons, plant origins, drying processes, and other related factors. Thus, it seems to be necessary to determine most of the phytochemical constituents of herbal products in order to ensure the reliability and repeatability of pharmacological and clinical research, to understand their bioactivities and possible side effects so as to enhance the quality of the herbal products [9]. Quality control of herbal medicines aims to ensure their quality, safety, and efficacy. The lack of chemical markers remains a major problem for the quality control of herbal medicines. In many cases, we do not have sufficient chemical and pharmacological data of chemical markers. Further, there are many technical challenges in the production of markers. For example, temperature, light, and solvents often cause degradation and/or

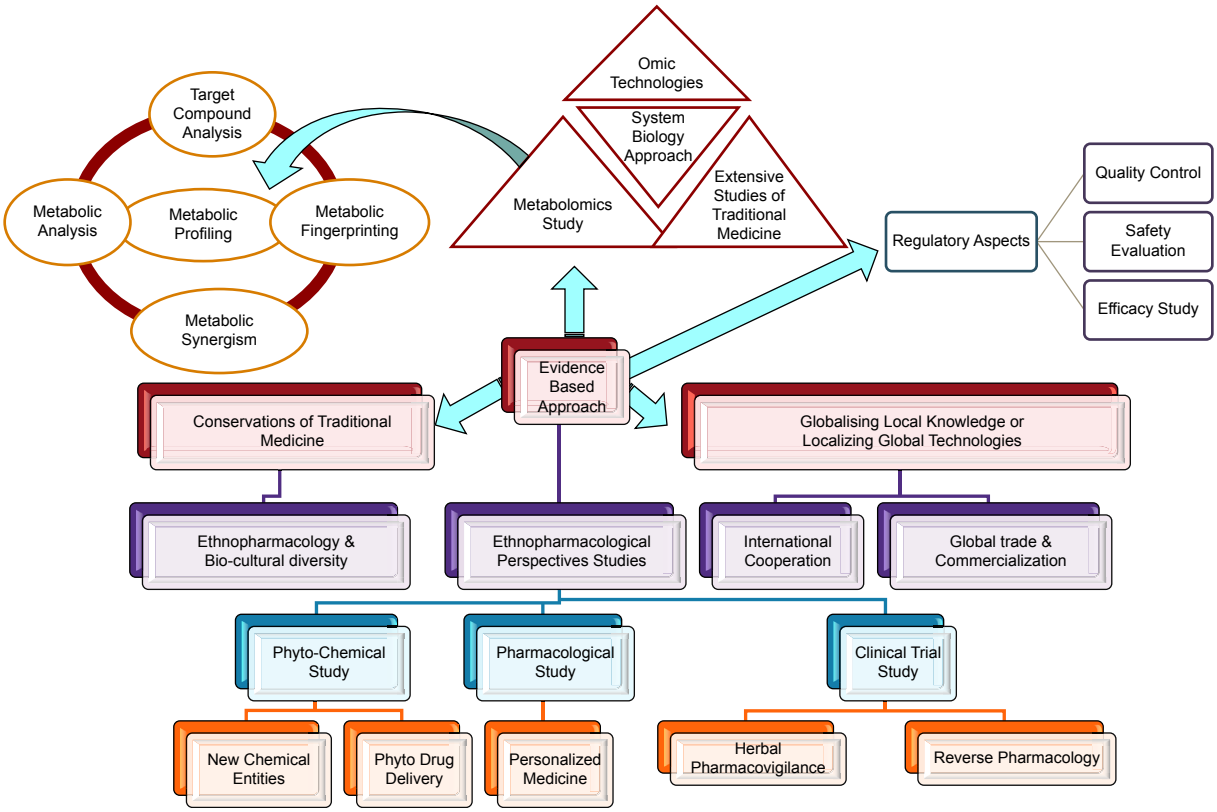


FIGURE 1.1 Integrated approaches in botanical research.

transformation of purified components; isomers and conformations may also cause changes in the markers. However, a concept of understanding the complex principles of herbal medicine must be developed through marker profiling and related approaches so as to develop evidence-based practice of herbal medicine [10]. Evidence-based submissions for regulatory approval and interlinking of various pharmacopoeial and monographs would be helpful for herbal manufacturers to the regulated markets across the world. A general comparison of the pharmacopoeial standards reveals that there is a wide variation in plant-specific parameters and quality standards of different nations. With respect to Southeast Asia, India is among the leading countries with respect to development of pharmacopoeial standards as well as modification of existing regulatory guidelines [11].

The major challenges for the development and promotion of TM include the chemoprofiling, safety evaluations, quality control, and effective regulatory guidelines for herbal medicines [12]. Wisdom and compassion-enhanced global collaboration and leadership are needed to change the contemporary paradigms and develop new strategies for the enhancement of TMs and dietary supplements. Research through collaboration and cooperation across the nation can help to a large extent in the promotion and development of the TM for the betterment health care globally [13]. Development and evaluation of medicinal plant-derived products are being controlled and implemented through various agencies in different countries. This provides unique advantages for researchers and pharmaceutical industries to enhance drug discovery and development [14].

1.2.2 Several Aspects for Revitalization of Medicinal Plants

In order to revitalize herbal medicine in line with modern medicine, various strategic areas in medicinal plant research are being considered. Scientists are convinced that the integration of herbal medicine with modern tools will not only benefit their own development but will also help to fight against many complex diseases through development of new entities [15]. It is a fact that a very large number of medicines are derived from plants or plant-derived synthetic analogs. Dedicated research would be beneficial only with support from advanced approaches and novel strategies [16]. Numerous methods exist to evaluate the quality of either natural or synthetic substances. Several in vitro, in vivo, and high-throughput screening methods are currently involved in traditional drug discovery approaches [17]. During the past decades, public interest

in natural therapies, namely, herbal medicine, has increased dramatically not only in developing countries but also mainly in industrialized countries [18]. This has increased the international trade in herbal medicine enormously and has attracted most of the pharmaceutical companies, including the multinationals. India is one of the few countries that are capable of producing most of the important plants used in modern as well as in traditional systems of medicine. In the modern era, combinatorial chemistry and high-throughput screening are very useful methods, and so many new drug molecules are emerging from herbal resources. The traditional use of medicinal plants needs to be systematically investigated and standardized from the perspective of quality, safety, and efficacy [14]. Although there has been an increase in interest in science-based research into herbal medicine, much of the research to date has been plagued by studies conducted using unauthenticated, uncharacterized products. One of the most important issues involved in any research study is the quality of the test material. A study cannot be considered scientifically valid if the material tested was not authenticated and characterized such that the material can be reproduced. In the case of botanicals, there may be misidentification of the collected plant, adulteration with other species, or contamination with extraneous ingredients [5].

1.3 USE OF HERBS IN TM

TM generally refers to those medical and health care systems that are practiced in a traditional manner from ancient times, and this discipline is not considered to be a part of conventional modern medicine. Over several years, this system has evolved on the basis of religious beliefs and social edifices of several indigenous peoples by exploiting the natural resources and more recently by developing a scientific method for validating therapeutic and preventive approaches [19]. However, TM is not always documented properly through evidenced-based scientific validation as in conventional modern medicine. TMs are more easily accepted by most people due to their strong beliefs faith, practical benefits, economical advantage, easy access, and many other reasons that have regional, religious, and social bases, etc. [20]. In almost all TM systems, botanicals as well as medicinal plants play a key role and constitute the backbone of TM. The Indian material medica includes approximately 2000 drugs of natural resources, nearly all of which are derived from different traditional systems of medicine and Indian folklore practices. Many conventional modern drugs originate from different natural sources especially medicinal plants: a century ago, most of the effective drugs were plant based [21]. Drug development from medicinal plants continues, with drug manufacturing companies engaged in large-scale pharmacological screening of herbs. In TM, some popular herbs such as Turmeric, Neem, Ginger, Holi Basil, Ashwagandha, and Rauwolfia., create a revival of interest in herbal products at a global level [22]. Around 60% of the global health care product market is dominated by medicinally useful formulations and other health products, derived or developed from botanicals. In India, around 25,000 traditional and folk medicinal effective plant-originated formulations are used. In India, more than 1.5 million consultants are using traditional medicinal systems for health care, and more than 7800 manufacturing units are involved in the production of natural health products (NHP) and traditional plant-originated formulations [11]. There is worldwide emerging interest in executing traditional practices in the health care system by exploring their therapeutic as well as preventive potential. In TM, various regulations and control on the use of botanicals have come up, which will not only help to cure different aliments through indigenous natural resources but will also help in the screening and evaluation of the medicinal plants in a better way to use them in traditional health care systems [23].

1.4 GLOBALIZATION OF TM

TM has been defined as skills and a practice based on the theories, beliefs, and experiences that are indigenous to different cultures. It is used in the maintenance of health care as well as in the prevention, diagnosis, and treatment of physical and mental illnesses [24]. Scientists around the world are highly emphasizing on medicinal plants as alternative medicine and their commercial potential in health care. Globalization of TM is necessary for the establishment of evidence-based health care, based on TM in consideration of its safety, efficacy, therapeutic, and clinical evidence [25]. Modern technology and science have developed many techniques and systems for core disciplines including ethnomedicine, ethnobotany, ethnopharmacology, and medical anthropology to promote TM compounds globally [10]. Establishment of global and/or regional regulatory harmonization is obligatory for its development and promotion through scientific validation. The development of TM and natural products requires the convergence of modern techniques and integrated approaches related to their evidence-based research in various fields of science through coordination and cooperation [26].

To combat the growing market demand, there is an urgency to expeditiously utilize and scientifically validate more medicinally useful plants globally, which

needs globalizing local knowledge and localizing global technologies, through international collaboration and cooperation. The major limits for the globalization of TMs are due to having different standards of TM products and practices, including varied terminology and philosophical approaches. Development of effective guidelines for safety, efficacy, and quality is regarded as a fundamental requirement in order to establish the evidence base for TM [27]. The International Union of Pure and Applied Chemistry (International of Pure and Applied Chemistry (IUPAC)) has published a series of protocols on quality control, safety, efficacy, standardization, and documentation of herbal medicine in which various significant aspects and features of phytochemistry and analytical chemistry have been described. If these strategies are fully implemented by the IUPAC, the World Health Organization (WHO) will explore TM from its pessimistic view to modern medicine [28].

1.4.1 Strategies for Globalization of TM

The term "globalization" means the increased mobility of individuals, information, goods, services, labor, technology, and capital throughout the world. There are huge databases of TM, which are used by ancient people as folk medicine, and this evidence was found in many written textbooks [29]. There are several strategies for the expansion of TM such as (1) addition in the health care system, (2) promotion of secure and valuable use, (3) increasing its access, (4) increasing communication, and (5) cooperation in generation and distribution of TM-related information. These strategies based on information, botany, chemistry, and biology of medicinal plant validation and quality control are essential [30]. In the era of modern research, some new drug molecules are emerging with the help of combinatorial chemistry and high-throughput screening from herbal resources.

A study cannot be considered scientifically valid if the material tested was not authenticated and characterized such that the material can be reproduced. In the case of botanicals, there may be misidentification of the collected plant, adulteration with other species, or contamination with extraneous ingredients. From the perspective of a regulatory action, these cases may range from simple misleading labeling to frank poisoning due to toxic contaminants. It can often be difficult to compare reported efficacy or toxicity studies even when "standardized" material has been used. Many studies refer to the use of standardized botanical material, which usually implies a chemical standardization [31]. Interdisciplinary approach of work on TM is to be explored for the discovery of novel bioactive compounds. Issues related to the appropriateness of conventional biomedical and clinical models for evaluating the efficacy of TM are sometimes very crucial. A holistic approach based on systems biology seems much more suited to study the therapeutic efficacy and pharmacodynamics of TM-based drug development [10]. Approaches for drug development based on traditional leads are described in Figure 1.2.

Most of the Indian and Chinese herbal formulations contain a mixture of herbs, and several methods are available to classify them. When two or more herbs

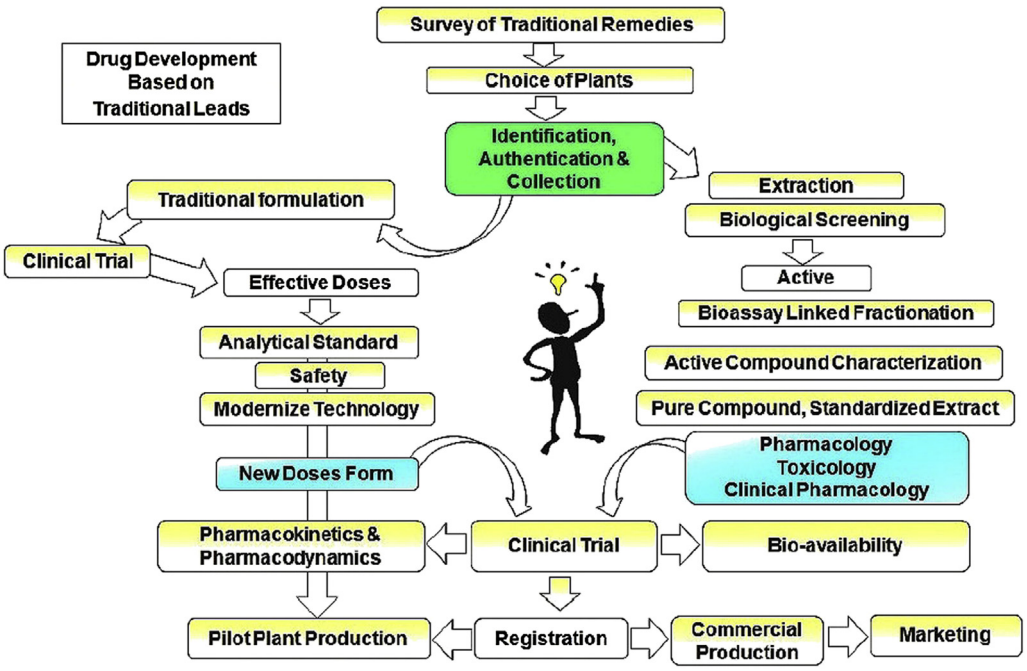


FIGURE 1.2 Drug development based on traditional claims.

with their bioactive compounds are combined to prepare formulations, they can be observed to have the following effects [32]:

- Synergistic significance
- Synergistic improvement
- Cocounteraction
- Mutual repression
- Communal aggression
- Communal inappropriateness

The main ingredient is a component that provides the main therapeutic action; the second ingredient potentiates the therapeutic actions of the other, which is known as synergistic reaction, and the component is synergism. The rest serve one of the following functions:

- Treat accompanying symptoms,
- Moderate the ruggedness or toxicity of the primary ones,
- Target the medicine to the proper organs,
- Exert a complementary effect.

In some cases, standardized bioactive compounds are subjected to animal models to correlate the presence of certain phytoconstituents with a pathophysiological condition of the human body [33]. In the context of modern biomedical research, there should also be necessary prerequisites for clinical trials.

1.5 TM INSPIRED DRUG DISCOVERY AND DRUG DEVELOPMENT

In order to revitalize herbal medicine in line with modern medicine, various strategic areas in medicinal plant research are to be considered of global importance [34]. Integration of herbal medicine and modern tools would not only benefit their own development but will also help to fight against many complex diseases through the development of new entities. Such dedicated research would be beneficial only with support from advanced approaches and novel strategies [35]. There are various thrust areas that play a very significant role for research and development of natural products as represented in Figure 1.3.

TM is helpful in all aspects of drug development from natural resources. A few examples of drugs from natural products would better explain the history of its own tradition. Several approaches on drug discovery and development from TM had been practiced by scientists for many years. Several therapeutically potential constituents were isolated from plants such as artemisinin (antimalaria), vincristine, vinblastine, camptothecin podophyllotoxin, etoposide, teniposide, and paclitaxel (anticancer) [36]. The development of drugs from ayurvedic plants is ongoing, with pharmaceutical companies engaged in large-scale pharmacologic screening of herbs. "Sushruta–Samhita," a Sanskrit text on Ayurveda written in 600 BC

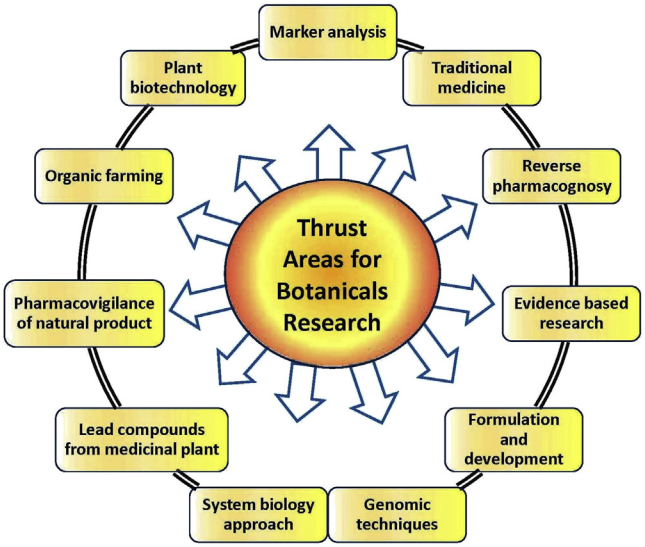


FIGURE 1.3 Thrust areas for botanical research.

noted that the plant *Commiphora mukul* Hook was useful in the treatment of obesity and related diseases. In recent years, a confluence of spectacular advances in chemistry, molecular biology, genomics, and chemical technology and the cognate fields of spectroscopy, chromatography, and crystallography may influence several therapeutically potent leading compounds from TM [11].

There are many approaches for the search of new biologically active principles from botanicals. One can simply look for new chemical constituents and find a biologist who will test the substance pharmacologically. This is not considered to be a very valid approach. A second approach is simply to collect every readily available plant, prepare extracts, and test each extract for one or more types of pharmacological activity. This testing will help in the standardization of extracts and the bioassay-guided isolation of the active constituents. The phytoconstituents obtained can then be taken further for structure–activity relationship studies [7]. Once all these factors are determined, the constituent/extract obtained can be further examined for its toxicity and safety evaluation, followed by clinical trials. This random collection and extensive screening method is a reasonable and the most effective approach that eventually should produce useful drugs, which can be well produced and formulated in industries. The classic method of pharmacologic screening involves sequential testing of herbal extracts or phytoconstituents from biological materials in isolated organs followed by testing in whole animals, mostly in rats and mice. Most of the drugs in use today as therapeutic agents have been found and evaluated with these methods [37]. However, for the evaluation of TM, we should not follow the reductionist approach, but go back to the holistic in vivo approach. This can be done in two different ways: one is through clinical trials; the

other is through animal experiments. Besides the classic physiologic observations that can be made by in vivo experiments, for example, blood pressure, analgesic activity, and sedation, nowadays, it is also possible to measure gene expression, the proteome, and the metabolome. These methods open up a completely new world of possibilities with several new technologies now giving a much better insight into the possible changes in the organism, in a holistic way. It will give us the possibility to better understand the mode of action by comparing the changes in the transcriptome, proteome, and metabolomic patterns when compared with those observed with known drugs. Such an approach is now known as the systems biology approach. The metabonomic approach requires the statistical analysis of large data sets by methods such as multivariate and principle component analysis to extract the information from these data [38]. Moreover, by using the systems biology approach for the organism combined with metabolomic data for the different extracts of the medicinal plant or fractions thereof, it should be feasible to make correlations between the occurrence of certain compounds in the extract and the activity.

Evidence-based medicine research should be conducted with the involvement of patients and funding bodies to establish a role of medical practitioners in decision making [39]. A widespread revolution in phytochemistry has been observed through strengthening its importance with the application of new technologies to enhance the original link between phytochemistry and TM. Evidence-based research includes developing policies, regulatory criteria, and technical guidelines that would ensure and provide the continued availability of quality, safety, and effective traditional medicinal products, which could support inclusion in health care systems, insurance programs, and on essential medicine lists [28]. Evidence-based submissions for regulatory authorization and interlinking of various pharmacopoeia and monographs would make it easy for herbal manufacturers and they will gain greater access to regulated markets across the world [40]. It is under these circumstances that some of the rationalists, scientists, scholars, and protagonists of alternative medicines dedicated themselves to the development of these alternative systems for drug development from natural resources, which required to be harmonized through international coordination.

1.6 ISSUES FOR QUALITY CONTROL AND QUALITY ASSURANCE OF HERBAL MEDICINE

Quality control and quality assurance of herbal medicines are very important to protect the integrity of the herbal extracts/products for the management of pharmaceutical quality. They have an important role for the reproducibility of the effect of the active ingredients from batch-to-batch uniformity. To maintain and comply with standard conditions with respect to quality, safety, and efficacy of herbal medicine, it is required to follow some important steps for the standardization [41]. This includes the (1) proper authentication and taxonomic assignment, such as through DNA fingerprinting and DNA bar coding; (2) structural elucidation of all isolated compounds of the medicinal plant; (3) identification and characterization of the bioactive constituents for the pharmacological activity; (4) standardization of the single extracts through spectroscopic analyses in the multicompound extracts; (5) international harmonization of specific standardization process under the umbrella of the International Federation of Pharmaceutical Manufacturers Associations. Therefore, it is very clear that major requisites for standardization of herbal products comply with international standards. There are several variables that can influence the standardization process. Therefore, it is compulsory to optimize all aspects of cultivation, harvesting, sample preparation, and sample processing to ensure reproducibility and eventually standardization of the herbal dugs. There are various new hyphenated technologies present such as chromatographic and spectroscopic analyses, which need to be effectively incorporated to ensure that sufficient quality control measures are implemented. By using several chromatographic and spectroscopic techniques, it is possible to analyze the full herbal product and thus generate a standardized "metabolic fingerprint" of specific herbal dugs. Metabolic profiling can then be incorporated to identify all the constituents [42]. The chemical fingerprints obtained from chromatographic or spectroscopic techniques should be similar in different samples. Spectroscopic and chromatographic techniques are now being used together, which leads to effective chemometric approaches. When these approaches are used in combination with chemometrics profiles, more precise data can be obtained that will be helpful in the establishment of the integrity of the herbal product and similarities and differences of the observed data will be produced [30].

Generally, it is believed that the risk associated with herbal drugs is very less, but reports on serious reactions indicate the need for the development of effective marker systems for isolation and identification of the individual components [43]. Standardization of herbal medicine includes the authentication of genuine drugs, harvesting of the best quality raw material, assessment of intermediate and finished product, and detection of harmful and toxic ingredients [44]. Several markers such as taxonomic, chemical, genomic, proteomic markers aid in the identification of herbal drug components. Chemical markers help in the identification of

adulterants, confirmation of collection site, and quality evaluation and diagnosis of herbal intoxication. As per the WHO definition, there are three kinds of herbal medicines that are obtained from raw plant material, processed plant material, and medicinal herbal products [38]. Herbal medicine products are dietary supplements that people take to improve their health and are marketed as tablets, capsules, powders, extracts, and fresh or dried plants. Herbals are traditionally considered harmless and increasingly being consumed by people, without any prescription. The evidence for the therapeutic actions of herbal drugs is documented in Indian, Chinese, European, and African systems of medicine [23]. There are several important aspects for quality control of herbal medicine that are shown in Figure 1.4.

The WHO has recognized the importance of the quality control of herbal medicine and developed a series of guidelines to assist several nations to develop their strategies for the quality control of herbal medicines and for conducting research on TMs [45]. The WHO had published the "Quality Control Methods for Medicinal Plant Materials," a collection of recommended test procedures for assessing the identity, purity, and content of medicinal plant materials to assist national laboratories engaged in drug quality control [46]. The WHO published the "Guidelines on good agricultural and collection practices (GACP) for medicinal plants" and in 2007, a new guideline "WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues" were formulated. The European Union, China, and Japan have developed regional and national guidelines for good agricultural and collection practices for medicinal plants that ensure that soil and irrigation water used for herbal material cultivation and propagation are within the limits or are free from harmful heavy metals, pesticides, herbicides, and toxicologically hazardous substances. The certification for this is based on parameters such as identification, water content, and chemical assay of active ingredients, inorganic impurities (toxic metals), microbial limits, mycotoxins, pesticides, and others [47]. From the cultivation to the final herbal product development of herbal products, there are so many significant factors that can influence the quality of herbal products. Some significant issues related to the quality control of herbal medicine are being described briefly in the subsequent section.

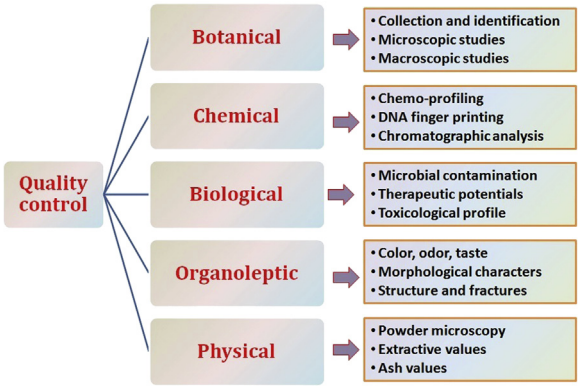


FIGURE 1.4 Important steps for quality control of herbs.

1.6.1 Contamination

There are so many contaminants mostly found in medicinal herbs including pesticides, heavy metals, microbes, and mycotoxins. Contaminations also present serious obstacles for the trade of herbal medicines [48]. Heavy metals have been found in herbal medicines with some regularity. Three most commonly detected toxic metals are mercury, arsenic, and lead. These contaminations may occur due to (1) the accumulation of heavy metals in the environment (e.g., from contaminated soil or atmosphere); (2) unintentional pollution during the production process; (3) deliberate addition. In some of the herbal products, residues of pesticides including their metabolites and depredated products remained in plants, and such residues have become a notable source of contamination for herbal medicines [49].

1.6.2 Adulteration

Adulteration in herbal medicine increases the impurity by adding some extraneous, improper, or inferior ingredients. Herbal medicines are adulterated with conventional drugs, and plant materials have repeatedly been documented. Adulterations can be done in the following way including addition of orthodox drugs, substitution of fake or inferior plant materials, and addition of foreign materials [35].

1.6.3 Misidentification

Conflicting to adulteration or substitutions, misidentification of herbal medicine mostly happens unintentionally. False identification can occur when an importer or retailer mistakes one herb for another, due to incorrect labeling and similar appearance of the herbal materials. Confusing nomenclature can be one of the reasons, because one herb may be known by many names: one or more common names, a Latin name, local names, and the brand name. Some different medicinal herbs of different plant species with different constituents may have similar names. The problem becomes even more complex through confusing terminologies and the use of different languages in different countries [50]. The common names of herbs usually do not reflect differences in scientific taxonomy; and the description and microscopic identification of n herb cannot identify its

constituents. Thus, a study of ancient documents and the use of modern analysis techniques are often necessary to properly authenticate herbal materials.

1.6.4 Nonuniform Chemical Constituents

The chemical composition of herbal products varies and depends on the growing conditions and geographic region. Several environmental factors that include atmospheric humidity, rainfall pattern, soil, altitude, seasonal variation, temperature, length of day light, may affect the concentration of chemical constituents in medicinal plants. Some other relevant factors, such as genetic make-up, seeding time, use of pesticides and fertilizers, planting density, also play a significant role. Various processing steps of raw materials can also change the pharmacological activity of the plant extract. Therefore, batch-to-batch standardization is very essential to maintain the uniformity of active constituents [51].

1.6.5 Pharmacopoeial Standards for Evaluation of Herbal Products

Safety and efficacy assessment for any pharmaceutical must be taken into account for the quality of the prepared formulation. Minimum standards for acceptable quality are generally laid down in pharmacopoeial monographs, which provide all the details of the acceptable substance and give the niceties of significant tests to determine its identity and purity. One type of pharmacopoeial monograph is found in the British or European pharmacopoeias, which give only details of the tests to be used to establish quality, with very concise notes about its therapeutic application. Another type of monograph is more concerned with the complete information about a medicinal plant and consists of all the information about its chemical constituents, pharmacology, toxicology, clinical studies and usage [52].

Pharmacopoeial monographs for the medicinal herbs deal with all types of pharmaceuticals and plant materials which have been included since the earliest editions with authorization at a national or international level. It is interesting to trace the evolution of a monograph for one particular medicinal plant because it reflects developments in analytical techniques, the increasing knowledge of the chemical compounds present, and the growing body of knowledge that links the compounds present to the desired biological or clinical effect [53]. More recent editions of the *British Pharmacopoeia* and *European Pharmacopoeia* have included monographs for many more herbal drugs and more sophisticated chromatographic methods, especially liquid chromatography (LC), have been introduced for both identity tests, impurities tests, and for assay procedures. Therefore, more attention should be given for the biological activity relevant to the reputation and claims for treating particular diseases associated with herbal medicines [35].

The Indian Pharmacopoeia 2007 includes pharmacopoeial specifications with monographs for some medicinal plants being most commonly used as therapeutic agents. The specifications include the name of the drug (along with its common name), its biological source (Latin name), the part of the plant under consideration, its description, macroscopic and microscopic study, identification, several quality control parameters, and assays with respect to the phytochemical reference standards or botanical reference standards [54]. The Ayurvedic Pharmacopoeia of India is another official compendium published by the Ministry of Health and Family Welfare, Government of India. This describes different methods for quality control and standardization of medicinal plants and herbal preparations. Several specifications for quality evaluation of natural products as prescribed in the Ayurvedic Pharmacopoeia include morphological study, determination of quantitative data (e.g., extractive values and foreign matter), limit tests, and different physical tests (e.g., boiling range, refractive index, and pH) [55].

1.7 MARKER ANALYSIS AND STANDARDIZATION OF BOTANICALS

Chemoprofiling of NHP helps in identifying the major metabolites and is useful to assess biological effects. The development of marker-based medicines requires a comprehensive understanding of plant systems including biological, chemical, genetic, and agronomic aspects. Chemical consistency at all stages of manufacturing processes is most important to ensure medicinal efficacy and consumer safety. This includes all the stages such as extraction, stability, shelf life, and purity of herbal medicines. Different methods for characterization of herbal drugs such as morphological identification, anatomical identification, and chemical analysis, such as thin layer chromatography (TLC), high-performance thin layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), Liquid chromatography–mass spectrometry (LC–MS), and protein analysis are extensively used [27].

According to the European Medicines Agency (EMEA), markers may be defined as chemical constituents or groups of constituents of a herbal medicinal product that are very important for quality control purposes regardless of whether they possess any therapeutic effect. Chemical markers are basically categorized into the analytical markers and active markers.

Analytical markers are the constituents or groups of constituents that solely serve for analytical purposes, whereas active markers are the constituents or groups of constituents that contribute to therapeutic activities [56]. Secondary metabolites as markers have been widely used in quality control and standardization of herbal medicines. Herbal products derived from botanicals are mostly obtained from wild sources and have the greatest challenge for ensuring consistent product quality. These are used for making medicines where the standardization and quality control with proper integration of scientific techniques and traditional knowledge are vital requirements [57]. Marker compound selection is generally based upon a variety of different factors including stability, ease of analysis, time and cost of analysis, relevance to therapeutic effect, and indicator of product quality or stability. Chemical markers are frequently used for assuring quality consistency of natural products derived from botanical sources [58]. An ideal chemical marker for a natural product should not only be a characteristic constituent but also a therapeutic constituent. 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, and analytical makers, while biomarkers may be defined as pharmacologically active [59]. Herbal manufacturers and researchers need to address these critical questions to aid in the harmonization of specifications and analytical methodologies for natural products. Usually, determination of single or several marker compounds by a developed method is required for quality control purpose [60]. Standardization methods through chemical fingerprinting should take into account all the aspects that contribute to the quality of the herbal medicine, including correct identification of sample, pharmacognostic evaluation, organoleptic evaluation, volatile matter evaluation, quantitative evaluation (ash values, extractive value, foreign matter), phytochemical evaluation, xenobiotic testing, toxicity testing, microbial load testing, and biological activity determination [39]. Medicinal plants contain several phytoconstituents in certain ratios and in standardized extracts. The ratio of these chemical constituents must be constant within narrow limits from one batch to another [61]. Chemical fingerprints obtained by chromatographic, spectroscopic, thermogravimetric analyses; CE; and polarography techniques have become the most important tools for the quality control and standardization of herbal medicines [12].

For ensuring consistent quality, the use of markers, standardization, chemical and DNA fingerprinting, bioassays, and the emerging field of phytomics are very important [62]. Some medicinally important plants are listed in Table 1.1. 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 [59].

A list of several therapeutically potent phytomarkers from plant species is shown in Table 1.1. Development of lead compounds from these medicinal plants and their evaluation may help to promote natural products based on their quality, efficacy, and safety. Marker analysis of several herbal drugs including polyherbal formulations from the Indian system of medicine has been performed. The fingerprint profiles of Emodin from *Aloe vera*, Gallic acid from *Terminalia chebula*, Boswellic acids from *Boswellia serrata*, Capsaicin from *Capsicum annum*, Glycyrrhizin from *Glycyrrhiza glabra*, epicatechin from *Camellia sinensis*, Eugenol from *Eugenia caryophyllata*, Ferulic acid from *Coffea Arabica*, Garlicin from *Allium sativum*, Genistein from *Glycine max*, Ellagic acid from

TABLE 1.1 Some Medicinally Important Plants and Their Known Phytomarkers

Scientific name	Family	Parts used	Marker compound
Aloe vera	Liliaceae	Leaves	Emodin
Terminalia chebula	Combretaceae	Fruit	Gallic acid
Boswellia serrata	Burseraceae	Resin	Boswellic acids
Capsicum annum	Solanaceae	Fruits	Capsaicin
Glycyrrhiza glabra	Leguminaceae	Root	Glycyrrhizin
Camellia sinensis	Theaceae	Leaves	Epicatechin
Eugenia caryophyllata	Myrtaceae	Flower bud	Eugenol
Coffea arabica	Rubiaceae	Seed	Ferulic acid
Allium sativum	Amaryllidaceae	Bulb	Garlicin
Glycine max	Fabaceae	Seed	Genistein
Punica granatum	Punicaceae	Fruit	Ellagic acid
Piper betel	Piperaceae	Leaves	Piperine
Tagetes erecta	Asteraceae	Leaves	Syringic acid
Paullinia cupana	Sapindaceae	Seed	Anthocyanidin
Matricaria recutita	Asteraceae	Flowering head	Apigenin
Citrus sinensis	Rutaceae	Fruit	Ascorbic acid
Berberis aristata	Berberidaceae	Berries	Berberine
Curcuma longa	Zingiberacease	Rhizome	Curcumin
Zingiber officinale	Zingiberacease	Rhizome	Gingerol
Citrus lemon	Rutaceae	Fruit	Naringenin
Vitis vinifera	Vitaceae	Fruit	Resveratrol

Punica granatum, and Piperine from *Piper betel*, Syringic acid from *Tagetes erecta*, Anthocyanidin from *Paullinia cupana*, Apigenin from *Matricaria recutita*, Ascorbic acid from *Citrus sinensis*, Berberine from *Berberis aristata*, Curcumin from *Curcuma longa*, Gingerol from *Z. officinale*, Naringenin from *Citrus lemon*, Resveratrol from *Vitis vinifera*, and their pharmacological activities have been reported. Marker analysis of Glycyrrhizin from *G. glabra* has been reported through HPTLC densitometry. This is a validated method as per the International Conference on Harmonization guideline where the amount of glycyrrhizin was determined in the extract of *G. glabra* through HPTLC. The method was validated in terms of specificity, linearity, precision, detection limit, and quantification limit [63].

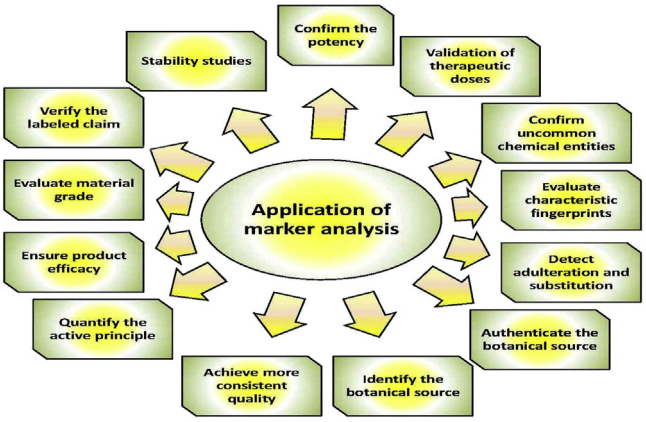


FIGURE 1.5 Application of marker analysis.

1.7.1 Applications of Marker Profiling

Identification, authentication, and quality evaluation of medicinal plants are fundamental requirements of industries and other organizations dealing with herbal health products. The fact must be taken into account that the plant material to be examined has a complex and inconsistent composition based on its content of secondary breakdown products or metabolites [18]. It is an accepted fact that the qualitative and quantitative analysis of major bioactive marker components of plant material is an important and reliable part of a quality control protocol because any change in the quality of the plant material directly affects the constituents. Medicinal plant materials that qualified within the requirements of the WHO guidelines and other regulatory affairs can be used to develop reference fingerprints of phytoconstituents. The presence of marker compounds may be detected by the densitometric scanning of the chromatograms [64]. The presence of these marker compounds in plant materials may be useful for quantifying the plant materials in formulations or herbal medicinal products and will be helpful for the quality control of single and polyherbal formulations. The marker profiling system is helpful as a tool in the quality control and standardization of the raw plant materials and finished herbal formulations. Marker analysis of phytoconstituents may also be helpful in phytoequivalence studies, including issues such as pharmacokinetics and other related parameters that can be recognized by studying the absorption, distribution, and metabolism of herbal drugs [16].

Further, medicinal plants do not have a constant chemical composition to their different parts such as roots, leaves, stems, flowers, and fruits. Therefore, each part needs individual chemoprofiling based on their different phytoconstituents. The use of marker profiling, standardization, DNA fingerprinting, bioassays, and related metabolomics studies, which is the new emerging field of phytomics can provide mechanisms for ensuring consistent quality. Marker profiling plays a vital role in several ways to evaluate quality control parameters so as to ensure the efficacy and safety of the herbal products Figure 1.5 [23]. There are numerous challenges in the isolation and identification of marker components of medicinal plants. Herbal manufacturers and researchers need to address these critical questions to aid in the harmonization of specifications and analytical methodologies for the development of natural products [65].

1.8 PHARMACOVIGILANCE OF HERBAL MEDICINE

Pharmacovigilance is the process of monitoring, evaluating, and communicating drug safety with profound implications that depend on the integrity and collective responsibility of all parties such as consumers, health professionals, researchers, academic, media, pharmaceutical industry, drug regulators, governments, and international organizations. The main objective of pharmacovigilance is to extend the safety monitoring and detect any adverse drug reactions that have previously been unrecognized in the evaluation of clinical trials. There is an ongoing problem with unexpected toxicity of herbal products due to quality issues, including the use of poor quality herbal materials, incorrect or misidentified herbs, incorrect processing methods, and supply of adulterated or contaminated herbs or products. The Medicines and Health care Products Regulatory Agency define some significant problems in the regulation of herbal medicines in the United Kingdom [66]. These include (1) Lack of knowledge about the products being used, (2) Limited use of yellow card adverse drug reporting-scheme, which represents underreporting rather than indicating an absence of adverse reactions, (3) Uniform manufacturing

standards mostly of unlicensed products, and (4) Herb–drug interactions of herbal medicines.

These quality issues can be addressed to some degree by improved regulation requiring good manufacturing practice (GMP) standards for manufacturing. Pharmacovigilance is a very essential tool for developing reliable information on the safety of herbal medicines. The existing systems were developed for synthetic medicines and require some modification to address the specific differences of herbal medicine. Systematic pharmacovigilance is essential to build up reliable information on the safety of herbal medicines for the development of appropriate guidelines for their safe and effective use [67].

1.8.1 Why Pharmacovigilance for Herbal Drugs?

The importance and significance of the pharmacovigilance of herbal medicines are increasing day by day. Presently, accessible surveillance system for herbal drug monitoring is not enough, and various cases of herbal toxicity are likely to be significantly underreported. There is no discrimination between chemically defined drugs and herbal drugs in the filing of procedure of adverse events. The recent information on "phytovigilance" (the term used for pharmacovigilance of herbal drugs) raises the suspicion that there is a tendency to unequal treatment of herbal medicine [68]. Botanicals are complex mixtures of multiple components or unknown active ingredients. This can change pharmacokinetic characteristics through various mechanisms of action. Because the process defines the product, extrapolation of scientific data across products from different manufacturers or sources is not possible. Defining the herb–drug interaction lies in the proper identification of plants, which includes Latin binominal and authority, identification of the plant and part(s) used in the preparation of herbal products, and the processes used to extract and isolate the desired active from plant resources [67].

Concomitant administration of herbal medicine with approved conventional medications can result in therapeutic failures or in adverse effects. Several research reports have suggested that St John's Wort decreases plasma levels of various other drugs [69]. There are no strict regulatory guidelines, and there are gaps in the inefficient regulatory processes that have allowed entry of unsafe products in the market. With prescription medicines, self-medication is a long-time practice that is unsafe and yet difficult to control, due to public assumptions that herbs are generally safe because of the long tradition of their usage and the concept of being natural. It is surprising that these are not recognized and if ever observed are attributed to the remedy's beneficial healing effect rather than harmful effects. Because of the scarcity of local data and lack of rigorous investigations on herbal traditional remedies, the promotion of the use of such products focused on claims of the beneficial effects and ignored the possible adverse effects. Therefore, there is now a need to revise the registration procedure for herbal products. The WHO has recognized the importance of the use of herbal medicines and developed some guidelines for monitoring herbal safety within the existing pharmacovigilance framework [68].

Herbal medicines are promoted in the market as natural and therefore as being safe and harmless. However, there is very less regulation control in the manufacturing of such products; consequently, quality control issues such as misidentification of herbs, mislabeling, contamination, standardization of dose, method of processing, product uniformity, batch-to-batch variation, and toxicity are the major problems in herbal drugs [70]. Manufacturing botanicals to meet analytical standards for marker compounds does not necessarily ensure product efficacy or generic equivalence with the products that have shown efficacy. Herbal medicines are complex mixtures of more than one active ingredient. Many times, it is unclear as to which or how many constituents are responsible for pharmacologically activity. This multitude of active ingredients increases the possibilities of interactions between conventional medicines and herb–herb interactions. The interaction of drugs with herbal medicine is a significant safety concern, especially for drugs with a narrow therapeutic index such as warfarin and digoxin [71]. In view of establishing the safety of herbs, initiating the pharmacovigilance program will assist in the understanding and prevention of adverse effects or any other possible drug-related problems. The strength and potency of herbal products are not easily quantified, and impurity and stability are habitually not easy to examine. Therefore, botanicals should be regulated as in western countries, and the necessities include labeling, GMP, packaging, marketing, and adverse effects reporting requirements, etc. [67]. Researchers, manufacturers, and regulatory agencies must apply precise systematic methodologies and clinical trials to ensure the quality, safety, and consistency of the herbal products, to gain public faith and confidence and to bring herbal products into the mainstream of health care systems.

1.8.2 Steps to Initiate Herbal Pharmacovigilance

Due to an increasing awareness at several levels of herbal medicine, it is compulsory to develop pharmacovigilance practices. Several models of pharmacovigilance and its associated tools have been developed in relation to synthetic drugs, and applying these methods to monitoring the safety of herbal medicines presents unique challenges in addition to those described for

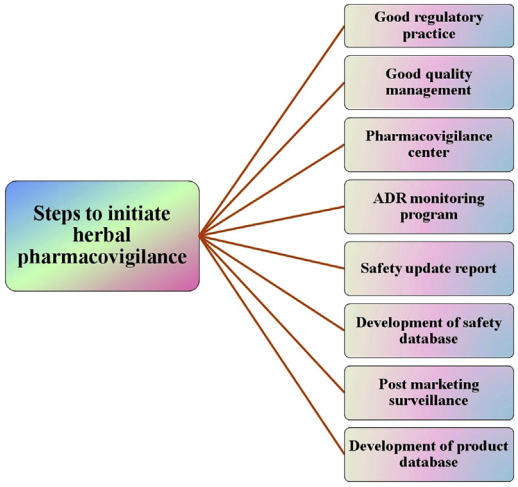


FIGURE 1.6 Steps to initiate herbal pharmacovigilance.

conventional medicines [72]. Several steps to initiate herbal pharmacovigilance to monitor the safety profiles of herbal medicines are shown in Figure 1.6.

1.9 SAFETY ISSUES ON HERBAL MEDICINE-CYTOCHROME P450 STUDY

It is a common belief that modern drugs are dangerous and produce side effects, while herbal medicines are natural and very safe. In fact, some herbs can also be unsafe and even cause serious adverse effects leading to death, if used inappropriately. The complexity of herbal medicine preparations and the interpretation of bibliographic data on safety and efficacy reflecting the experience gathered during long-term use are best addressed by involving specific expertise and experience. Without the knowledge of the prescriber, the consumer tends to consume the herbal products along with prescription medicine, which may lead to herb–drug interaction [73]. The use of complementary and substitute medical therapies has now become a very common trend, and their use has been documented widely during 2004–2014. The increased usage of herbs as dietary supplements and over-the-counter products suggested the need for the development of clinical and scientific data for quality and safety evaluation [74].

Toxicity may be due to the interaction of the herbal material with conventional drugs. Besides there are large number of clinical drugs reported to have potential hepato, renal, cardio toxicity, etc. during epidemiological and other prospective studies. Other agents, such as excipients present in formulations and herbal medicines that are consumed and often not disclosed should also be considered for safety evaluation [75]. Several phytoconstituents have been identified as inhibitors or inducers of cytochrome resulting in herb–drug interaction. Interaction of active compounds including allicin, quercetin, silymarin compounds, etc., has also been reported. Conventional pharmacokinetic literature generally deals with drug–drug interactions, but recently, such interactions between phytoconstituents and prescription drugs have drawn attention, because of increasing physician awareness of the widespread adverse effects of undisclosed herbal use by the patients [11].

Cytochrome P450 (CYP450) enzymes are the major drug-metabolizing enzymes and responsible for the metabolism of a variety of xenobiotics including therapeutic drugs and some important endogenous substances, and most of the herb–drug interactions occur due to either induction or inhibition of these enzymes. CYP450 enzymes are necessary for the production of cholesterol, steroids, prostacyclins, and thromboxane [76]. These enzymes also play an important role in the detoxification of foreign chemicals and the metabolism of drugs. There are more than 50 CYP450 enzymes, but the CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 enzymes metabolize about 90% of drugs. These isozymes are predominantly found in the liver, and are also present in the small intestine, placenta, lungs, and kidneys [77]. An abundance of several cytochrome isoforms has been determined as 30% CYP3A4, 20% CYP2C, 13% CYP1A2, 7% CYP2E1, 4% CYP2A6, 2% CYP2D6, and 1% CYP2B6, which appears to be most relevant for the metabolism of drugs [78]. Repeated administration of drugs can induce CYP enzymes by enhancing the rate of enzyme synthesis. Induction of enzymes leads to an increase in the rate of metabolite production and hepatic biotransformation and decreases in serum drug half-life, response and can also lead to alteration of the pharmacokinetic profile of the drug. CYP enzyme inhibition can be classified into reversible inhibition and irreversible inhibition based on the enzymatic mechanism. Reversible inhibition occurs as a result of direct competition for the binding site on a CYP enzyme between a substrate and an inhibitor, whereas irreversible inhibition is caused by reactive metabolites generated from CYP-catalyzed reactions. Thus, modulation of CYP450 enzyme metabolism is the key to change systemic drug concentrations.

With the increasing consumption of herbal extracts along with prescription medicine, issues related to the safety of herbs have become a key concern. The medical and scientific literature is abundant in in vitro and in vivo reports; this suggests that the concomitant oral administration of natural products and prescription drugs or over-the-counter products may affect human drug metabolism and significantly increase the risk of serious

(clinical) adverse reactions. When conventional drug substances are co-administered along with dietary substances or herbal components, herb–drug interaction may occur by CYP450 isozymes in several ways [76].

- A herbal constituent can be a substrate of one or several isoforms of CYP enzymes. Therefore, one substrate can compete for another substrate for metabolism by the same CYP isoenzyme, resulting in higher plasma concentrations of the drug due to competitive inhibition.
- A herbal constituent can also be an inducer of one or several CYP isoforms, and thereby lower plasma concentrations of the drug due to a higher rate of metabolism. Such interactions may produce subtherapeutic plasma drug concentrations.
- A compound can also be an inhibitor of CYP450 enzymes and result in reduced activity of one or several isoforms of CYP450.

Several attempts have been made to evaluate the inhibitory effects of medicinal plants on CYP enzymes. The potential for herbal remedies to induce or inhibit CYP levels has been examined. By using in vitro and in vivo methods, several herbs have been studied for their potential inhibitory effects on human liver microsomes, rat liver microsomes, and cDNA expressed human liver microsomes [79]. Studies on drug-metabolizing enzymes enhance our understanding of the possibilities for herb–drug interactions. Several existing reports on the role of drug metabolism enzymes, mainly CYP450, in herb–drug interactions are summarized in Table 1.2 with reference to individual herbs.

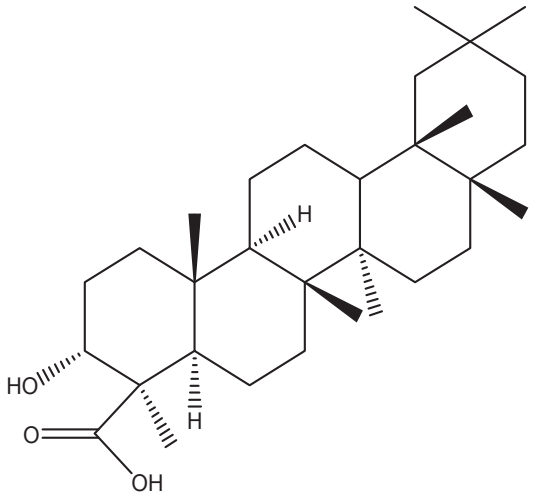
Several therapeutically active plant extracts have been reported to interact with drugs leading to clinically relevant adverse drug reactions. Interaction potentials of some medicinal plants are being described in the following section.

1.9.1 *Cimicifuga racemosa* (Family: Ranunculaceae)

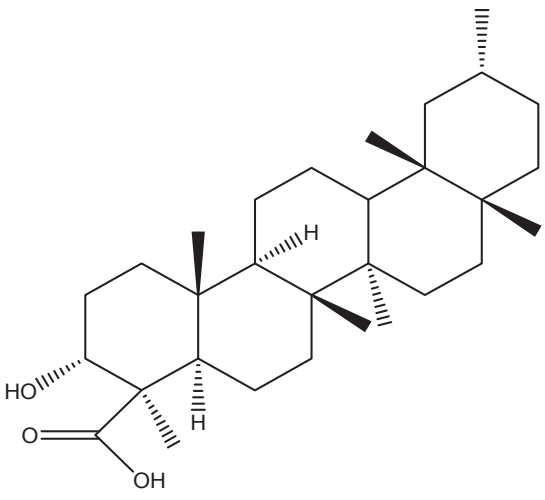
A commercially available dietary supplement made from black cohosh was identified as CYP3A4 inhibitor [90]. The polar fraction from the extract showed 44% inhibition at 5 μg/ml, which was as potent as the inhibition produced by ketoconazole 58% at 5 μg/ml. The IC_{50} values of these six compounds were in the range of 0.10–7.78 mM [76].

1.9.2 *Boswellia serrata* (Family: Burseraceae)

The main constituents of salai guggal are volatile oils, polysaccharides, triterpene acids such as α, β-boswellic acids (**1-2**). *Boswellia carteri*, *Boswellia frereana*, *Boswellia sara*, and *Boswellia serrata* (Family: Burseraceae) herbal extracts were studied for their potential inhibitory activity on CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and 3A4 [91]. The aqueous methanolic (MeoH 80% v/v) extracts of Boswellia species showed potential inhibitory activities. CYP1A2 and CYP2D6 were inhibited at a concentration of 10 μg/ml. CYP2C8/9/19 and 3A4 were strongly inhibited at this concentration (IC_{50} values between 1 and 10 μg/ml). In order to evaluate the contribution of boswellic acids, selected boswellic acids were evaluated for their inhibition activity. From the study, boswellic acids were identified as moderate to potent inhibitors of the CYP enzymes (IC_{50} values between 5 and 120 μM) [76].



α-Boswellic acid (**1**)



β-Boswellic acid (**2**)

TABLE 1.2 Effects of Herbal Extracts on CYP450 Enzymes [76]

Name of the plant	Part used in the study	Type of extract	Study method	CYP isoforms used	Effects on CYP450	IC50 value/ percentage inhibition	Reference
Acorus calamus	Root	Hydroalcohol	Fluorimetry	Human CYP3A4 and CYP2D6	Inhibition	46.84 µ/ml and 36.81 µg/ml	[80]
Capsicum annuum	Fruit	Methanolic	Fluorimetry	Human CYP3A4 CYP2D6 CYP2C9 and CYP2C19	Inhibition	99.69 µg/ml 68.25 µg/ml 88.03 µg/ml and 84.16 µg/ml	[81]
Centella asiatica	Whole plant	Aqueous	HPLC	Human CYP2C9 CYP2D6 CYP3A4	Inhibition	599.0 µg/ml 413.1 µg/ml 229.5 µg/ml	[82]
Centella asiatica	Whole plant	Ethanolic	HPLC	CYP2C9 CYP2D6 and CYP3A4	Inhibition	28.3 µg/ml 418.9 µg/ml and 465.8 µg/ml	[82]
Curcuma longa	Rhizome	Aqueous	HPLC	Human CYP2C9 CYP2C19 CYP2D6 CYP3A4	Inhibition	82.3% 92.7% 48.6% 92.8%	[83]
Cymbopogon nardus	Aerial part	Methanolic	Radiometry	Rat CYP3A4	Inhibition	370 µg/ml	[84]
Emblica officinale	Fruit	Hydroalcohol	Fluorimetry	Human CYP3A4 and CYP2D6	Inhibition	152.11 µg/ml and 109.96 µg/ml	[85]
Origanum vulgare	Leaves	Aqueous	HPLC	Human CYP2C9 CYP2C19 CYP2D6 CYP3A4	Inhibition	35.4% 80.2% 94.6% 98.6%	[83]
Piper longum	Fruit	Methanolic	Fluorimetry	Human CYP3A4 and CYP2D6	Inhibition	164.81 µg/ml and 210.60 µg/ml	[86]
Piper nigrum	Fruit	Methanolic	Fluorimetry	Human CYP3A4 and CYP2D6	Inhibition	178.34 µg/ml and 234.90 µg/ml	[86]
Rheum palmatum	Root	Methanolic	Radiometry	Rat CYP3A4 and CYP2D6	Inhibition	467 µg/ml and 385 µg/ml	[84]
Rhodiola rosea	Root	Ethanolic	Spectrofluorimetry	CYP2D6 and CYP3A4	Inhibition	32 µg/ml and 67%	[87]
Rhododendron groenlandicum	Leaf	Ethanolic	Spectrofluorimetry	Human CYP3A4	Inhibition	48%	[87]
Salvia officinalis	Leaves	Aqueous	HPLC	Human CYP2C9 CYP2C19 CYP2D6 CYP3A4	Inhibition	97.2% 99.9% 99.8% 97.0%	[83]

Continued

TABLE 1.2 Effects of Herbal Extracts on CYP450 Enzymes [76]—cont'd

Name of the plant	Part used in the study	Type of extract	Study method	CYP isoforms used	Effects on CYP450	IC50 value/ percentage inhibition	Reference
Sanatalum album	Wood	Methanolic	Radiometry	CYP3A4 and CYP2D6	Inhibition	337 μg/ml and 886 μg/ml	[84]
Strychnos ligustriana	Wood	Methanolic	Radiometry	Rat CYP2D6	Inhibition	637 μg/ml	[84]
Strychnos ligustrina	Leaf	Methanolic	Radiometry	Rat CYP2D6	Inhibition	302 μg/ml	[84]
Syzygium aromaticum	Flower	Methanolic	Radiometry	Rat CYP3A4 and CYP2D6	Inhibition	219 μg/ml and 249 μg/ml	[84]
Tanacetum parthenium	Leaves	Hydroalcohol	HPLC	Human CYP2C9 CYP2C1 CYP2D6 and CYP3A4	Inhibition	51.5% 46.2% 54.1% and 64.7%	[83]
Terminalia bellerica	Fruit	Hydroalcohol	Fluorimetry	Human CYP3A4 and CYP2D6	Inhibition	77.94 μg/ml and 90.20 μg/ml	[85]
Terminalia chebula	Fruit	Hydroalcohol	Fluorimetry	Human CYP3A4 and CYP2D6	Inhibition	95.52 μg/ml and 102.35 μg/ml	[88]
Thonningia sanguinea	Root	Aqueous	Spectrophotometry	Rat CYP1A1 CYP2B1 CYP2B2 CY1A2	Inhibition	19% 18% 18% 40%	[89]
Tinospora crispa		Methanolic	Radiometry	CYP3A4 and CYP2D6	Inhibition	428 μg/ml 488 μg/ml	[84]
Zingiber aromaticum	Rhizome	Ethanolic	Radiometry	Rat CYP3A4 and CYP2D6	Inhibition	102 μg/ml and 693 μg/ml	[83]
Zingiber officinale	Fruit	Methanolic	Fluorimetry	Human CYP3A4 and CYP2D6	Inhibition	286.49 μg/ml and 249.52 μg/ml	[86]

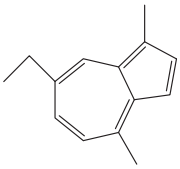
1.9.3 *Gardenia jasminoids* (Family: Rubiaceae)

The effects of *Gardenia jasminoids* extract on liver CYP-450-dependent monooxygenases, glutathione, and glutathione-S-transferase were investigated using rats treated orally with the iridoid glycoside (0.1 g/kg body weight/day) or the crude extract of its fruits (2 g/kg/day) for 4 days [92]. *Gardenia jasminoides* decreased the CYP450 content in liver microsomes and demonstrate that geniposide from *G. jasminoides* has the ability to inhibit a CYP3A4 monooxygenase and increase glutathione content in rat liver. Further, immunochemical data using immunoblotting studies using antibodies revealed that geniposide treatment markedly decreased the protein immunorelated to CYP3A in rat liver [76].

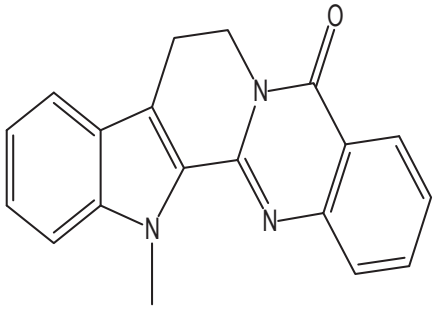
1.9.4 *Matricaria recutita* (Family: Asteraceae)

The inhibitory effect of *Matricaria recutita* essential oil and its major constituents such as chamazulen **(3)** and a-bisabolol **(4)** on four selected human CYP450 enzymes (CYP1A2, CYP2C9, CYP2D6, and CYP3A4) demonstrated the inhibition of these enzymes, with CYP1A2 being more sensitive than the other isoforms. Chamazulene (IC_{50} = 4.41 μM), *cis*-spiroether (IC_{50} = 2.01 μM), and *trans*-spiroether (IC_{50} = 0.47 μM) were shown to be potent inhibitors of this enzyme and also active toward CYP3A4, CYP2C9, and CYP2D6. Chamazulene (IC_{50} = 1.06 μM) and a-bisabolol (IC_{50} = 2.18 μM) caused a significant inhibition of CYP2D6. As indicated by these in vitro data, chamomile preparations contain

constituents inhibiting the activities of major human drug-metabolizing enzymes [93].



Chamazulene (**3**)



α-Bisabolol (**4**)

1.9.5 *Echinacea purpurea* (Family: Asteraceae)

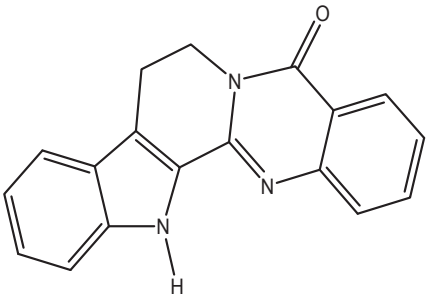
In vitro CYP3A4 inhibition profiles of *Echinacea purpurea* and ketoconazole were evaluated by different substrates and showed a much lower CYP3A4 inhibition by *E. purpurea* (IC_{50} = 5394 μg/ml) compared to that by fluorescent substrates (IC_{50} 354 and 452 mg/ml, respectively). From the study, it was suggested that the substrate/assay-dependent effects may occur due to the complex nature of *E. purpurea* constituents, involving different CYP3A4 substrate binding sites. A weak inhibition potential of *E. purpurea* toward CYP3A4-mediated metabolism in vitro was confirmed by the use of three different substrates [94].

1.9.6 *Ginkgo biloba* (Family: Ginkgoaceae)

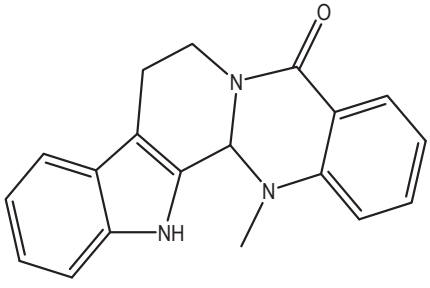
Ginkgo is believed to improve cerebral and peripheral blood flow through nitric oxide-induced vasodilation and possesses antioxidant activity [95]. Certain components of *Ginkgo biloba* are potent in vitro inhibitors of human CYP2C9. Another study was undertaken to clarify the influence of repeated oral administration of ginkgo extract on CYP2C9 and CYP3A4. A combination of *G. biloba* and tolbutamide is to be administered cautiously in terms of potential interactions, especially in elderly patients or patients treated with drugs exerting relatively narrow therapeutic windows. Greenblatt et al. (2006) investigated the effect of *G. biloba* on the activity of CYP2C9 when administered with warfarin in humans [96].

1.9.7 *Evodia rutaecarpa* (Family: Rutaceae)

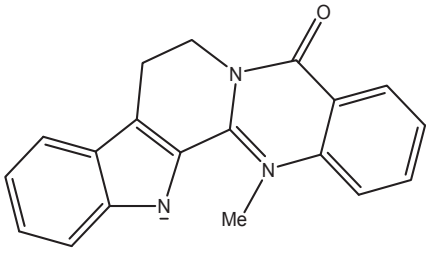
Rutaecarpine **(5)**, a quinazolinocarboline alkaloid that is a major constituent of Evodia fruit, caused the most dramatic decrease in residual CYP3A4 activity. It was further identified as a mechanism-based inhibitor of CYP3A4. Rutaecarpin also showed potent inhibition to CYP1A1 and CYP1A2 (IC_{50} 0.90 and 0.06 μM). An analysis showed that methanol extract increased the levels of CYP1A1, CYP1A2, CYP2B, and glutathione-S-transferase Yb immunoreactive proteins and aqueous extract increased CYP1A2 protein level. Three major bioactive alkaloids, that is, rutaecarpine, evodiamine **(6)**, and dehydroevodiamine **(7)** at 25 mg/kg increased hepatic ethoxyresorufin-O-deethylase (EROD) activity. These results demonstrated that *Evodia rutaecarpa* methanol and aqueous extracts could affect drug-metabolizing enzyme activities [76].



Rutaecarpine (**5**)



Evodiamine (**6**)



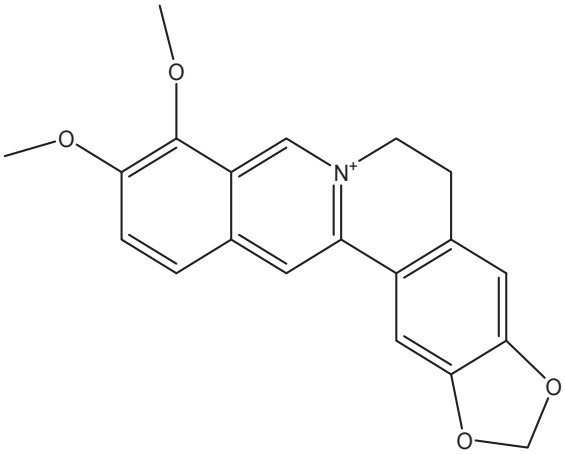
Dehydroevodiamine (**7**)

1.9.8 *Hydrastis Canadensis* (Family: Ranunculaceae)

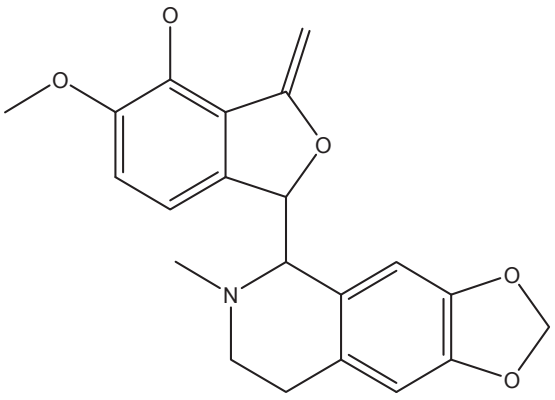
This contains the alkaloids berberine **(8)** and hydrastine **(9)**, hydrastinine **(10)**, and canadine **(11)**. Its extract contains approximately equal concentrations (~17 mM) of two ethylenedioxyphenyl alkaloids, berberine and hydrastine, inhibited with increasing potency the isoform CYP2C9 (diclofenac

4b-hydroxylation), CYP2D6 (bufuralol 1b-hydroxylation), and CYP3A4 (testosterone 6β-hydroxylation) activities in human hepatic microsomes with interpolated IC_{50} values of 0.98%, 0.66%, and 0.18%, respectively [76].

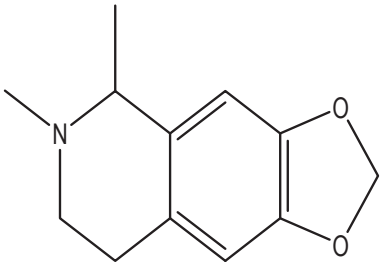
inhibitors of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP4A9/11 and studied the effect of kava rhizome extracts and kava alkaloids, pipermethystine **(12)** in rat liver microsomes. Pipermethystine alone demonstrated a nonsignificant increase in CYP1A2,



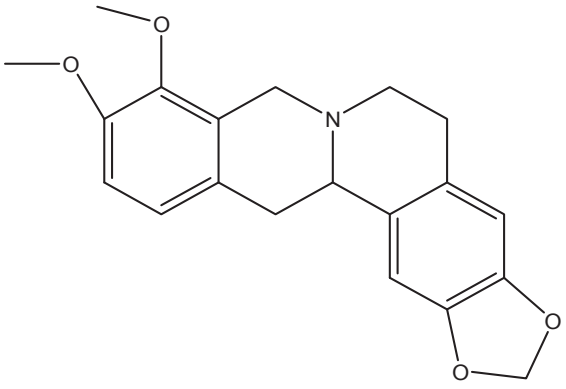
Berberine (**8**)



Hydrastine (**9**)



Hydrastinine (**10**)

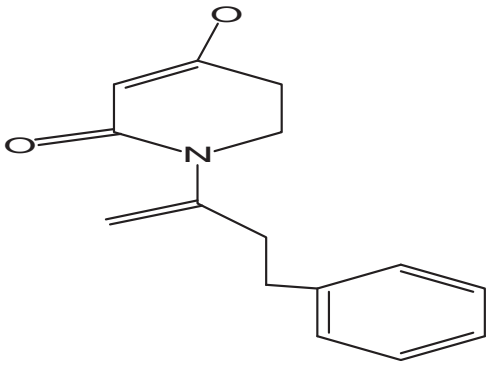


Canadine (**11**)

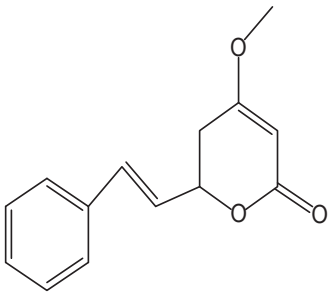
1.9.9 *Piper methysticum* (Family: Piperaceae)

Whole kava extract (normalized to 100 1 M total kavalactones) caused concentration-dependent decreases in CYP450 activities, with significant inhibition of the activities of CYP1A2 (56% inhibition), CYP2C9 (92%), CYP2C19 (86%), CYP2D6 (73%), CYP3A4 (78%), and CYP4A9/11 (65%) following preincubation for 15 min. These data indicated that kava has a high potential for causing drug interactions through inhibition of CYP450 enzymes. Jennifer and Ramzan and Jennifer (2004) [97] reported that several kavalactones are potent

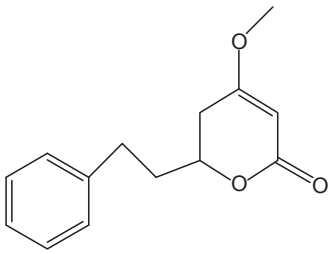
while kava rhizome extracts alone and in combination with pipermethystine increased hepatic CYP1A2 protein levels by 98%. From the study, it is understood that kava kava may have a potential to produce drug interactions. Zou et al. (2002) [98] investigated the influence of isolated kavalactones kavain **(13)**, dihydrokavain **(14)**, methysticin **(15)**, yangonin **(16)**, and desmethoxyyangonin **(17)** on recombinant human CYP isoforms such as CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4. They calculated IC50 values between 0.43 and 153.20 μM from the mean of four determinations for the potent inhibitory active compounds.



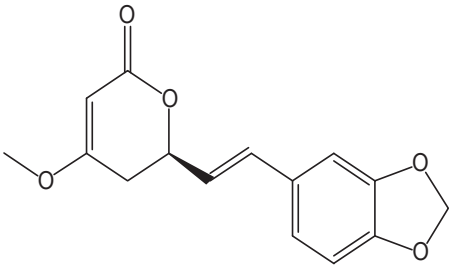
Pipermethystine (**12**)



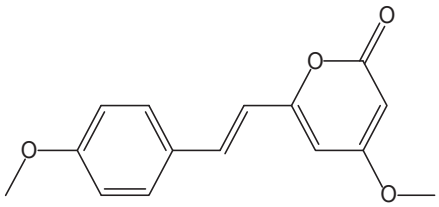
Kavain (**13**)



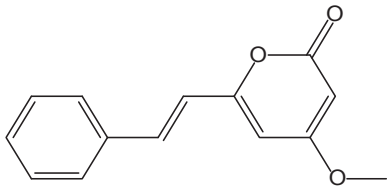
Dihydrokavain (**14**)



Methysticin (**15**)



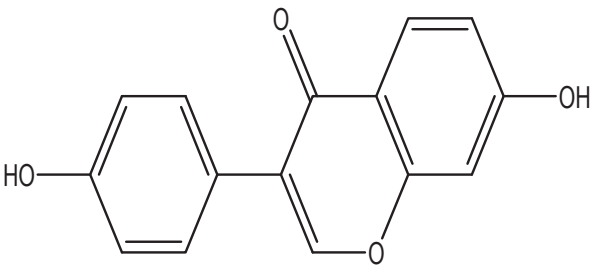
Yanogonin (**16**)



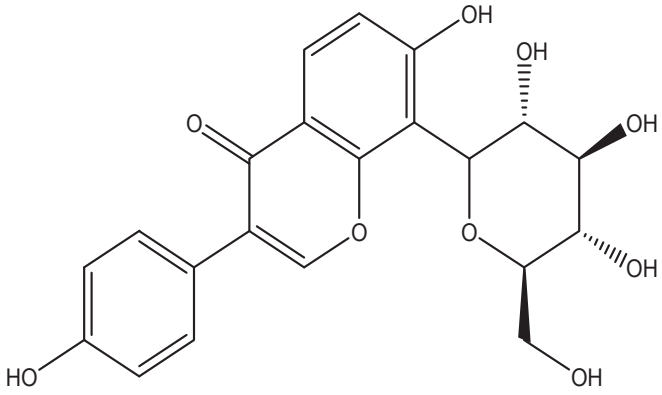
Desmethoxyyanogonin (**17**)

1.9.10 *Radix pueraria* (Family: Fabaceae)

This possesses a high content of flavonoids, coumarins, and isoflavones such as daidzein **(18)** and puerarin **(19)**. Crude extracts containing puerarin inhibited in a dose-dependent fashion. Although both CYP content and reduced nicotinamide adenine dinucleotide phosphate-(CYP)-c-reductase activity were significantly increased, a complex pattern of CYP modulation was observed, including both induction (puerarin: CYP2A1, CYP1A1/2, CYP3A1, CYP2C11; Ge-gen: CYP1A2, CYP3A1, CYP2B1) and inactivation (Ge-gen and puerarin: CYP3A, CYP2E1, CYP2B1) [99].



Daidzein (**18**)



Puerarin (**19**)

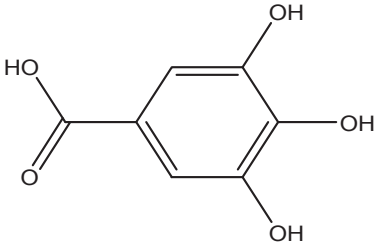
1.9.11 *Phyllanthus amarus* (Family: Euphorbiaceae)

All the CYP450 enzymes were significantly inhibited by the *Phyllanthus amarus* extract in a concentration-dependent manner. The concentrations needed for 50% inhibition of CYP1A1, CYP1A2, and CYP2B1/2 were 4.6 μg/ml, 7.725 μg/ml, and 4.18 μg/ml, respectively. Oral administration of *P. amarus* (250 mg/kg) was found to reduce the activity of these enzymes and an increased concentration of the extract (750 mg/kg) [100].

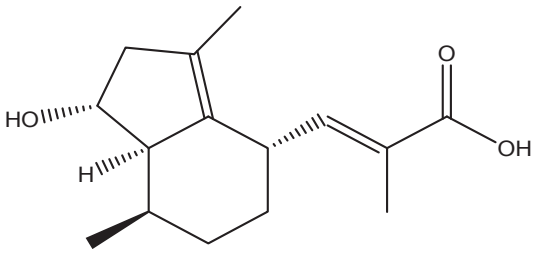
1.9.12 *Valeriana officinalis* (Family: Valerianaceae)

Its essential oil contains terpenoid-like hydroxylvalerenic acid **(20)**, and valerenic acid **(21)**. CYP3A4-mediated metabolism tended to be slightly lower in aqueous extracts and slightly higher in those extracted with acetonitrile. In most cases, the ethanolic extracts were slightly less active than the corresponding aqueous or acetonitrile extracts. From the study, it was concluded that valerian extracts exhibited a marked inhibition of CYP3A4-mediated metabolism [76].

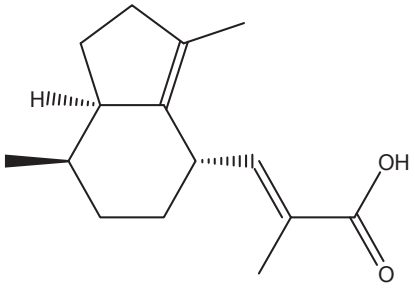
extracts showed higher IC_{50} values (<0.1 mg/ml) compared to those of gallic acid (0.09 mg/ml) against CYP2D6. On the other hand, quinidine and ketoconazole showed relatively strong inhibition with an IC50 value of <0.007 mg/ml. The results indicated that the test substances have much less interaction potential with coadministered drugs than do the known inhibitors. Triphala formulation has less interaction potential when compared with individual plant extracts and bioactive molecules. Triphala formulation and its individual ingredients may likely interact with drug-metabolizing enzymes, but are less likely to produce significant drug interactions [85].



Gallic acid (**22**)



Hydroxyvalerenic acid (**20**)



Valerenic acid (**21**)

1.9.13 Triphala

"Triphala" is a well-known polyherbal formulation from Indian Ayurveda called "Rasayana" in Sanskrit. It consists of dried fruits of *Emblica officinalis*, *Terminalia belerica*, and *Terminalia chebula* in equal proportions (1:1:1). Traditionally, this formulation has been prescribed for several ailments and used as a laxative, detoxifying agent, digestive agent, and rejuvenator [101]. It has been reported that *E. officinale* extract showed the highest IC_{50} value (152.11 ± 2.18 μg/ml) and *Terminalia bellerica* showed the lowest IC50 value (69.89 ± 2.50 μg/ml) against CYP3A4. The inhibitory activity against CYP3A4 with IC_{50} values <0.1 mg/ml were found with the three fruit extracts and the formulation, while gallic acid **(22)** was found to produce inhibitory activity with approximately 0.09 mg/ml. Likewise, all the fruit

1.9.14 Trikatu

Trikatu is a very well-known "Rasayana" in Ayurveda and widely used as a polyherbal ayurvedic formulation in India. Trikatu means the mixture of three acids. It consists of three well-known plants, namely, *Piper longum*, *Piper nigrum*, and *Zingiber officinale*, in an equal ratio. Trikatu has been prescribed for cough, cold, fever, asthma, respiratory problems, and improvement of digestive disorders. It is reported that the trikatu and its biomarkers have very less inhibitory effect on CYP450 enzymes. Different concentrations of the trikatu formulation and its individual components showed significantly ($P < 0.001$) less inhibitory activity on individual isoenzymes as compared to that shown by the positive control [86].

1.9.15 *Murraya koenigii* (Family: Rutaceae)

Murraya koenigii extract has a higher IC_{50} value (160.47 ± 5.45, 206.63 ± 1.99, and 156.56 ± 3.77 μg/ml of CYP3A4, 2D6, and 2C9 isozymes, respectively) than do the standard biomarkers. *M. koenigii* extract and its bioactive compounds have an inhibitory effect on drug metabolism enzymes when consumed along with conventional medicine. The IC_{50} values were higher than those of the positive control and indicated that the test extracts and constituents have moderate interaction in drug metabolism [81].

1.9.16 *Glycyrrhiza glabra* (Family: Fabaceae)

The major bioactive constituents of *Glycyrrhiza glabra* are glycyrrhizin, glabranin A and B, glycyrrhetol, glabridin, formononetin, glabrone, etc. [62]. A research report on the CYP450 interaction profiles of *G. glabra* and its bioactive compound glycyrrhizin showed that the extract and glycyrrhizin have a higher interaction potential with CYP2D6 compared with CYP3A4. The licorice extract showed a comparably higher IC_{50} value with both the isozymes, which may be related to the synergistic effects for the presence of other constituents in the extract. The IC_{50} values that are higher than those of the positive inhibitors indicated that the test samples have only a weak interaction potential in drug metabolism. An IC_{50} value of the extract that is lower than that of the pure compound indicates that care should be taken when administering the extract with other CYP450-interacting compounds, particularly those with a low therapeutic index [102].

1.10 HERB-DRUG INTERACTIONS

The plant material as an active ingredient in herbal products may also be related to health risks, 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 [103]. Another problem associated with the use of herbal products is underreporting of observed adverse reactions and herb–drug interactions. In a study, it was found that 58% of users do not inform their physician when they buy any herbal medicinal products. It has been reported that 69% of Britishers and 61.7% of Italians that use herbal product do not consult their physicians [104].

Many medicinal herbs have a long history of use, and this may have generated a significant amount of published toxicological information including scientifically accepted monographs, clinical experience, and epidemiological studies, as well as data from postmarketing surveillance programs. This information may be used as a basis for a simplified registration procedure and may serve as a substitute for animal experiments and reduce the number of clinical trials in humans [105]. Prescribers and consumers of herbal products will be able to recognize and report on major acute adverse events, such as dermatological reactions, nausea, and disturbances of the gastrointestinal tract. Consequently, data on traditional use are unlikely to provide information on chronic toxicity and carcinogenic, mutagenic, and teratogenic effects [76]. Several regulatory bodies have acknowledged this problem and have given the right to national authorities to demand such supplementary safety testing when bibliographic evidence is deemed to be insufficient to prove safety before marketing authorization of the herbal products [78].

Consumption of herbal products that are capable of modulating CYP metabolism can cause clinically relevant herb–drug interactions and can alter drug bioavailability. Any inhibitory effect of herbal extracts on CYP may result in enhanced plasma and tissue concentrations leading to toxicity, while any inductive effect may cause reduced drug concentrations leading to decreased drug efficacy and treatment failure [104]. The complexity of herbal medicine preparations and the interpretation of bibliographic data on safety and efficacy reflecting the experience gathered during long-term use are best addressed by involving specific expertise and experience.

1.10.1 Synergistic Effects of Herbs

The mechanisms of synergistic actions of herbal ingredients can be explored for designing new multitarget drugs and drug combinations and for discovering potent drug combinations that are individually subtherapeutic but efficacious in combination. Synergistic actions involve interactions with multiple sites, targets, and pathways that are sensitively influenced by genetic, environmental, behavioral, and scheduling profiles [106]. It is claimed that combinations of herbs have synergistic effects. There is much in vitro and/or in vivo evidence to support the occurrence of synergism between constituents in certain herbal extracts. Synergy is also taken to mean an attenuation of undesirable effects, another key theory of herbalism being that the toxicity of plant extracts is less than that of a single isolated constituent.

Synergism has a major role in therapeutic efficacy of medicinal plants and plant-derived formulation. This cannot be easily distinguished from additive effects and usually relies on high margins of variation. In fact, the mechanism of action of many phytomedicines is still unknown, and there are several instances of a

total herb extract showing a better effect than an equivalent dose of an isolated compound [107]. Generally, synergistic effects are considered to be positive, with the low doses used perceived as a benefit, although it is obvious that there may also be negative aspects. Pepper contains the alkaloid piperine, which is known to increase the bioavailability of a number of clinically used drugs. Unwanted interactions, for example, would be the presence of tannins in herbal drugs, which may hinder the absorption of proteins and alkaloids, or the induction of enzymes, such as CYP450, which may accelerate drug metabolism resulting in blood levels of actives too low for a therapeutic effect [101]. The issues concerning the safety of herbs with increasing consumption of herbal extracts along with prescription medicine have become a major concern. This leads to several studies on evaluation of their effects on drug-metabolizing enzymes. These studies will help to understand and ensure the possibilities for herb–drug interactions.

1.11 SYSTEM BIOLOGY AND METABOLOMICS

System biology intends to recognize the biological complexity of different measurements without any hypothesis. The major focus of systems biology is to enquire the dynamics of all genetic, regulatory, and metabolic processes in a cell and to understand the complexity of cellular networks [108]. Adoption of the systems biology approach would be more helpful for exploring the scientific implication of herbal medicine and the modernization of TM.

There are several technological platforms of system biology, such as genomics, proteomics, and metabolomics, that provide powerful tools for the study on the essence and function of herbal drugs (Figure 1.7). Scientifically and technologically validated herbal products can be explored on a fast track using various innovative approaches such as reverse pharmacology and systems biology, which are based on a knowledge of TM. TM

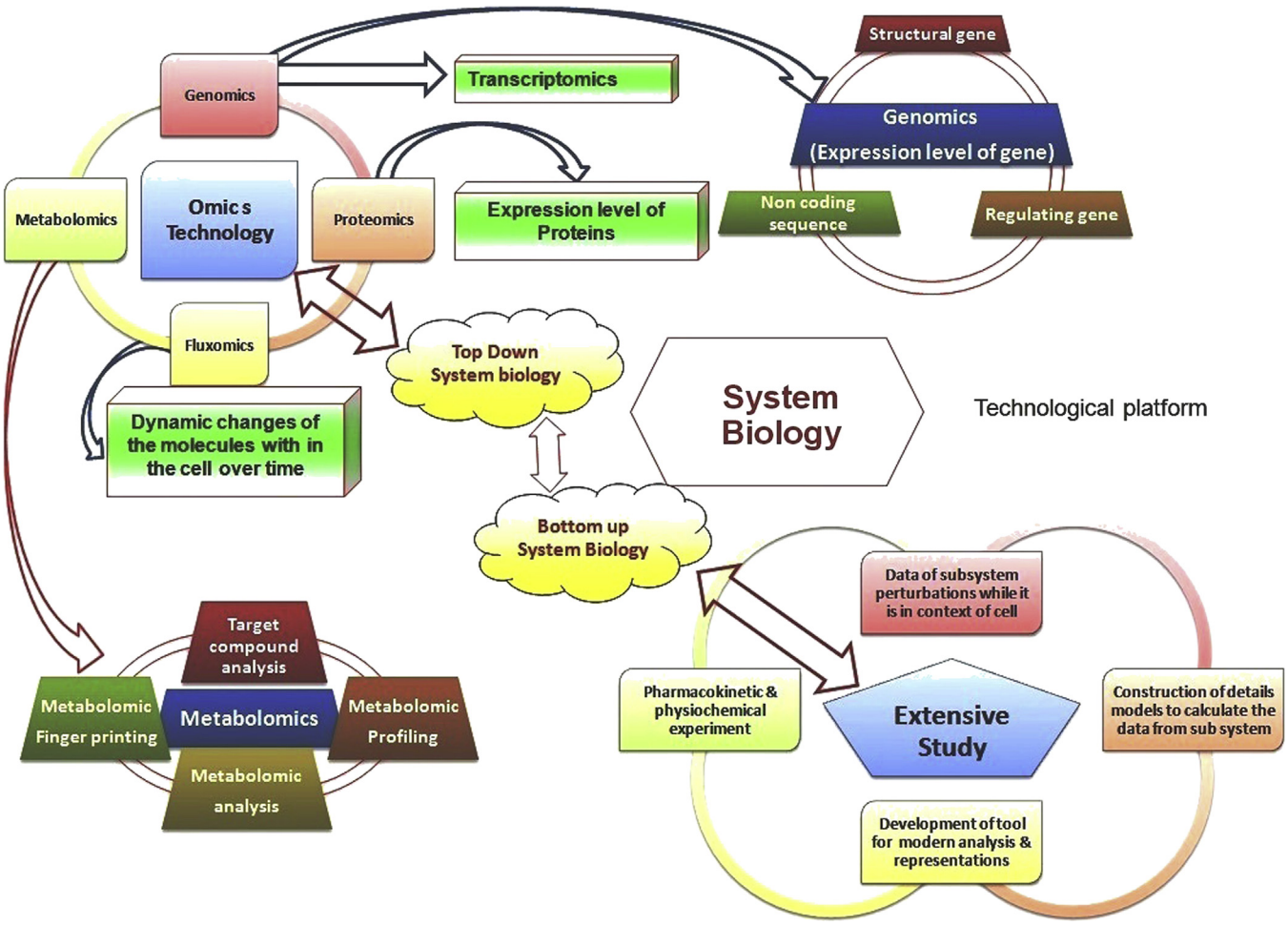


FIGURE 1.7 System biology in drug discovery.

comprises of evolutionary process as communities to discover practice transforming techniques. The methods for carrying out metabolic modeling by means of collecting, storing, and analyzing metabolomic data are considerably different, which will generally be performed by individuals or in laboratories with different skill sets, and yet necessarily will deal with the same molecules [109]. It is therefore very essential to timely bring together the known or conditional metabolic maps of suitable organisms with measurements of their metabolomes to provide a system level understanding of the metabolic fluxes and metabolite concentration in these organisms, and their way of changing under different conditions [110].

LC−MS is an analytical technique that combines the physical separation capabilities of LC with the mass analysis capabilities of MS. LC−MS is a powerful technique used for many applications and has a very high sensitivity and selectivity. LC−MS consists of an ultra-high LC combined with a mass spectrometer that contributes to achieve high-throughput studies in metabolomic analysis. LC−MS is frequently used in drug development at many different stages including natural product dereplication, metabolomic stability screening, metabolomic identification, impurity identification, quantitative analysis, and quality control. Generally, its application is oriented toward the specific detection and potential identification of phytoconstituents in a plant extract. LC−MS becomes a powerful approach for the rapid identification of phytochemical constituents in botanical extracts, and it can avoid the time-consuming isolation of all compounds to be identified. LC−MS is most commonly used for metabolomic analysis of plant extract where secondary metabolite masses may overlap even with a high-resolution mass spectrometer. Profiling of secondary metabolites in plants or food, such as phenolics, can be achieved with LC−MS [111].

Metabolomics aims at qualitatively and quantitatively determining as many compounds as possible. This can not only be in extracts of tissues but also in body fluids such as serum or urine in the case of humans. Chromatographic methods in combination with MS, MS with nuclear magnetic resonance (NMR) spectrometry, are used for such analyses [112]. By combining the result of such analyses with other parameters, novel correlations can be found, for example, a relationship between the occurrence of certain compounds in extracts and a biological activity. Analysis of metabolites in urine by means of 1H-NMR is already extensively applied for studying the toxicity of drugs [113]. The metabolomics approach is also a very promising tool for the quality control of botanicals. A study on the metabolic profiling of *G. biloba* L. in pharmaceutical preparation by means of 1H-NMR has been reported. Besides a recognizable pattern, the quantitative analysis of ginkgolides and bilobalide could be done with a 5-min acquisition time of the spectrum, without the need for any elaborate sample preparation. Also, for other preparations, it was found that this method is very suitable; among other preparations studied were strychnos, ephedra, and cannabis. Such studies are the first steps in the long way to a better understanding of the activity of medicinal plants [114].

Metabolomic study reveals the quantitative and qualitative estimation of "whole" metabolite found in a cellular or organism system. It can be defined as the systemic study of the individual chemical fingerprints that a definite cellular process leaves behind and even more particularly the technique of the metabolite profile of the "whole" small molecules in an organism. The combined data of all the metabolites in a biological system, which are the final products of its gene expression, are known as the metabolome. These approaches deal with the study of genomics, transcriptomics, and proteomics of biological systems. It involves four major steps of analysis of the unknown compound present in the herbal medicine, which includes the following:

- Targeted investigation of the compound: This deals with quantitative estimation of definite metabolites.
- Metabolic documentation: Quantitative and qualitative data for the estimation of the unknown compound or of definite metabolic pathways.
- Metabolomic fingerprinting: This is the process of sample classification by rapid global investigation.
- Metabolomic examination: This pertains to the quantitative and qualitative analysis of "whole" metabolites.

Metabolomic analyses use a particular set of analytical techniques such as Fourier transformed infrared spectroscopy, gas chromatography−mass spectrometry, LC−MS, NMR, CE−MS, and TLC. Recent advances made in analytical chemistry for small mass compound detection and characterization, such as MS and high-field NMR, coupled with modern multivariate statistics, have led to a highly efficient system for the comprehensive analysis of the metabolite data matrices generated by metabolomic experiments [9]. In the past decades, several attempts have been made to solve these problems using metabolomics. Metabolomics is a relatively new field of "omics" research concerned with the high-throughput identification and quantification of small-molecule metabolites in the metabolome. Analysis of a large number of samples might facilitate the identification of patterns or metabolite markers that are characteristic of a species, a cultivar, a certain stage of development or conditions, such as disease state, stress, or daily and seasonal changes. Therefore, the high-throughput global analysis of a metabolome through

hyphenated technologies is a key factor in this field [115]. Thus, metabolomics is now emerging as a powerful tool for the characterization of complex phenotypes affected by both genetic and environmental factors. Nevertheless, metabolomic fingerprinting often lacks robustness, so targeted or profiling approaches may be useful techniques for the validation of herbal medicine with the necessary specificity, precision, accuracy, linearity, sensitivity, recovery, and stability in the presence of potentially interfering compounds [116].

1.12 INTERNATIONAL HARMONIZATION

To ensure homogeny of quality, safety, and efficacy of the herbal medicines across countries, harmonizing efforts have been initiated on pharmacopoeial specifications, standardization, and classification of herbal drugs. Different specifications have been found in different pharmacopoeias of Korea, Japan, and China for a single herbal medicine. The same crude plant material may be described, but the family or species of the original plant may be different [117]. The Western Pacific Regional Forum for the Harmonization of herbal medicine tried to harmonize the crude drug monographs in the pharmacopoeias of six Asian countries (Japan, China, Korea, Singapore, Vietnam, and Hong Kong) in order to help in promoting commercialization of safe and effective herbal drugs across countries. Harmonization process and herbal product registration were initiated in 2000 among different countries. International harmonization would help in developing evidence-based clinical practice guidelines on TM. India has nearly 8000 herbal drug companies, among which about five thousand companies have GMP-compliant manufacturing units, and most of them are of small and medium size. Seventy percent of the Indian exports from the herbal sector consist of only raw materials, and 30 percent consist of finished products including herbal extracts. There are 55 major herbal drugs exporting companies in India [28]. As discussed in the monographs of the American Herbal Pharmacopoeia, the use of single or multiple chemical markers was important for quality control. Protocols and guidance documents on safety and toxicity testing of TMs have been issued by the International Life Sciences Institute, the Institute of Medicine, National Research Council (2004), the Union of Pure and Applied Chemistry, the EMEA (e.g., EMEA, 2009) [118], and by the European Food Safety Authority (EFSA, 2009) [119] These regulation documents tell us about the assessment of the safety, efficacy, and quality of herbs for food and medicine purposes. Standards for medicinal plants are being developed worldwide, but as yet there is no consensus as to how these should be adopted. There are several publications: US Pharmacopoeia, British Herbal Compendium, British Herbal Pharmacopoeia, Chinese Pharmacopoeia, and Physician's Desk Reference for herbal medicines, Ayurvedic Pharmacopoeia of India have monographs for herbal raw materials [24]. For a single plant, the monograph may vary in different publications, different country standards with respect to a single formulation, which creates difficulties for manufacturers in herbal drug trade. Thus, the need to establish global regulatory mechanisms for regulating herbal drugs seems obvious. An improvement in the processes of regulation and global harmonization is desirable and necessary, which combines scientific data and traditional knowledge [34].

Several challenges and issues on promotion and development of herbal drug have been identified, which should be solved through international coordination and collaboration. Considering the widespread use and popularity of herbal products, there is a need for adequate evidence in the quality, safety, and efficacy of herbal products, which is mandatory. Therefore, there is a need for coordination and harmonization of research and development of medicinal plants as both pharmaceuticals and food supplements.

1.13 CONCLUSION

The existing knowledge of herbal medicines needs to be validated and documented through regulatory guidelines of quality control, standardization, and manufacturing process. Chemical consistency at all stages of manufacturing processes such as extraction, standardization, quality control, quality assurance, stability, shelf life, and purity is of utmost important to ensure medicinal efficacy and safety. Considering the widespread use and popularity of herbal medicines, proper standardization and validation methods have been developed for the promotion of natural products. Medicinal plants are most commonly used in TMs and can potentially influence the bioavailability and pharmacokinetics of some pharmaceuticals by affecting CYP450 drug metabolism. Evidence-based submissions for regulatory approval and interlinking of various pharmacopoeial monographs would be helpful for herbal manufacturers to regulate markets across the world. Research through collaboration and cooperation can help to a large extent in the promotion and development of TM for the betterment of health care globally. This may bring scientists and other stakeholders together to discuss global issues and implications of new strategic terms, with a new set of goals, a new agenda, but most importantly, a new vigor is vital for global development.

The different systematic approaches of TMs are not only about a single science or technique but also an amalgamation of these concomitant areas, which are mutually interrelated.

Acknowledgments

The authors wish to express their gratitude to the National Medicinal Plant Board (NMPB), Department of AYUSH, Ministry of Health and Family Welfare, Government of India, for providing financial assistance to the School of Natural Product Studies, Jadavpur University, through project grant [F. No.: Z. 18017/187/CSS/R&D/WB-01/2009-10-NMPB].

References

[1] Tistaert C, Dejaegher B, Heyden YV. Chromatographic separation techniques and data handling methods for herbal finger prints: a review. Anal Chim Acta 2011;690:148–61.
[2] Zhang J, Wider B, Shang H, Li X, Ernst E. Quality of herbal medicines: challenges and solutions. Complement Ther Med 2012;20:100–6.
[3] Mukherjee PK, Sahoo AK, Narayanan N, Kumar NS, Ponnusankar S. Lead finding from medicinal plants with hepatoprotective potentials. Expert Opin Drug Discov 2009;4: 545–76.
[4] Shinde VM, Dhalwal K, Potdar M, Mahadik KR. Application of quality control principles to herbal drug. Int J Phytomed 2009; 1:4–8.
[5] Booker A, Johnston D, Heinrich M. Value chains of herbal medicines—research needs and key challenges in the context of ethnopharmacology. J Ethnopharmacol 2012;140: 624–33.
[6] Weathers PJ, Arsenault PR, Covello PS, McMickle A, Teoh KH, Reed DW. Artemisinin production in Artemisia annua: studies in planta and results of a novel delivery method for treating malaria and other neglected diseases. Phytochem Review 2011; 10:173–83.
[7] Mukherjee PK, Wahile A. Perspectives of safety for natural health products. In: Sharma RK, Arora R, editors. Herbal drugs—a twenty first century perspectives. New Delhi: Jaypee Brothers Medicinal Publishers Ltd; 2006. p. 50–9.
[8] Choi DW, Kim JH, Cho SY, Kim DH, Chang SY. Regulation and quality control of herbal drugs in Korea. Toxicology 2002;58: 181–2.
[9] Liang YZ, Xie P, Chan K. Quality control of herbal medicine. J Chromato B 2004;812:53–70.
[10] Patwardhan B, Mashelkar RA. Traditional medicine-inspired approaches to drug discovery: can Ayurveda show the way forward? Drug Discov Today 2009;14:804–11.
[11] Mukherjee PK, Venkatesh P, Venkatesh M, Ponnusankar S, Khan MY. Strategies for revitalization of traditional medicine. Chin Herb Med 2010;2:1–15.
[12] Mukherjee PK. Exploring botanicals in Indian system of medicine-regulatory perspectives. J Clin Res Reg Affairs 2003; 20:249–64.
[13] Mukherjee PK. Evaluation of Indian traditional medicine. Drug Inf J 2001;35:631–40.
[14] Mukherjee PK, Ponnusankar S, Venkatesh M. Ethnomedicine in complementary therapeutics. In: Chattopadhyay D, editor. Ethnomedicine: a source of complementary therapeutics. Trivandrum: Research Signpost; 2010. p. 29–52.
[15] Nema NK, Dalai MK, Mukherjee PK. AYUSH herbs and Status Quo in herbal industries. The pharma review. New Delhi: Kongposh Publications; 2011. 141.
[16] Mukherjee PK, Houghton PJ, editors. Evaluation of herbal medicinal products—perspectives of quality, safety and efficacy. UK: Pharmaceutical Press, Royal Pharmaceutical Society of Great Britain; 2009. p. 501.
[17] Mukherjee PK. Quality control of herbal drugs: an approach to evaluation of botanicals. 1st ed. India: Business horizons; 2002. 113–119.
[18] Mukherjee PK, Venkatesh M, Gantait A. Ayurveda in modern medicine: development and modification of bioactivity. In: Mander L, Lui HW, editors. Comprehensive natural products II chemistry and biology, vol. 3. Oxford: Elsevier Science; 2009. p. 479–507.
[19] Mukherjee PK, Bahadur S, Harwansh RK, Nema NK, Bhadra S. Development of traditional medicines: globalizing local knowledge or localizing global technologies. Pharma Times 2013;45: 39–42.
[20] Mukherjee PK, Wahile A. Integrated approaches towards drug development from Ayurveda and other Indian system of medicines. J Ethnopharmacol 2006;103:25–35.
[21] Mukherjee PK, Sahu M, Suresh B. Indian herbal medicines. In: The Eastern pharmacist, 42; 1998. p. 21–4.
[22] Mukherjee PK, Saha BP. Quest for GMP for the production of quality botanicals. In: Mukherjee PK, Verpoorte R, editors. GMP for botanicals—regulatory and quality issues on phytomedicine. New Delhi, India: Eastern Publishers, Business Horizons Ltd; 2003. p. 165–90.
[23] Mukherjee PK, Nema NK, Venkatesh P, Debnath PK. Changing scenario for promotion and development of Ayurveda—way forward. J Ethnopharmacol 2012;143:424–34.
[24] Mukherjee PK, Maity K, Mukherjee K, Houghton PJ. Leads from Indian Medicinal Plants with hypoglycemic potentials. J Ethnopharmacol. 2006;106:1–28.
[25] Bilello JA. The agony and ecstasy of "OMIC" technologies in drug development. Curr Mol Med 2005;5:39–52.
[26] Sahoo N, Manchikanti P, Dey S. Herbal drugs: standards and regulation. Fitoterapia 2010;81:462–71.
[27] Cordell GA. Phytochemistry and traditional medicine—a revolution in process. Phytochem Lett 2011;4:391–8.
[28] Cordell GA, Colvard MD. Natural products and traditional medicine: turning on a paradigm. J Nat Prod 2012;75:514–25.
[29] Mukherjee PK, Rai S, Kumar V, Mukherjee K, Hylands PJ, Hider RC. Plants of Indian origin in drug discovery. Expert Opin Drug Discov 2007;2:633–58.
[30] Guo R, Canter PH, Ernst E. A systematic review of randomized clinical trials of individualized herbal medicine in any indication. Postgrad Med J 2007;83:633–7.
[31] Homma M, Oka K, Yamada T, Niitsuma T, Ihto H, Takahashi N. A strategy for discovering biologically active compounds with high probability in traditional Chinese herb remedies: an application of Saiboku-To in bronchial asthma. Ana Biochem 1992; 202:179–86.
[32] Mukherjee PK, Kumar V, Mal M, Houghton PJ. In vitro acetylcholinesterase inhibitory activity of essential oil and its main constituents of *Acorus calamus*. Planta Med 2007;73:285–8.
[33] Mukherjee PK, Verpoorte R. GMP for botanicals—regulatory and quality issues. New Delhi: Business Horizons; 2003.
[34] Mukherjee PK, Houghton PJ. The worldwide phenomenon of increased use of herbal products: opportunity and threats. In: Houghton PJ, Mukherjee PK, editors. Evaluation of herbal medicinal products—perspectives on quality, safety and efficacy. Pharmaceutical Press, Royal Pharmaceutical Society of Great Britain; 2009. p. 3–12.

[35] Cragg GM, Schepartz SA, Suffness M, Grever MR. The taxol supply crisis. new NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. J Nat Prod 1993;56:1657–68.
[36] Sharma RK, Arora R. Herbal drugs—a twenty first century perspective, 141. New Delhi: Jaypee Brothers, Medical Publishers (P) Ltd; 2006. 319–320, 527.
[37] Verpoorte R, Choi YH, Kim HK. Ethnopharmacology and systems biology: a perfect holistic match. J Ethnopharmacol 2005;100:53–6.
[38] Mukherjee PK, Ponnusankar S, Venkatesh P, Gantait A, Pal BC. Marker profiling: an approach for quality evaluation of Indian Medicinal Plants. Drug Inf J 2011;45:1–14.
[39] Evans S. Changing the knowledge base in Western herbal medicine. Soc Sci Med 2008;67:2098–106.
[40] Ulrich-Merzenich G, Zeitler H, Jobst D, Panek D, Vetter H, Wagner H. Application of the "-Omic-" technologies in phytomedicine. Phytomedicine 2007;14:70–82.
[41] Tyler VE. Phytomedicines: back to the future. J Nat Prod. 1999;62: 1589–92.
[42] Venkatesh P, Mukherjee PK, Kumar NS, Bandyopadhyay A, Fukui H, Mizuguchi H, et al. Anti-allergic activity of standardized extract of Albizia lebbeck with reference to catechin as a phytomarkers. Immunopharm Immunotoxicol 2010;32:272–6.
[43] Mukherjee PK, Wahile A, Kumar V, Rai S, Mukherjee K. Marker profiling for a few botanicals used for hepatoprotection in Indian system of medicine. Drug Infor J 2006;40:131–9.
[44] Raja S, Nazeer HA, Kumar V, Mukherjee K, Bandyopadhyay A, Mukherjee PK. Exploring the effect of *Asclepias curassavica* on markers of oxidative stress in rats. Evidence Based Int Med 2005;2:87–9.
[45] World Health Organization. Quality control methods for medicinal plant materials. 1998. 9241545100.pdf, Last accessed on 17th April 2014.
[46] WHO. Guidelines for assessing quality of herbal medicines with reference to contaminants and residues. 2007.
[47] Xue J, Liu DJ, Chen SL, Liao YH, Zou ZM. Overview on external contamination sources in traditional Chinese medicines. World Sci Techn 2008;10:91–6.
[48] World Health Organization. Guidelines on good agricultural and collection practices (GACP) for medicinal plants. 2003.
[49] World Health Organization. WHO monographs on selected medicinal plant. 2004.
[50] Bent S, Ko R. Commonly used herbal medicines in the United States: a review. Am J Med 2004;116:478–85.
[51] Anonymous. Markers and natural health products. Technology Watch 2006;3:2–4.
[52] Mukherjee PK, Kumar SN, Heinrich M. Plant made pharmaceuticals (PMPs)—development of natural health products from biodiversity. Indian J Pharm Educ Res 2008;42:113–22.
[53] Ministry of Health and Family Welfare. Department of ISM and H. Part I The Ayurvedic Pharmacopoeia of India 2001;III.
[54] Li S, Han Q, Qiao C, Song J, Cheng CL, Xu H. Chemical markers for the quality control of herbal medicines: an overview. Chinese Med 2008;3:1–16.
[55] Chaudhary N, Sekhon BS. An overview of advances in the standardization of herbal drugs. J Pharm Edu Res 2011;2:55–70.
[56] Mukherjee PK, Rai S, Bhattacharya S, Wahile A, Saha BP. Marker analysis of polyherbal formulation, Triphala: a well known Indian traditional medicine. Indian J Trad Knowl 2008;7:379–83.
[57] Mukherjee PK, Rai S, Kumar V, Mukherjee K, Hyland PJ, Hider RC. Plants of Indian origin in drug discovery. Ex Opin Drug Dis 2007;2:633–57.
[58] Song j, Li S, Zhou Y, Qiao C, Chen S, Xu H. A novel approach to rapidly explore analytical markers for quality control of radix salviae miltiorrhizae extract granules by robust principal component analysis with ultra-high performance liquid chromatography–ultraviolet–quadrupole time-of-flight mass spectrometry. J Pharm Biomed Anal 2010;53:279–86.
[59] Liu Y, Shi X, Liu E, Sheng L, Qi L, Li P. More accurate matrix-matched quantification using standard superposition method for herbal medicines. J Chromato A 2012;1254:43–50.
[60] Guliyev VB, Gul M, Yildirim A. Hippophae rhamnoides L: chromatographic methods to determine chemical composition use in traditional medicine and pharmacological effects. J Chromato B 2004;812:291–307.
[61] Mukherjee PK. Problems and prospects for the GMP in herbal drugs in Indian systems of medicine. Drug Inf J 2002;63: 6635–44.
[62] Gantait A, Pandit S, Nema N, Mukherjee PK. Quantification of glycyrrhizin in *Glycyrrhiza glabra* extract by validated HPTLC densitometry. J AOAC Inter 2010;93:492–5.
[63] Mukherjee PK, Nema NK, Bhadra S. Shifting paradigm for validation of traditional medicine: need and reality, Jatmed. 2013. 1–9.
[64] Busse W. The significance of quality for efficacy and safety of herbal medicinal products. Drug Infor J 2000;34:15–23.
[65] Srinivasan VS. Challenges and scientific issues in the standardization of botanicals and their preparations. United States Pharmacopoeia's dietary supplement verification program—a public health program. Life Sci 2006;78:2039–43.
[66] Kayne S. Problems in the pharmacovigilance of herbal medicines in the UK. Pharm J 2006;276:543–5.
[67] Huang S, Hall SD, Watkins P, Love AA, Singh CS, Betz JM, et al. Drug interactions with herbal products and grapefruit juice: a conference report. Clin Pharmacol Ther 2004;75:1–12.
[68] World Health Organization. WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems. Geneva: World Health Organization; 2004.
[69] Bauer S, Stormer E, Johne A, Kruger H, Budde K, Neumayer H, et al. Alterations in cyclosporine A pharmacokinetics and metabolism during treatment with St. John's wort in renal transplant recipients. Brit J Clin Pharmaco 2003;55:203–11.
[70] Ponnusankar S, Venkatesh P, Venkatesh M, Mandal SC, Mukherjee PK. Herbal drug require pharmacovigilance study—need and reality. Pharm Rev 2007;12:113–20.
[71] Zhou S, Zhou Z, Li C, Chen X, Yu X, Xue CC, et al. Identification of drugs that interact with herbs in drug development. Drug Discov Today 2007;12:664–73.
[72] Barnes J. Pharmacovigilance of herbal medicines: a UK perspective. Drug Saf 2003;26:829–51.
[73] Bagnais CI, Deray G, Baumelou A, Le, Quitrec M, Venherweghem JL. Herbs and the kidney. Am J Kidney Dis 2004;44:1–11.
[74] Mukherjee PK, Ponnusankar S, Badra S, Pandit S, Venkatesh M. Confluence of strategies for the development of botanicals. Pharm Rev 2008;12:114.
[75] Larrey D, Pageaux GP. Drug-induced acute liver failure. Eur J Gastroen Hepat 2005;17:141–3.
[76] Mukherjee PK, Ponnusankar S, Pandit S, Hazam PK, Ahmmed M, Mukherjee K. Botanicals as medicinal food and their effects on drug metabolizing enzymes. Food Chem Toxicol. 2011;49:3142–53.
[77] Lynch T, Price AB. The effect of cytochrome P450 metabolism on drug response, interactions, and adverse effects. Am Fam Phy 2007;76:391–6.
[78] Zhou S, Gao Y, Jiang W, Huang M, Xu A, Paxton JW. Interactions of herbs with cytochrome P450. Drug Metabol Rev 2003;35:35–98.
[79] Crespi CL, Penman BW. Use of cDNA expressed human cytochrome P450enzymes to study potential drug–drug interactions. Adv Pharmacol 1997;43:171–88.

[80] Pandit S, Mukherjee PK, Ponnusankar S, Venkatesh M, Srikanth N. Metabolism mediated interaction of a-asarone and *Acorus calamus* with CYP3A4 and CYP2D6. Fitoterapia 2011;82: 369–74.
[81] Pandit S, Mukherjee PK, Mukherjee K, Gajbhiye R, Venkatesh M, Ponnusankar S, et al. Cytochrome P450 inhibitory potential of selected Indian spices—possible food drug interaction. Food Res Intern 2012;45:69–74.
[82] Pan Y, Abd-Rashid BA, Ismail Z, Ismail R, Mak JW, Pook PC, et al. In vitro modulatory effects on three major human cytochrome P450 enzymes by multiple active constituents and extracts of *Centella asiatica*. J Ethnopharmacol 2010;130:275–83.
[83] Foster BC, Vandenhoek S, Hana J, Krantis A, Akhtar MH, Bryan M, et al. In vitro inhibition of human cytochrome-P450 mediated metabolism of marker substrates by natural products. Phytomedicine 2003;10:334–42.
[84] Usia T, Iwata H, Hiratsuka A, Watabe T, Kadota S, Tezuka Y. CYP3A4 and CYP2D6 inhibitory activities of Indonesian medicinal plants. Phytomedicine 2006;13:67–73.
[85] Ponnusankar S, Pandit S, Babu R, Bandyopadhyay A, Mukherjee PK. Cytochrome P450 inhibitory potential of *Triphala rasayana* from Ayurveda. J Ethnopharmacol 2011;133:120–5.
[86] Harwansh RK, Mukherjee K, Bhadra S, Kar A, Bahadur S, Mitra A, et al. Cytochrome P450 inhibitory potential and RP-HPLC standardization of trikatu—a Rasayana from Indian Ayurveda. J Ethnopharmacol 2014;153:674–81.
[87] Scott LM, Leduc RI, Burt AJ, Marles RJ, Arnason JT, Foster BC. The inhibition of human cytochrome P450 by ethanol extracts of North American botanicals. Pharm Biol 2006;44:315–27.
[88] Ponnusankar S, Pandit S, Venkatesh M, Bandyopadhyay A, Mukherjee PK. Safety evaluation of standardized extract of *Terminalia chebula* Retz by Cytochrome P450 inhibition assay. Phytother Res 2011;25:151–4.
[89] Gyamfi MA, Hokama N, Oppong-Boachie K, Aniya Y. Inhibitory effects of the medicinal herb *Thonningia sanguine* on liver drug metabolizing enzymes of rats. Human Exp Toxicol 2000;19:623–31.
[90] Tsukamoto S, Aburatani M, Tomihisa O. Isolation of CYP3A4 inhibitors from Black Cohosh (*Cimicifuga racemosa*). eCAM 2005;2:223–6.
[91] Frank A, Unger M. Analysis of frankincense from various Boswellia species with inhibitory activity on human drug metabolizing cytochrome P450 enzymes using liquid chromatography mass spectrometry after automated on-line extraction. J Chromatogr A 2006;21:255–62.
[92] Kang JJ, Wang HW, Liu TY, Chen YC, Ueng TH. Modulation of cytochrome P450 dependent monooxygenases, glutathione and glutathione S-transferase in rat liver by geniposide from *Gardenia jasminoides*. Food Chem Toxicol 1997;35:957–65.
[93] Ganzera M, Schneider P, Stuppner H. Inhibitory effects of the essential oil of chamomile (*Matricaria recutita* L.) and its major constituents on human cytochrome P450 enzymes. Life Sci 2006;78:856–61.
[94] Hansen TS, Nilsen OG. In vitro CYP3A4 metabolism: inhibition by Echinacea purpurea and choice of substrate for the evaluation of herbal inhibition. Basic Clin Pharmacol Toxicol 2008;103: 445–9.
[95] Bent S, Goldberg H, Padula A, Avins AL. Spontaneous bleeding associated with *Ginkgo biloba*. J Gen Intern Med 2005;20:657–61.
[96] Greenblatt DJ, Von Moltke LL, Luo Y, Perloff ES, Horan KA, Bruce A, et al. *Ginkgo biloba* does not alter clearance of flurbiprofen, a cytochrome P4502C9 substrate. J Clin Pharmacol 2006;46: 214–21.
[97] Jennifer A, Ramzan I. Pharmacokinetic and pharmacodynamic drug interactions with Kava (*Piper methysticum* Forst. F). J Ethnopharmacol 2004;93:153–60.
[98] Zou L, Harkey MR, Henderson GL. Effects of herbal components of cDNA expressed cytochrome P450 enzyme catalytic activity. Life Sci 2002;71:1579–89.
[99] Guerra MC, Speroni E, Broccoli M, Cangini M, Pasini P, Minghetti A, et al. Comparison between Chinese medical herb *Pueraria lobata* crude extract and its main isoflavone puerarin antioxidant properties and effects on rat liver CYP catalyzed drug metabolism. Life Sci 2000;67:2997–3006.
[100] Harikumar KB, Kuttan R. Inhibition of drug metabolizing enzymes (cytochrome P450) in vitro as well as in vivo by *Phyllanthus amarus* Schum and Thonn. Biol Pharm Bull 2006;29: 1310–3.
[101] Peng CC, Glassman PA, Trilli LE, Hayes-hunter J, Good CB. Incidence and severity of potential drug–dietary supplement interactions in primary care patients. Arch Intern Med 2004; 164:630–6.
[102] Pandit S, Ponnusankar S, Bandyopadhyay A, Ota S, Mukherjee PK. Exploring the possible metabolism mediated interaction of *Glycyrrhiza glabra* extract with CYP3A4 and CYP2D6. Phytother Res 2011;25:1429–34.
[103] De Smet PA. Health risks of herbal remedies: an update. Clin Pharmacol Ther. 2004;76:1–17.
[104] Colalto C. Herbal interactions on absorption of drugs: mechanisms of action and clinical risk assessment. Pharmacol Res 2010;62:207–27.
[105] Anonymous. Directive 2004/24/EC of the European Parliament and of the Council of 31 March 2004, amending, as regards traditional herbal medicinal products, Directive 2001/83/EC on the Community code relating to medicinal products for human use. Off J Eur Union 2004;136:85–90.
[106] Barnes JA. Close look at synergy and polyvalent action in medicinal plants. In pharma 1999;1185:3–4.
[107] Wagner H. New targets in the phytopharmacology of plants. In: Herbal medicine, a concise overview for health care professionals. Butterworth-Heinemann; 1999. p. 34–42.
[108] Kitano H. Computational systems biology. Nature 2002;420: 206–10.
[109] Kell DB. Systems biology, metabolic modelling and metabolomics in drug discovery and development. Drug Discov Today 2006;11:1085–92.
[110] Li P, Yang LP. Application of systems biology method in the research of traditional Chinese medicine. J Chin Int Med. 2008; 6:454–7.
[111] Li F, Gonzalez FJ, Ma X. LC–MS-based metabolomics in profiling of drug metabolism and bioactivation. Acta Pharmaceu Sini B 2012;2:118–25.
[112] Hood L, Perlmutter RM. The impact of systems approaches on biological problems in drug discovery. Nat Biotech 2004;22: 1215–7.
[113] Holmes E, Nicholls AW, Lindon JC, Connor SC, Connelly JC, Haselden JN, et al. Chemometric models for toxicity classification based on NMR spectra of biofluids. Chem Res Toxicol 2000;13:471–8.
[114] Choi YH, Choi HK, Hazekamp P, Bermejo YDC, Schilder C, Erkelens C, et al. Quantitative analysis of bilobalide and ginkgolides from Ginkgo biloba leaves and Ginkgoo products using 1HNMR. Chem Pharm Bull 2003;51:158–61.
[115] Choi YH, Kim HK, Hazekamp A, Erkelens C, Lefeber AMW, Verpoorte R. Metabolomic differentiation of *Cannabis sativa* cultivators using 1H-NMR and principal component analysis. J Nat Prod 2004;67:953–7.
[116] Park HW, In G, Kim JH, Cho BG, Han GH, Chang IM. Metabolomic approach for discrimination of processed ginseng genus (*Panax ginseng* and *Panax quinquefolius*) using UPLC–QTOF MS. J Ginseng Res 2014;38:59–65.

[117] Jordan SA, Cunningham DG, Marles RJ. Assessment of herbal medicinal products: challenges, and opportunities to increase the knowledge base for safety assessment. Toxicol Appl Pharmacol 2010;243:198−216.
[118] EMEA. Committee on herbal medicinal products. Guideline on selection of test materials for genotoxicity testing for traditional herbal medicinal products/herbal medicinal products (Draft). Committee on herbal medicinal products. London: EMEA; 2009.
[119] EFSA. European food safety authority, guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements. 2009.

LIST OF ABBREVIATIONS

AYUSH Ayurveda, Yoga, Unani, Siddha and Homeopathy
CDSCO Central Drugs Standard Control Organization
CYP450 Cytochrome P450
EMEA European Medicines Agency
GACP Good Agricultural and Collection Practices
IUPAC International Union of Pure and Applied Chemistry
TM Traditional medicine
USFDA United States Food and Drug Administration
WHO World Health Organization

CHAPTER

2

Value Chains of Herbal Medicines—Ethnopharmacological and Analytical Challenges in a Globalizing World

Anthony Booker[1], *Deborah Johnston*[2], *Michael Heinrich*[1]

[1]Centre for Pharmacognosy and Phytotherapy/Research Cluster Biodiversity and Medicines, UCL School of Pharmacy, London, UK; [2]Department of Economics, School of Oriental and African Studies, University of London, London, UK

OUTLINE

2.1 INTRODUCTION

According to the World Health Organization, in 2008, world trade in herbal medicine was estimated at US$83 billion [1]. Much of the trade in medicinal plants has been based within national market systems. While global trade in high-value products, such as spices and medicines, has a long history (e.g., the spice route [2]), during 1984–2014, international trade has flourished, and a main thoroughfare of this trade exists between Asia and Europe, the United States and Australia.

From a global perspective and particularly with regards to products sold with a specific medical claim, Europe has been leading the way in terms of supplying high-quality herbal medicinal products (HMPs). First, with diverse national initiatives and with the development of quality standards, for example, in the European Pharmacopoeia and, in 2004, with the introduction of the EU-wide, Traditional Herbal Medicinal Products Directive (THMPD), which requires well-defined standards of quality and safety to be ensured before a product can be released onto the market. Until December 2012, there has been >1000 traditional use registration licenses granted in Europe [3].

The effect of these new regulations on products originating outside of the EU and particularly in Asia has been substantial, with protests being voiced from trade associations and government officials. This has been particularly noticeable in China and India, two countries

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00002-7

that have had a long history of trade in HMPs with the UK and the rest of Europe [4,5].

The main problems highlighted and voiced by manufacturers are that the more sophisticated inputs involved in meeting the standards and the costs attached to these registered products are seen as being too high for many companies that operate within Asia and particularly within India [6].

These concerns are similar to those held generally on the impact of production standards [7]. However, changes in regulatory requirements or the setting of higher entry bars, although presenting many challenges to the industry, can also offer opportunities. An increase in quality standards is often regarded as a positive step by consumers, particularly when related to food or medicines, and these improvements in quality and safety can be regarded as adding value to a product. If companies are able to use these added requirements to their advantage, it may be possible for new and more sustainable enterprises to be established, particularly if there is cooperation between Asian and European actors.

The creation of vertically integrated value chains (VIVCs) is one way to achieve this cooperation and for producers in less economically developed countries (LEDCs) to gain better access to highly regulated markets.

An introduction to value chains, the impact that these chains have on the quality of finished HMPs, the impact on livelihoods, and an outline of the potential effects that overharvesting may have on the industry, forms the main aims of this chapter.

2.2 THE CONCEPT OF VALUE CHAINS

The literature on global commodity chains has moved away from the term "commodity chain or supply chain" in favor of the term "value chain." The latter is thought to better describe a wider variety of products and services, while also focusing on the distribution of benefits. As a result, the global commodity chain approach is now known as global value chain analysis [8].

> Value chain analysis describes the activities within and attached to an organisation, and relates them to an analysis of the competitive strength of the organisation. Therefore, in economic terms, it evaluates that value each particular activity adds to the organisations' commodities or services. This idea was founded upon the insight that any company is more than a random assembly of machinery, people and finance and only if these things are arranged into definable systems will it become possible to produce something for which customers are willing to pay a price. Therefore, it is argued that the ability to perform particular activities and to manage the linkages between these activities is a source of competitive advantage [9].

Changes in production, distribution, and financial systems, in synergy with the globalization of markets and the spread of information and communication technologies, suggest that more attention needs to be paid to both external and internal linkages within a company structure. The concept of the value chain allows us to shift the focus from manufacturing only to the other activities involved in the supply of goods and services, including distribution and marketing.

> Value chain research focuses on the nature of the relationships among the various participants involved in the chain, and on their implications for development. At any point in the chain, some degree of governance or coordination is necessary in order to take decisions on how the chain should be managed effectively [10].

However, value chain analysis has also been criticized for not giving enough attention to wider social, economic, and political factors. Its strength lies in describing the value chain linkages, but it does not explain how and why these linkages have developed. Therefore, any research investigating the value chain should also include a wider exploration of these surrounding issues.

2.3 THE MEDICINAL PLANT VALUE CHAINS—RESEARCH NEEDS

Value chain analysis has been applied to a variety of consumer goods, but only in the last 10 years has it been applied to medicinal plant production [11–17].

By examining medicinal plant value chains (MPVCs), it is possible to gain a better understanding of the role of different actors and inputs in the chain and their influence over chain management. Understanding the process is a vital step toward suggesting any meaningful strategies for improvement. The cultivation of medicinal plants is a relatively new industry, and wild collection represents the main route of supply in terms of the number of species collected [18,19]. On many occasions, wild medicinal plants are preferred by traditional healers and consumers over cultivated ones [20], as there is a general feeling that wild plant species are more clinically effective.

Worldwide, medicinal plant species are getting depleted rapidly due to overcollection from their natural habitats [21,22]. The collection and marketing of medicinal plants from the wild is an important source of livelihood for many of the poor in LEDCs. In Nepal, >300,000 households are engaged in the collection of medicinal plants [12]. It is claimed that up to 15,000 species of medicinal plants are globally threatened [23], and two ways are identified to conserve threatened species: first by

tightening restrictions on collection practices, and second by cultivation on a large scale [24].

2.4 MEDICINAL PLANT VALUE CHAINS IN ASIA

Kala et al. [21] assert that the marketing system in India is largely unregulated and inequitable. The medicinal plant cultivators are generally marginal farmers and laborers. They receive a cash income to meet their basic requirements of food, health, and the education of their children. They are often unaware of the real market prices of many of the medicinal plant species. It is often difficult for farmers to sell certain herbs due to their lack of knowledge of the marketing system, and conversely, many medicinal plants are destined to be traded through illegal channels. Other constraints are the slow rate of production, a long gestation period, shortage of cultivation technology, low yields, unscientific harvesting, poor processing, lack of quality control, scarcity of good manufacturers, and poor marketing infrastructure.

In a review [15] of fieldwork conducted in Uttaranchal, one of India's poorest states, van de Kop et al. document that resource-poor people collect plants from the wild to supplement their low income. They point out that high risks, transaction costs, and a lack of trust among chain actors prevent small-holder producers from taking up the cultivation of medicinal plants and suggest public–private collaboration as a way of reducing these constraints and secure market accessibility for small producers. They analyze the opportunities for and the constraints on developing medicinal plant chains and aim to identify the role of medicinal plant chains in poverty reduction.

Most of the medicinal plants in Uttaranchal are collected from the wild. Permits are issued to cooperative groups in the area, which in turn employ contractors to organize the collection. The contractors employ collectors, usually land owning farmers or landless laborers. The contractor can sell the collected plants either to the local cooperatives or directly to independent traders after paying royalties to the cooperative.

The cooperatives sell either to local agents or wholesalers, traders in large cities, or to drug manufacturers. The traders supply the domestic market and international markets.

Van de Kop et al. suggest that in the value chain, the collectors and local contractors are in a weak position as they cannot sell directly to the largest trading companies in the cities, and depend on the local traders for marketing. This weak position often results in them receiving a considerably lower price than the true market price.

Cultivation is seen as a way of breaking free from some of these ties, and small-scale projects have commenced in the area. There are difficulties and risks attached to cultivation, and public–private collaborations are suggested as a way of minimizing these constraints. However, these collaborations have remained small and further promotion, regulation, and investment are needed if these ventures are to produce a meaningful result [12].

The work of Alam and Belt focused on a project in Uttarakhand, northern India, where 80% of the population relies on agriculture as their main economic activity, and 40% of people live below the poverty line. It was proposed that the cultivation of a medicinal plant, *kutki*, *Picrorhiza kurroa* Royle ex Benth. Plantaginaceae, would benefit the farmers financially, provide social benefits, and help preserve wild species. Moreover, the European buyer would have a secure supply of the plant from a fully traceable source.

The Uttarakhand project produced disappointing results, and in their paper, the authors highlighted the reasons for this as being poor quality of planting material, planting on small, poorly irrigated plots, and the emergence of apples as a profitable cash crop, resulting in farmers switching over from kutki to apples.

Alam and Belt concluded that the cultivation of medicinal plants is more difficult than usually suggested in the scientific literature and government promotional material and stress the importance of agencies and nongovernmental organizations (NGOs) taking these difficulties into account and taking steps to minimize these. The authors further argue that a thorough technical and economic feasibility study of the value chain, long-term involvement of governmental and NGOs, and an understanding of the prevalent farming system are necessary to ensure the success of the chain.

Conversely, a project conducted in Bangladesh [14] appears to be more optimistic with respect to the economic potential of medicinal plants and suggests vertical integration as a vehicle to benefit and empower producers and processors at the beginning of the value chain. The primary and wholesale secondary markets were dominated by middlemen, but their study challenged the view that medicinal plant cultivation was only appropriate for relatively well-off people with better access to land, capital, and information.

The authors built on previous work by researchers [15] and argue that some of the mechanisms employed in developing and sustaining institutional relationships may also apply equally well to defining the MPVC and list *contracts*, *quasivertical integration* (an especially close and long-term relationship), *tapered vertical integration* (when a company sources inputs externally from independent suppliers as well as internally within the same company), *cost plus agreements* (where the

contractor is paid a negotiated amount regardless of incurred expenses), *joint ventures*, and *strategic alliances* as examples of these potential relationships.

Moreover, they argue that the benefits of an integrated value chain are numerous.

> It enables primary producers to become active participants in the process, it removes market access barriers, and it results in better commercialization of products and is attractive to companies as they can have greater control over quality and supply.

In their study, Shahidullah and Haque, contrary to previous views, found that the cultivation or production of medicinal plants could play an important role in improving the livelihoods of those poor or very poor people who may own only small pieces of land.

They argue that to sustain growth in medicinal plant production,

> A fair distribution of the gross margin to the primary producers is necessary.

In the value chain system examined in [14], it was found that downstream buyers, especially manufacturers and consumers, pay most of their money for the value additive opportunistic pricing of middlemen due to inherent weaknesses in the chain.

A vertically integrated chain, with only producers and processors as commercial actors and NGO's as promoters, could create a better and more equitable situation.

However, there are some studies that suggest that vertically integrated chains, particularly ones that are dominated by a powerful company, can lead to negative effects on the livelihoods of small producers.

This has been illustrated in the work of van Niekerk and Wynberg [25], in which the authors present evidence to suggest that (based on their assessment) the monopolistic behavior of one large European company threatened the livelihoods of farmers in South Africa (for a single botanical drug) and that there were major inequalities between the bargaining powers of the local manufacturers and the primary producers.

2.5 MEDICINAL PLANT PRODUCTION IN CHINA AND INDIA

2.5.1 China

China has a long and relatively well-recorded history of using plants for medicinal purposes [26]. There are hundreds of state-owned and increasingly shared ownership companies producing traditional Chinese medicines (TCMs), many of which export their products internationally [27].

China has partly addressed its own difficulties relating to the manufacture and supply of TCM products by modernizing its traditional medicines profession with government-sponsored good agricultural practices and good manufacturing practices (GMPs). All manufacturers of TCM products must comply with standards set down by the China Food and Drug Administration to gain GMP certification. Only around 1500 companies have achieved this standard.

However, TCM products originating in China are of particular concern to many regulatory authorities such as in the UK and instances of poor quality and adulterated material are commonly reported [28–30].

To satisfy the requirements of an international market, some companies have found it necessary to put in place systems that help address the quality concerns of foreign customers. For example, Taiwanese herbal product manufacturers, which supply herbs to distributors in many parts of the world, had to find an alternative strategy for buying herbs at local or provincial herbal markets to better assure their customers that they can source good quality herbal ingredients that can be traced back to the areas of cultivation. The belief is that by having direct links to the farms it is easier and less costly to ensure the quality and traceability of the herbs in comparison to using the herbal markets that tend to dominate herbal medicinal trade. In China, it is difficult for non-Chinese organizations to make direct links with individual farmers, so herbal sourcing companies have emerged to provide this missing link in the export market supply chain.

2.5.2 India

Kala et al. [21] assert that of the 17,000 higher plant species to be found in India, 7500 are known for medicinal uses, with Ayurvedic medicine claiming to use 2000 of these. Most plants used in Indian systems of medicine are collected from the wild. More than 60 species are in great demand, and the "tribal belt" of India is abundant in these plants, and minority groups mainly depend on this trade for their livelihoods [31]. The annual turnover of the Indian herbal medicine industry has been estimated by different authors to be between US$377 million and US$1 billion per annum [21,32], between 0.5 and 0.8 per cent of world trade.

The globalization of Ayurvedic practices gained momentum during the 1990s and onward into the 2000s, and Ayurvedic products are commonly used as food supplements in North America, Australia, New Zealand, Europe, and Japan [33]. While Indian exports, valued at US$132 million in 2008, contributed less than one per cent to the global herbal market, industry observers suggest that growth is rapid and that Indian

companies are fast emerging as key international suppliers of medicinal plants [34].

A study commissioned by India's National Medicinal Plants Board and conducted by the Bangalore-based institution, the Foundation for the Revitalization of Local Health Traditions has estimated that 177,000 tonnes of medicinal plants are used each year by India's domestic herbal industry, that 86,000 tonnes are used within rural Indian households, and that 56,500 tonnes are exported through international trade [35].

The structure of the industry is quite diverse. HMPs are produced by several thousand companies in India, most of whom are quite small, including numerous neighborhood pharmacies that compound ingredients to make their own remedies [36]. The products of these companies are included within the broad category of "fast moving consumer goods," which mainly involves foods, beverages, toiletries, cigarettes, and may also contain certain types of pharmaceuticals [37]. Most of the larger HMP suppliers provide materials other than herbal medicines, particularly in the areas of foods and toiletries [36].

The exact number of manufacturers is unclear. Subrat et al. [38] suggest that there are approximately 6000 licensed manufacturers and about the same number of unlicensed ones, with about 70 per cent of the market share belonging to Ayurveda. However, Polshettiwar [39] proposes that about 1200 licensed small manufacturers in India are on record, with about 20 well-recognized manufacturers of HMPs and 140 small- to medium-sized manufacturers. However, what is generally agreed upon is that the formal sector of the industry is dominated by less than a dozen major companies. In 2012, Emami was the leading company, with a market value share of 17 per cent, followed by Dabur with 16 per cent and Proctor and Gamble, the American multinational consumer goods company, with 11 per cent, indicating an increase in market presence from non-Indian companies [40].

However, according to Seale [34], there are around 30 other companies that produce US$1 million or more of Ayurvedic products each year, including small pharmacies and family-owned enterprises that formulate their own products and guard their remedy recipes closely. But although Ayurveda has traditionally been the province of home remedies and naturalist producers, like everything in India, Ayurvedic and other traditionally based products are going increasingly high-tech [34].

These manufacturers are supplied by local or national markets, directly from farms, or commonly through middlemen. The vast majority of medicinal plants used in India to make HMPs are still collected from the wild although there is evidence to suggest that some companies are developing more sustainable cultivation strategies [35].

Exports of Indian herbal products increased from US$69 million in 2005–2006 to US$128 million in 2009–2010, and recorded a compounded annual growth rate of 16.8 per cent. North America, Pakistan, Germany, Japan, the United Kingdom, Spain, China, France, Vietnam, and Mexico were the top 10 export destinations for India's herbal exports during 2011–2014. The United Kingdom remained the fifth largest overseas market for Indian herbal products with exports worth US$3.7 million in 2007–2008, and US$5 million in 2008–2009 (Table 2.1). The UK had a 2.5 per cent share in the country's total herbal exports in 2009–2010 [41]. If these figures are accurate, it indicates that UK imports in 2009–2010 declined to around US$3.2 million, possibly as a result of the implementation of the THMPD which, it has been argued, raises the entry bar for producers of these types of plant-based medicines to enter the European market [42].

The number of professional practitioners of traditional Indian medicine (TIM), including Ayurveda, are scarce in the UK compared with that of Western herbalists or TCM practitioners, and Indian HMPs are generally sought out by the general public in retail health food shops or by using online websites. Ayurvedic products are also available as food items in supermarkets in the form of teas. This is a juxtaposition of how these plants are administered in India, where many herbal products are regarded as potent medicines often superior in efficacy to pharmaceuticals and with fewer side effects [31].

Despite some healthy growth in Indian HMP exports, adulteration and contamination are commonplace, so the supply of good quality raw materials is limited [31]. This has in turn stunted the industry's growth in previous years.

TABLE 2.1 Estimated Annual Values of Herbal Medicines and Some Health Foods

Global value of herbal medicine	**US$83 billion (2008)**
Value of Indian herbal trade	US$1 billion (2006)
Value of Indian herbal exports	US$128 million (2010)
Value of UK imports of Indian herbal medicine	US$5 million (2009)
Value of herb trade at Bozhou market, China	US$735 million (2011)
Value of Canadian ginseng trade	US$68 million (2001)
Value of US turmeric imports	US$4 million (2008)

Refs [1,21,32,41,44–46].

Increasingly more species are being gradually added to the Indian herbal *materiamedica*, and the standards for purity and identification do not always keep pace with this expansion process [21]. This potentially lucrative position has led to the overexploitation and depletion of medicinal plants. According to Kala et al. (2006), >95 per cent of the 400 species used in the herbal industry are wild collected, and development of agrotechnology should be a research priority. Consequently, farming will foster the production of uniform material from which products with a chemically well-defined composition can be readily obtained. Moreover, by cultivating plant species, identification can be controlled from the seed stage. Arguably, the medicinal plant sector can be improved if the agricultural support agencies would come forward to help strengthen the medicinal plant growers and if research institutions would aid the plant growers by improving their basic knowledge of plant cultivation practices [21].

Vaidya and Devasagayam [43] are more positive about the condition of the industry and propose that evidence-based herbals are widely used and manufactured, as per the guidelines, by a well-organized industry and suggest that newer approaches, using collaborative research and modern technology in combination with established traditional health principles, will yield rich dividends in improving health.

However, as indicated below (the tea and ginseng value chains), there is some debate and no firm resolution concerning the long-term effects of a move to cultivation, the impact of closer regulation, and of the implementation of VIVCs.

According to Patwardhan et al. [31], India should take the lead from China in developing a more quality-driven ethos towards medicinal plant production. However, Gong [47] points out that despite the fact that China outpaces India in economic development, the Indian pharmaceutical industry excels in the international market while Chinese companies lag behind and argues that the critical success factors of Indian pharmaceuticals can be explained from both the macrolevel of the industry environment and the microlevel of enterprises (Table 2.2).

Pharmacoeconomic studies on TIM and TCM are rare but can help in understanding cost effectiveness and cost benefit of traditional medicine. Patwardhan et al. [31] argue that in all such attempts, Chinese medicine regulation can help India at various levels, including with policies, quality standards, research models, and integration into the health system.

There is no doubt that China has considerable experience in this arena, but it may also be productive for India to make collaborations with the countries in which their products are destined in order that they can fully comply with international standards and regulations. As discussed above, India has already achieved this in the allopathic pharmaceutical arena in which it appears ahead of China in terms of its achievements.

TABLE 2.2 Success Factors for the Indian Pharmaceutical Industry [47]

Macrolevel	Microlevel
The development of the pharmaceutical industry is driven by societal development and innovation.	Well-established privately owned enterprises are commonplace in India.
The Indian government provides incentives for exporting active pharmaceutical ingredients and reduces taxation to promote trade.	Resources are well used and enterprises are efficiently managed.
To Investors, India is more attractive than China because of better corporate governance, more regulated finances, and more transparent intellectual property rights protection, and a more business friendly legal system.	Indian companies have a longer history and a deeper understanding of international markets than their Chinese counterparts have.
Exceptional patent protection exists in India.	Indian companies place a high priority on applying technologies and expertise to business management.

2.6 SUPPLY, DEMAND, AND SUSTAINABILITY

As experienced in connection with the expansion of traditional medical systems globally, rapid growth in the traditional Asian medicine industry has led to the overexploitation and depletion of medicinal plants, not only affecting biodiversity and the ecology but also having a serious detrimental impact on the livelihoods of the indigenous forest peoples.

It has been estimated that >2000 medicinal and aromatic plant species are used commercially in Europe, of which 1200–1300 are European native species. Approximately 90 per cent of the European species are collected from the wild, with eastern Europe and the Mediterranean regions being the main suppliers [48].

In India, it has been estimated that approximately 7500 species are used for medicinal and veterinary purposes [49]. Over 10,000 species are used medicinally in China [50], with 1000 species commonly used in medicinal preparations, of which 80 per cent are wild collected [51].[1]

[1] These figures are difficult to obtain and to validate, and thus, there may well be a high degree of conflicting data.

Kala [21] argues that developing agrotechnology should be a research priority.

> Farming will foster the production of uniform material from which standardised products can be readily obtained. Moreover, by cultivating plant species, identification can be controlled from the seed stage. The medicinal plant sector can be improved if the agricultural support agencies would come forward to help strengthen the medicinal plant growers and if research institutions would aid the plant growers by improving their basic knowledge of plant cultivation practices.

Opportunities for governments to develop legislation to control and monitor harvest and trade of medicinal plant species and to consider conservation and sustainable use of medicinal plants as a priority in establishing protected areas have been greatly improved through the addition of medicinal plant species to the Convention on International Trade of Endangered Species and the entry into force of the Convention on Biodiversity [18].

The following value chain case studies consider some aspects and exemplify opportunities and challenges. While not specifically focusing on species that yield herbal medical products, the debate centers on how private companies and other agencies are likely to be able to support agricultural initiatives and whether this leads to better income for farmers and workers and improved working conditions.

2.7 THE TEA VALUE CHAIN

The tea industry, including green tea and other niche products, provides a good example of how value may be added to a product. Tea has uses as a food, a medicine, and also in the cosmetic industry [52,53]. It is a product that has a history of being exported and has been subject to value chain analysis. Moreover, the cultivation of this product can have a considerable impact on the livelihoods of farm workers in LEDCs [54].

It is widely reported that tea cultivation in the countries where it is prevalent has historically demonstrated a positive impact on the economy of poorer rural areas [55,56].

Tea cultivation often requires companies to provide employment to large numbers of the least privileged segments of society, mostly in remote areas where there is little other infrastructure. As a result, roads, electricity, water, etc., become more widely available to the general populace in these isolated districts.

In India, for instance, the major tea plantation companies provide affordable housing, medical care, and education to their employees and their employees' families. Wage agreements are usually industrywide, and salaries are above the national average [57].

Green tea production is chiefly located in southeast Asia and particularly in China, which is responsible for 75 per cent of all green tea produced [56]. Although much of Chinese tea production is conducted on small farms rather than on large tea plantations, Unilever has invested tens of millions of Yuan in establishing a research center for tea and TCM in Anhui province [58]. The plant has a production capacity of >100 million Yuan (US$16.5 million) every year.

Although this seems to be a beneficial situation for rural workers, as outlined below, there is a counterargument suggesting that it is the tea companies that are the biggest winners through their exploitation of the farmers and their workers, who have few alternatives other than to subjugate themselves either to the multinational or state-run companies, finding themselves increasingly dependent on a wide range of exterior inputs ranging from seed to fertilizers to pesticides.

The bargaining power of producers at the cultivation end of the value chain appears relatively weak compared to that of the processors and retailers who are able to exert control on both the price paid for the raw crops and the essential inputs needed to produce economically viable yields [59].

One initiative that is claimed to have a beneficial effect on both the incomes of farmers and the quality of produce is the Fairtrade® initiative [55].

This has been particularly noticeable in the tea and coffee markets and the broad-based aim of the Fairtrade® movement is to offer a better deal for farmers by paying above the market rate for the commodity in question, and in return, the farmers are expected to adhere to the Fairtrade® policies on production and follow quality-driven requirements in key areas, particularly in the cultivation and collection stages.

Although basically this is a positive step forward, the scheme is far from a panacea as it only represents a small section of the total market and may be more suitable in some countries than others [60].

Fairtrade® schemes tend to favor larger companies and have little influence on the wages of the workers [61]. The market price for tea in China and the "Fair" price, according to the Fairtrade® database was US$1.20 per kilogram and US$1.70, respectively. According to Ref. [62], even low-quality Chinese tea typically costs >US$1.70, indicating that the bar is set very low for Fairtrade® certification. Hodge asserts that in China, Fairtrade® is not relevant at all in relationship to good quality tea and argues that the producers of this level of quality tea do financially well compared to peasant farmers that are growing other crops. Chinese agriculture does not tend to be based on the corporate, plantation model, as in the rest of the world and, therefore, many Fairtrade® initiatives are less implementable

into the Chinese framework than they are for the huge plantations of South America and to a lesser extent, for those of East Africa.

Although consumer support is needed, it appears that the issues are much more complicated than just price. Since virtually all farming in China is done by small farmers, organizational issues are very different from those for an international plantation model. This view appears to have some credibility; the Fairtrade® website makes little mention of Chinese Fairtrade® tea, whereas it does list out bean products from Inner Mongolia as one of their success stories in terms of supporting poorer communities. However, in terms of using Fairtrade® as a vehicle to help develop organic, sustainable farming, there are a few small specialist farms in China beginning to emerge.

Moreover, in a 10-year review of the Fairtrade® literature [60], the authors conclude that although the evidence base is patchy, there are producer and gender inequalities and Fairtrade® has not shown to be a solution to eliminate rural poverty. The information gathered indicated that Fairtrade® was valuable in providing organized small export producers with the stability and security they needed to make longer term investments and that most Fairtrade® cooperatives are becoming stronger, particularly where producer ownership further along the chain is achieved.

One of the major reasons for the price of some tea being kept so low is due to the domination of the tea sector by a few companies, and it is seen in the breakdown of who accrues the largest share of the value chain that the highest earners are traders and retailers. Approximately 40 per cent of the retail price of tea accrues to the tea traders and manufacturers, and a further 40 per cent goes to the processors and blenders, packagers, and retailers, based mainly in rich countries [55]. In tea-producing countries, around 15 per cent of the retail price goes to the plantation and factory, and less than one per cent goes to the auction broker. The plantation worker is likely to earn one per cent or less of the retail value.

This unappreciation and underinvestment of the agricultural workforce as a valuable human resource is not only grossly unfair and a cause of huge social inequity but may also lead to quality problems that will be carried through the length of the production process. This can potentially result in a finished product that will be difficult to sell as an aid to health in the hugely competitive functional food and nutraceutical arena.

The future for the growth of tea and particularly green tea production is inextricably linked to its health claims, but it is not only the health of consumers in Europe that are affected but also the workers and farmers in the countries of origin. In Vietnam, tea producers often benefit from better living standards than do producers of other crops, with reports of incomes doubling through tea production; this benefit appears mainly dependent on the ability of producers to connect to a value chain, which in turn opens up access to a more lucrative export market [54].

However, one of the more concerning reports suggests that the overuse of pesticides by producers in Darjeeling, India, leads to pesticide residue rates, which far exceed international limits, and exposes tea pickers to high levels of toxins that are hazardous to their health [63].

One pesticide, which is of particular concern, is the organochlorine pesticide, Endosulfan®. Although Endosulfan® has been banned or severely restricted in >60 countries, it is still widely used in many LEDCs, including India and China, due to its high effectiveness and low application cost. Endosulfan® is a "persistent organic pollutant" as defined under the Stockholm Convention: it is persistent in the environment, bioaccumulative, demonstrates long range environmental transport, and causes adverse effects to human health and the environment [64].

One central question is how the tea industry can be developed sustainably to optimize the health benefits at both ends of the value chain. Workers and small farmers have historically been weakened and marginalized, and today, they hold a relatively minor position in the tea value chain. A downward pressure on the price paid for tea to the farmers and subsequently on the daily wages of the workers causes poverty and distress among hundreds of thousands of people whose living depends entirely on tea production. At the same time, traders and tea packers are continuing to realize large profits. It is claimed that large companies have a policy of deliberately reducing differences in quality among the different teas produced worldwide, enabling teas to be purchased at the lowest cost and maximizing profits from the blending, packaging, and marketing stages that tend to be in the hands of the large tea oligopolies [65].

2.8 THE GINSENG VALUE CHAIN

Another value chain, which has been well documented, and that gives a good account of how value can be added to a product is that of *Panax ginseng* C.A. Mey. and some related species. It is of particular relevance as the literature shows that buyers' perceptions of what constitutes a good quality and desirable product can make huge value additions to a product. Moreover, it is seen through an examination of this literature that the leading firms in a value chain are not always at the retail end of the chain and more power can sometimes be held by farmers.

During the 14-year period from 1989 to 2003, approximately 17 tonnes of dried American ginseng (*Panax*

quinquefolius L.) root were harvested from Pennsylvania. Approximating a conservative price of US$600 per kilogram paid to collectors, the contribution from the ginseng trade to Pennsylvania's economy can be estimated at >US$10 million during this period and this figure does not include income received from downstream and value added processing [66]. In 2012, the United States exported approximately 20 tonnes of wild or wild-simulated[2] American ginseng, worth US$27 million [67]. When the market was at its peak, ginseng root was one of the world's most profitable legal crops (in terms of its value per kilogram), selling for as much as US$770 per kilogram for semiwild woodland crops [45].

Ginseng values are dependent on the production method. The lowest investment and production costs are for wild-simulated ginseng, while the greatest expenses are required for intensively cultivated field grown ginseng under artificial shade. Wild ginseng is considered the most profitable, and was sold at US$990 per kilogram of dry weight in 1999, compared with wild simulated at US$550 per kilogram and woods cultivated at US$330 per kilogram. This translates to revenues of US$ 17,700 per hectare for wild-simulated over an 8- to 10-year period and US$31,500 per hectare for woods cultivated over a five-year period and so although the wild-simulated approach requires less input, the longer cultivation period reduces overall revenue [68].

Field-cultivated ginseng had a value of under US$44 per kg in the same year [69]. This difference in price is at first difficult to comprehend, but it may be linked in some way to the ginsenoside content of the herbal material and consequently to its effectiveness. The potency of herbal products can vary from manufacturer to manufacturer and from batch to batch, partly because of nonstandard processing methods and also due to the variability of cultivation conditions, for example, soil, temperature, moisture, length of cultivation, and harvest season [70].

Asian buyers consider wild ginseng to be more potent than cultivated ginseng and connect value to morphology (wild roots can look more human in shape). Consequently, the root value is highly dependent on its appearance. However, in a study conducted by Schlag and McIntosh [93], the authors fail to find a significant difference between wild and cultivated plants with reference to total ginsenoside content. This could indicate that Asian traditional beliefs surrounding ginseng are based on the subjective value of a wild crop or linked to experiences of taste, color, etc., or it could mean that total ginsenoside content is not a clinically relevant measure of ginseng potency, and other metrics such as relative ginsenoside composition may prove to be more meaningful. In the Schlag and McIntosh study, significant variations were found between the ginsenoside composition of wild and cultivated ginsengs, and two distinct chemotypes were identified. However, chemotypical differences in composition were also observed between wild ginsengs grown in different areas of the United States, and it is not such a simple picture of wild versus farmed.

Although the price paid for semiwild ginseng doubled from 1999 to 2009, the price for farmed ginseng dropped by 75 per cent. Current prices for farmed roots are below the cost of production, and consequently, the artificial shade cultivated industry in North America is in a state of collapse. One of the primary reasons for this decline in prices in North America is increased cultivation of *P. quinquefolius* in China. It is unlikely that this situation will extend to wild-simulated ginseng or even woods-cultivated as China has long since gone through a process of deforestation.

Although high yielding, the main problem for farmed ginseng is that it is susceptible to disease and requires the heavy use of pesticides and fungicides in order to thrive [72]. This can be a real drawback when presented as an aid to good health, especially in a market that historically sets high entry bar standards for non-EU commodities. China seems to be responding in typical fashion and along the highways one can now see small trees several rows deep. These are part of China's reforestation program, and once they reach a suitable age, they are transferred to where forests once stood for replanting. It may take some time for these new forests to mature, but there is a real prospect of wild ginseng growing once again in the country that made it a global commodity.

2.9 PLANT METABOLOMICS AND ANALYTICAL CHALLENGES

Our research in recent years has used a mixed methods approach combining a value chain analysis with a study of the chemical composition of the starting materials sourced from diverse regions of India with a metabolomics analysis. Here, we outline two examples of analytical platforms, ^{1}H-Nuclear magnetic resonance (NMR) spectroscopy and High-performance thin layer chromatography (HPTLC), which may be used to better understand the composition and chemical variability of HMPs. Both techniques provide different and complementary data, and together they can be used to

[2] Plants are cultivated in areas and under conditions that reproduce as closely as possible a wild environment. Whereas woods-cultivated are farmed in a forest environment.

effectively differentiate between a wide variety of crude drug powders and HMPs. ^{1}H-NMR spectroscopy coupled with multivariate analysis provides a quick method of separating different samples but the statistical software is sometimes confounded by multicomponent products or by the addition of excipients. Comparing results against HPTLC helps to avoid this pitfall, and when combined the two techniques provide a clearer picture of sample composition, and offer new ways to understand how chemical quality may potentially be linked to local livelihoods and the fast developing market links of ethnopharmacological commodities [17].

Metabolomics is the term used for the comprehensive, nonbiased, high throughput analyses of complex metabolite mixtures such as those typically seen in plant extracts. Achievement of a broad overview of metabolic composition requires the establishment of a fully integrated approach for the optimization of sample extraction, metabolite separation/detection/identification, automated data gathering, processing and analysis, and quantification [71]. Metabolomics has become a well-recognized method for the study of all types of organisms, and complements the data obtained by the other omics technologies: genomics, transcriptomics, and proteomics [73].

One of the main problems associated with metabolomics is that the metabolome consists of a wide range of compounds at very different concentrations and with different polarities and other chemical characteristics. At present, there is no single solvent capable of solubilizing the whole range of chemical constituents. The choice of extraction solvent is thus limiting the view on the metabolome. In general, metabolomic studies should be designed to detect as many metabolites as possible in an organism [74].

It is widely accepted that a single analytical technique will not provide sufficient visualization of the metabolome, and therefore, multiple techniques are needed for a comprehensive view. However, practical reasons can force us to choose an optimum analytical tool for metabolomic profiling. Consequently, it may be preferable to use a wide spectrum chemical analysis technique, which is rapid, reproducible, and stable in time, while needing only the very basic sample preparation. NMR spectroscopy is potentially an analytical tool that could meet these requirements [75]. NMR spectroscopy is a physical measurement of the resonances of magnetic nuclei, such as ^{1}H, ^{13}C, or ^{15}N, in a strong magnetic field.

Each compound has a highly specific spectrum. The only variables are the solvent used and the magnetic field strength [76].

NMR spectroscopy is an effective tool for the quality control of medicinal plants or HMPs [77]. The advantages of NMR spectroscopy over other techniques such as mass spectrometry (MS) for metabolomics applications include the relative ease of sample preparation, nondestructive analysis, potential to identify a broad range of compounds, enhanced capacity for definitive chemical compound identification, and provision of structural information for unknown entities [78].

Recent advances in analytical chemistry, combined with multivariate analysis techniques, have brought us closer to the final goal of metabolomics: the comprehensive evaluation of all the metabolites, both qualitatively and quantitatively in living organisms.

NMR spectroscopy and MS have been successfully used for metabolic fingerprinting analysis. These two techniques have their respective advantages and limitations; however, as a tool for metabolomics, NMR spectroscopy has some unique advantages over MS-based methods. It can provide a detailed analysis on the biomolecular composition very quickly with relatively simple sample preparation. It is a universal detector for all molecules containing NMR-active nuclei. Using a proper internal standard, the real concentration of metabolites can be easily calculated and because NMR spectroscopy is based on the physical characteristics of compounds, it has a very high reproducibility [79]. In any metabolomics application, the robustness and reproducibility of data collection are vitally important.

^{1}H-NMR spectroscopy is an ideal tool for large-scale plant metabolomics data collection. In a study by Ward et al. [80], it was concluded that "with attention to experimental design and careful set up, data collection for large scale plant metabolite fingerprinting using ^{1}H-NMR spectroscopy can be carried out as a dispersed activity across laboratories, using different NMR spectroscopy instruments." Moreover, many techniques have now been devised to develop ^{1}H-NMR spectroscopy as a fingerprinting tool for the quality assessment of crude plant materials.

Multivariate or pattern recognition techniques, such as principal component analysis (PCA), are valuable techniques for the analysis of data obtained by ^{1}H-NMR spectroscopy. In combination with PCA, ^{1}H-NMR spectroscopy has been applied to the metabolomic profiling of plants and herbal medicines [81]. In the case of feverfew, ^{1}H-NMR spectroscopy and PCA was used to differentiate between 14 commercial batches of samples based on their multicomponent metabolite profile [82]. In the case of *Hypericum perforatum* L. (St John's Wort) the PCA of the NMR spectra from different commercial extracts was used to differentiate between various preparations according to their metabolomic profile. This included differentiation between various batches obtained from the same supplier. This highlighted the potential to use the method for assessing whether the species extract variability

TABLE 2.3 Comparison of Key Strengths and Weaknesses of ^{1}H-NMR Spectroscopy and HPTLC

^{1}H-NMR Spectroscopy	HPTLC
Equipment expensive	Equipment relatively cheap
High throughput technique with sample preservation	High throughput technique with sample preservation
One system needed to view an entire range of extractable metabolites	Several systems needed to view an entire range of extractable metabolites
Small peaks may be difficult to see or obscured by larger peaks	It is possible to view compounds that are present in very small quantities
Multivariate analysis is able to group the samples according to the metabolite composition	Grouping of samples is achieved manually by visual inspection
Multivariate analysis can be confounded by multiingredient samples leading to errors in groupings	Separate systems and visual inspection reduces grouping errors
Manipulation of data allows for individual samples to be compared in detail against other samples or to the whole group	Comparisons are generally achieved visually

versus the manufacturing process accounts for the variability [83].

Further studies carried out on herbal tinctures have shown that ^{1}H-NMR spectroscopy and adjuvant techniques can potentially be applied to the quality control of plant extracts, including batch-to-batch consistency and stability studies [84].

HPTLC also has a use in metabolomic analysis and can provide complementary and different data to other analytical platforms. HPTLC is limited in that it is not possible to see the same range of metabolites in a single test, as is possible for NMR spectroscopy. However, by using solvent systems of different polarities, it is possible to view a metabolomic fingerprint that is comparable to NMR spectroscopy spectra.

An important advantage of HPTLC is that it is possible to visualize compounds directly that are present only in very small concentrations that are not readily seen using NMR spectroscopy due to baseline noise or the presence of other peaks. A wider comparison of the advantages and disadvantages of NMR spectroscopy and HPTLC is given in Table 2.3.

2.10 DISCUSSION

Farmers and pickers are beginning to move away from the traditional route of selling their herbs at the local or provincial herb markets and are beginning to form alliances with local pharmaceutical companies or with regional sourcing companies to gain access to the more lucrative export market [21,85,86].

The perceived benefits to the farmer to this approach are mainly market price stabilization and a fixed-term contractual arrangement that allows for longer term planning, where the buyer is able to provide target crop yields for the farmer and agree a contractual price that will not be easily affected by many of the market shocks that may negatively impact the market traders. Moreover, the farmer has access to a wide range of inputs that are necessary to help cultivate medicinal plant crops effectively.

However, it is the very nature of an unstable and fluctuating market that continues to attract and sustain interest in the medicinal plant trade at the traditional market level, and it appears by far to be the more common route of supply [86].

It may be that there is room for both approaches; the free market for those who want to speculate, chiefly supplying to other traders and the Asian HMP manufacturing market, and those who are more inclined toward receiving a steady income with price guarantees and regular orders and who are amenable to modifying their working practices to satisfy the needs of international buyers.

Moreover, there is some evidence from the literature to suggest that VIVCs can have a detrimental effect on livelihoods and lead to primary producers being exploited by large multinational companies. Work on the effect on livelihoods from the cultivation and supply of *Catharanthusroseus* (L.) G. Don (often known as *Vincarosea* L. or Madagascan periwinkle) [87] serves as an illustration of this. However, this is not a totally new phenomenon as bioprospecting and the extraction of active drug substances from plants has been born from a long history of the exploitation of other countries' natural resources and the development of drugs from them, for example, curare, caffeine, and opium. For the farmers and collectors, changing working practices and changes in the governance of the regulation of medicinal plant cultivation are a priority. If higher quality raw materials can be produced for the export market, this may lead to a shift in equity toward the actors who operate at the beginning stages of the value chain. Moreover, by forming partnerships and alliances with well-established European companies, small farmers can expect greater benefits in terms of total remuneration, governance, training, supply of essential inputs, and better business stability for the long term.

The cultivation and supply of medicinal plants and the manufacture of HMPs in India, although having a long history of trade, both domestically and internationally, has failed to become a major industry in terms of European exports. India has to some extent attempted

to follow the Chinese initiative, with the globalization of its traditional medical system and particularly with the export of food supplements and HMPs [31].

However, quality-related problems inherent throughout the supply chains of manufacturing industries in India and China have resulted in finished products that are unable to rival similar products produced within the EU. This has resulted in a poor export profile for these commodities. This deficiency in export trade potential has been further compounded by the introduction of more stringent EU regulations for herbal medicines and according to some industry stakeholders, pressures being exerted by European manufactures,[3] on medicine regulators to enforce these new regulations and standards and to close any loopholes that may have allowed for less restrictive trade between Europe and LEDCs [88].

A totally Eurocentric approach, where plants are grown in the EU, and subsequently manufactured and distributed by European companies, may be easier to regulate and have less inherent dangers attached, but in an increasingly globalized market, this is clearly not an option. There should be a means to make products of a suitable standard available from farther afield. This attitude would not only result in a wider variety of products for consumers, but may also have a positive impact on the lives and livelihoods of primary producers in LEDCs.

European consumers have already experienced food products, including tea, coffee, and cocoa originating in these countries and being produced to acceptable EU standards. The difference for HMPs is that, "not only are the requirements for testing different to that of foods," but unlike the food sector products, "they are yet to be established as global commodities" and are entering the market at a time of great change and uncertainty. This makes these products, and the companies that produce them, far more vulnerable to changes in legislation, increases in consumer expectations and market shocks than products produced by more well-established industries.

In the global pharmaceutical industry, the quality of raw materials has been an important factor for many years [89], but in the traditional medicines industry, the focus has been directed more toward the end product, with companies investing more heavily toward packaging, marketing, and advertising and other end stage activities that are seen as a means to add value to a product. However, the source and quality of the plant material, and the integrity of the initial processing stages, have in many instances, been largely ignored.

Quality remains a key concern. China has been plagued by instances of substandard quality in many of its industries, including pharmaceuticals and herbal medicines [90]. Although India is regarded as a country that can produce acceptable quality drugs, there are major concerns regarding the production of HMPs, especially regarding authentication of raw materials, heavy metal content, and pesticide use [31]. As found in TCM, Indian systems of herbal medicine still advocate the practice of ingesting heavy metals [91], a practice that will never be acceptable to European medicine regulators.

The THMPD has introduced EU-wide standards that will help ensure the quality and safety of HMPs, but its implementation has so far favored European companies who have the infrastructure and hardware analytical and manufacturing platforms in place to deliver this without incurring massive costs. Manufacturers and retailers have intimated that the introduction of the THMPD has decreased their profit margins as in order to achieve the standards far more quality related expenditure is required [42].

However, for the farmers and collectors, changing working practice may be more important than heavy investment, and if high-quality raw materials can be produced, then it may lead to a shift in equity toward the beginning stages of the value chain. Moreover, by forming partnerships and alliances with well-established European companies, small farmers may expect greater benefits in terms of governance, training, and supply of essential inputs.

There is evidence to suggest that there are differences in chemical composition in plants obtained from a VIVC compared with the traditional routes and that safety and quality can be better ensured through the management of such a chain. Products obtained through a VIVC are less likely to be spoiled or contaminated through microbial growth, and paradoxically fewer chemical treatments are needed throughout the chain steps [17].

For the end user, the customer in previously unregulated or poorly regulated markets like the UK, there are now safeguards in place to help ensure the safety and quality of HMPs through the introduction of the THMPD. However, apart from acknowledging the plausibility of a herb to treat a particular condition, the directive does not address efficacy.

In the context of these discussions about quality assurance, the metabolomic profile of a plant becomes of importance. It is generally not considered relevant in orthodox testing protocols, where a crude drug or

[3] European manufacturers of registered HMPs have complained that many herbal products continue to be found in the retail market available as "food supplements."

product is generally assessed as suitable or not suitable depending on the concentration of one, or sometimes several, marker compounds and on the absence of impurities. Metabolomics can offer a whole new approach to quality assessment and one that is particularly relevant to medicinal plants, whereby the active ingredient is often undefined, and any therapeutic benefits may be due to the synergistic action of multiple plant components. It also offers opportunities to study the composition of the materials from source to consumer, that is, along the different value chains.

As plants are depleted in the wild; their scarcity often leads to an increase in their desirability, which in turn results in an increase in the price. The impact at a grassroots level is that they become more economically attractive. This process can continue until the plant is endangered or even made extinct in the wild. It is often the poorest of people who make their living from foraging plants from the wild; however, it is these same people who are most open to exploitation from middlemen and frequently only receive about six per cent of the retail price of a herbal medicament [24].

There seems little doubt that the Indian cultivated medicinal herb industry is some way behind that of China with over 90 per cent of medicinal plants in India still being collected from the wild [92]. As demand for Indian medicinal plants increases, it is unlikely that wild collection will be sustainable in the long term, and new strategies will be needed if the industry is to succeed.

There is already some evidence of strategic alliances and global partnerships manifesting but sometimes with mixed results [12,14,86].

Professionals, producers, and academics should work together to share what strategies succeed and where the problems lie. Poor seed quality, unsuitable environment, and lack of knowledge base have already been identified as contributory factors for failure, and it is important that measures are taken to avoid repetition.

Farmers and primary producers need incentives and guarantees if they are to invest finance, time, and resources into the cultivation of medicinal plants. A robust regulatory system is needed to be in place to control the manufacture and delivery of plant medicines through all stages of production. The European THMPD is an example of how a pragmatic approach can be utilized to improve the quality and safety of HMPs; however, one weak point is that it fails to regulate the appropriate sourcing of plants in a meaningful way.

This situation is even more relevant to the Asian medicinal plant industry, and if this industry is to develop its export capability, it will be necessary for it to address issues such as endangered species, sustainability, and economic viability in parallel to those of quality, safety, and effectiveness. From the manufacturers' perspective, to implement a sustainable business model, the contracts that are put in place need to be managed effectively from both sides.

Although a VIVC may seem to be more stable and less of a risk to the farmer, there are some instances where the farmer may not benefit. Once contracted into a chain, a degree of freedom is sacrificed, and the farmer is no longer free to sell on the open market. Large companies may be able to exert a downward pressure on the farmers as highlighted with the tea farmers whose livelihoods can become totally dependent on a single company. In other circumstances, as pointed out by Neimark [87] on his investigation into the *Catharanthus-roseus* (*Vincarosea*) value chain and the production of chemotherapeutic agents, although the finished product end of the market may be modern and well regulated, the beginning stages of the market may be quite unregulated, even when the farmer is integrated into a value chain, allowing the lead company to dominate the farmer.

When selling to one or two companies, which may be the main buyers for a raw material, the farmer may find himself with no alternative than to make contracts with these big corporations, who are less likely to be interested in the livelihoods of small farmers.

For the buyer too, some risks exist, even when under contract, there is no guarantee that the farmer will not sell his crop on the open market if the price is right and there is unlikely to be any redress for the buyer in this situation. There are also a myriad of economic, political, and climatic risks associated with the cultivation of medicinal plants and their manufacture into HMPs that need to be overcome to create a successful industry.

Albeit with the caveat that there are risks in any business and that no system is fool proof or without its own unique set of exogenous and endogenous problems. For small- to medium-sized businesses that are prepared to build strong relationships with farmers over time and that are proactive in supply chain management strategies, and for primary producers who are prepared to adapt their working practices to comply with standards of different countries than their own, there are mutual benefits to be attained and importantly that these benefits can be measured through an interdisciplinary understanding of the strategic issues.

A thorough investigation of MPVCs can provide the industry with a far more comprehensive picture of the cultivation, processing, and storage history of a given HMP. This information can be used to help justify, encourage, and promote the continued development of VIVCs together with the implementation and management of better contractual relationships between

manufacturers of HMPs and the primary producers that supply them—a process that ultimately will benefit both producers (through better income and thus better livelihoods) and consumers (though products with a reproducible high quality).

Acknowledgments

This work has been supported by the Leverhulme Trust through a grant to the LIDC (London International Development Centre) for establishing the Leverhulme Centre for Integrative Research on Agriculture and Health (LCIRAH).

References

[1] Robinson MM, Zhang X. The world medicines situation 2011. 3rd ed. Geneva: WHO; 2012.
[2] Freedman P. Spices: how the search for flavors influenced our world, in history of globalization. Yale Global Online, Yale Centre For The Study Of Globalization; 2003. Published on the internet.
[3] EMA. Uptake of the traditional use registration scheme and implementation of the provisions of directive 2004/24/EC in EU member states. In: Patient health protection. London, UK: European Medicines Agency; 2013.
[4] Das S. India to move WTO against EU herbal drug order. In: The financial express. New Delhi: The Indian Express Group; 2013.
[5] InfoseekChina. Traditional Chinese medicine presence challenged by EU herbal rule. 2011. Published on the internet.
[6] Patel P. Impact assessment of THMPD on trade of Ayurveda products. In: Ayurveda and its scientific aspects: opportunities for globalisation. New Delhi, India: Council of Scientific and Industrial Research; 2006.
[7] Henson S, Humphrey J. Understanding the complexities of private standards in global agri-food chains as they impact developing countries. J Dev Stud 2010;46:1628–46.
[8] Gibbon P, Ponte S. Trading down: Africa, value chains and the global economy. Philadelphia: Temple University Press; 2005.
[9] Recklies D. The value chain. In: Management models and tools. Recklies Management Project GmbH; 2001. Published on the internet.
[10] Giuliani E, Pietrobelli C, Rabellotti R. Upgrading in global value chains: lessons from latin American clusters. World Dev 2005;33:549–73.
[11] Wynberg R, Laird SA, Shackleton S, Mander M, Shackleton C, du Plesis P, et al. Marula policy brief: marula commercialization for sustainable and equitable livelihoods. For Trees Livelihoods 2003;13:203–15.
[12] Alam G, Belt J. Developing a medicinal plant value chain: lessons from an initiative to cultivate Kutki (*Picrorhiza kurrooa*) in Northern India. In: KIT working papers series C5. Amsterdam: KIT; 2009.
[13] Kala C. Commercial exploitation and conservation status of high value medicinal plants across the borderline of India and Nepal in Pithoragarh. Indian For 2003;129:80–4.
[14] Shahidullah AKM, Haque CE. Linking medicinal plant production with livelihood enhancement in Bangladesh: implications of a vertically integrated value chain. J Transdiscipl Environ Stud 2010;9:1–18.
[15] van de Kop P, Alam G, de Steenhuijsen Piters B. Developing a sustainable medicinal plant chain in India. Linking people, markets and values. In: Ruben R, Slingerland M, Nijhoff H, editors. Agro food chains and networks for development. Netherlands: Springer; 2006. p. 191–237.
[16] Booker A, Johnston D, Heinrich M. Value chains of herbal medicines—Research needs and key challenges in the context of ethnopharmacology. J Ethnopharmacol 2012;140:624–33.
[17] Booker A, Frommenwiler D, Johnston D, Umealajekwu C, Reich E, Heinrich M. Chemical variability along the value chains of turmeric (*Curcuma longa*): a comparison of nuclear magnetic resonance spectroscopy and high performance thin layer chromatography. J Ethnopharmacol 2014;152:292–301. http://dx.doi.org/10.1016/j.jep.2013.12.042.
[18] Schippmann U, Leaman D, Cunningham AB. A comparison of cultivation and wild collection of medicinal and aromatic plants under sustainability aspects. In: Bogers RJ, Craker LE, Lange D, editors. Medicinal and aromatic plants. Dordrecht: Springer; 2006. p. 75–95.
[19] Kuipers SE. Trade in medicinal plants. In: Bodeker G, Bhat KKS, Burley J, Vantomme P, editors. Medicinal plants for forest conservation and health care. Rome: FAO; 1997. p. 45–59.
[20] Giblette J. Chinese medicine and species conservation. In: Call E, editor. Mending the web of life. The International Fund for Animal Welfare (IFAW) and the Foundation for Education and Research on Botanicals, American Herbal Products Association (AHPA-ERB); 2006.
[21] Kala C, Dhyani P, Sajwan B. Developing the medicinal plants sector in northern India: challenges and opportunities. J Ethnobiol Ethnomed 2006;2:32.
[22] Manish M. Conservation of biodiversity in the natural forests of central India. Biosci Disc 2011;2:299–308.
[23] Hawkins B. Plants for life: medicinal plant conservation and botanic gardens. Richmond, UK: Botanic Gardens Conservation International; 2008. p. 1–49.
[24] Schippmann U, Leaman D, Cunningham A. Impact of cultivation and gathering of medicinal plants on biodiversity: global trends and issues. In: Biodiversity and the ecosystem approach in agriculture. Rome, Italy: Food and Agriculture Organization of the United Nations; 2002. p. 1–21.
[25] van Niekerk J, Wynberg R. The trade in Pelargonium sidoides: rural livelihood relief or bounty for the 'bio-buccaneers'? Dev South Afr 2012;29:530–47.
[26] Petrovska BB. Historical review of medicinal plants' usage. Pharmacogn Rev 2012;6:1–5.
[27] PMMI. China Industry sector report: outlook on China's pharmaceutical Industry. Packaging Machinery Manufacturers Institute; 2001.
[28] MHRA. Public health risk with herbal medicines: an overview. In: Herbal documents. MHRA Policy Division; 2008. Published on the internet.
[29] MHRA. Warning over dangerous traditional Chinese medicines. Press Release 2013. August 20.
[30] MHRA. Warning over potentially toxic Chinese herbal medicine. Press Release 2013. April 13.
[31] Patwardhan B, Warude D, Pushpangadan P, Bhatt N. Ayurveda and traditional Chinese medicine: a comparative overview. Evid Based Complement Alternat Med 2005;2:465–73.
[32] Sharma A, Shanker C, Tyagi LK, Singh M, Rao V. Herbal medicine for market potential in India: an overview. Acad J Plant Sci 2008;1:26–36.
[33] Ravishankar B, Shukla VJ. Indian systems of medicine: a brief profile. Afr J Trad Copl Alt Med 2007;4:319–37.
[34] Seale S. Annual ayurvedic medicine production in India. In: Culture and society. eHow; 2011. Published on the internet.
[35] Cavaliere C. Efforts to increase sustainability of ayurvedic plants in India. Her Geneal 2010;87:22–3.
[36] Dharmananda S. The ayurvedic medicine industry in India. Portland, Oregon: Institute for Traditional Medicine; 2003.

[37] LBS. Fast moving consumer goods (FMCG). ISD Library, London Business School; 2009.
[38] Subrat N, Iyer M, Prasad R. The ayurvedic medicine industry: current status and sustainability. p. 1–176. New Delhi, India: Ecotech Services (India) Pvt. Ltd. and International Institute for Environment and Development; 2002.
[39] Polshettiwar S. Indian herbal drug industry—future prospects: a review ayurveda emphasis relationship between man and plants throughout the development of human culture. In: Reviews. Pharmainfo.net; 2006.
[40] Euromonitor. Herbal/traditional products in India. In: Consumer health. Euromonitor International; 2013. Published on the internet.
[41] Scindia JM. India's herbal product exports rising at a compounded annual rate of 16.8 per cent news. In: Industry. Domain-B.com; 2010. Published on the internet.
[42] Patwardhan B. European Union ban on ayurvedic medicines. Ayurveda Integr Med 2011;2:47–8.
[43] Vaidya ADB, Devasagayam TPA. Current status of herbal drugs in India: an overview. J Clin Biochem Nutr 2007:1–11.
[44] Brion. Herbs cultivation site tour—Anhui, Bozhou. Taiwan: Brion Research Institute; 2011. p. 5–6.
[45] UPA. American ginseng. In: Gaspé Peninsula's non-Timber Forest products. Canada Economic Development and Natural Resources; 2003. Published on the internet.
[46] Hallquist A, Jansen S, Mielke S. Global trade: transportation of turmeric from India to the United States. In: Global trade assignment executive summary; 2010.
[47] Gong A. Indian experience & Chinese Perspective: international expansion of pharmaceutical Industry. In: CEIBS knowledge. CEIBS; 2006. Published on the internet.
[48] Lange D. Europe's medicinal and aromatic plants: their use, trade and conservation. Cambridge, United Kingdom: TRAFFIC International; 1998.
[49] Uniyal RC, Uniyal MR, Jain P. Cultivation of medicinal plants in India: a reference book. Delhi, India: TRAFFIC India; 2000.
[50] He SA, Cheng ZM. The role of Chinese botanical gardens in conservation of medicinal plants. In: Akerele O, Heywood V, Synge H, editors. The conservation of medicinal plants. Switzerland: WHO, IUCN-The World Conservation Union and WWF, Geneva and Gland; 1991.
[51] He SA, Sheng H. Utilization and conservation of medicinal plants in China with special reference to *Atractylodes lancea*. In: Medicinal plants for forest conservation and healthcare. Rome, Italy: FAO; 1997.
[52] Cabrera C, Artacho R, Giménez R. Beneficial effects of green tea—a review. J Am Coll Nutr 2006;25:79–99.
[53] Katiyar SK, Ahmad N, Mukhtar H. Green tea and skin. Arch Dermatol 2000;136:989–94.
[54] Agrifood. Linking the poor with tea value chains. In: The participation of the poor in agricultural value chains. Hanoi, Viet Nam: Agrifood Consulting International; 2004. p. 1–4.
[55] Fairtrade. Stirring up the tea trade: can we build a better future for tea producers?. In: A Fairtrade foundation briefing paper. London, UK: Fairtrade Foundation; 2010. p. 1–24.
[56] Groosman M. Tea: sector overview. Utrecht, The Netherlands: IDH The Sustainable Trade Initiative; 2011. p. 1–14.
[57] Menon N, Rodgers Y. International trade and the gender wage gap: new evidence from India's manufacturing sector. World Dev 2008;37:965–81.
[58] Liu J. Green tea from unilever. In: Business weekly. China Daily; 2010.
[59] Hilary J, Dromey J. A bitter cup: the exploitation of tea workers in India and Kenya supplying British supermarkets. In: Fighting global poverty. War on Want; 2010. Published on the internet.
[60] Nelson V, Pound B. The last ten years: a comprehensive review of the literature on the impact of fairtrade. Natural Resources Institute (NRI), University of Greenwich; 2009.
[61] Cramer C, Johnston D, Oya C, Sender J. Fairtrade, employment and poverty reduction in Ethiopia. School of Oriental and African Studies; 2013. p. 1–145.
[62] Hodge A. Fair trade in tea in China. In: Tea Industry news. Green Dragon Enterprises; 2009. Published on the internet.
[63] Gurusubramanian G, Rahman A, Sarmah M, Somnath R, Bora S. Pesticide usage pattern in tea ecosystem, their retrospects and alternative measures. J Environ Biol 2008;29:813–26.
[64] PAN. Information for the consideration of Endosulfan. In: Provision of information to the Stockholm Convention Secretariat for use by the POPs Review Committee. Penang, Malaysia: Pesticide Action Network (PAN) Asia & Pacific; 2008. p. 1–46.
[65] Oldenziel J. Sustainabilities: the Dutch Tea market and corporate social responsibility. Utrecht, The Netherlands: SOMO LIW/ ICN– India Committee of the Netherlands; 2006. p. 1–81.
[66] Burkhart EP, Jacobson M. Non timber forest products (NFTPs) from Pennsylvania: American ginseng (*Panax quinquefolius* L.). In: Agricultural research and cooperative extension. Pennsylvania, USA: The College of Agricultural Sciences; 2004. p. 1–12.
[67] Schulz H. Planning needed to ensure health of wild US ginseng stocks, experts say. In: News. Nutraingredients—USA; 2013. Published on the internet.
[68] Burkhart EP, Jacobson MG. Transitioning from wild collection to forest cultivation of indigenous medicinal forest plants in eastern North America is constrained by lack of profitability. Agroforest Syst 2007;76:437–53.
[69] Lucio A. Ginseng. In: UK cooperative extension service. Frankfort, Kentucky, USA: University of Kentucky, College of Agriculture; 2002. p. 1–6.
[70] Yuan C, Wu J, Osinski J. Ginsenoside variability in American ginseng. Am J Clin Nutr 2002;75:600–1.
[71] Hall R, Beale M, Fiehn O, Hardy N, Sumner L, Bino R. Plant metabolomics: the missing link in functional genomics strategies. Plant Cell Online 2002;14:1437–40.
[72] Hankins A. Producing and marketing wild simulated ginseng in forest and agroforestry systems. In: Virginia cooperative extension. Virginia State University, Virginia State University; 2009.
[73] Schripsema J. Application of NMR in plant metabolomics: techniques, problems and prospects. Phytochem Anal 2010;21:17–21.
[74] Kim HK, Verpoorte R. Sample preparation for plant metabolomics. Phytochem Anal 2010;21:4–13.
[75] Choi YH, Tapias EC, Kim HK, Lefeber AWM, Erkelens C, Verhoeven JTJ, et al. Metabolic discrimination of *Catharanthus roseus* Leaves infected by phytoplasma using ^{1}H-NMR spectroscopy and multivariate data analysis. Plant Physiol 2004;135: 2398–410.
[76] Verpoorte R, Choi Y, Kim H. Ethnopharmacology and systems biology: a perfect holistic match. J Ethnopharmacol 2005;100: 53–6.
[77] Shyur L-F, Yang N-S. Metabolomics for phytomedicine research and drug development. Curr Opin Chem Biol 2008;12:66–71.
[78] Zulak KG, Weljie AM, Vogel HJ, Facchini PJ. Quantitative ^{1}H NMR metabolomics reveals extensive metabolic reprogramming of primary and secondary metabolism in elicitor-treated opium poppy cell cultures. BMC Plant Biol 2008;8.
[79] van der Kooy F, Maltese F, Hae Choi Y, Kyong Kim H, Verpoorte R. Quality control of herbal material and phytopharmaceuticals with MS and NMR based metabolic fingerprinting. Planta Med 2009;75:763–75.
[80] Ward JL, Baker JM, Miller SJ, Deborde C, Maucourt M, Biais B, et al. An inter-laboratory comparison demonstrates that [^{1}H]-

NMR metabolite fingerprinting is a robust technique for collaborative plant metabolomic data collection. Meta 2010;6:263–73.
[81] Kim HK, Choi YH, Erkelens C, Lefeber AW, Verpoorte R. Metabolic fingerprinting of Ephedra species using ^{1}H-NMR spectroscopy and principal component analysis. Chem Pharm Bull (Tokyo) 2005;53:105–9.
[82] Bailey NJC, Sampson J, Hylands PJ, Nicholson JK, Holmes E. Multi-component metabolic classification of commercial feverfew preparations via high-field 1H-NMR spectroscopy and chemometrics. Planta Medica 2002;68:734–8.
[83] Heinrich M. Ethnopharmacy and natural product research—multidisciplinary opportunities for research in the metabolomic age. Phytochem Lett 2008;1:1–5.
[84] Politi M, Zloh M, Pintado ME, Castro PML, Heinrich M, Prieto JM. Direct metabolic fingerprinting of commercial herbal tinctures by nuclear magnetic resonance spectroscopy and mass spectrometry. Phytochem Anal 2009;20:328–34.
[85] Prahalathan S. Export potential of Indian medicinal plants and products. Fin Agric 2004;36:33–6.
[86] Booker AJ. The transformations of traditional Asian medical knowledge into international commodities—the link between traditional medicines and the international market. In: Centre for pharmacognosy and phytotherapy, school of pharmacy. London: UCL; 2014. p. 488.
[87] Neimark B. Green grabbing at the "pharm" gate: rosy periwinkle production in southern Madagascar. J Pesant Stud 2012;39: 423–45.
[88] Starling S. MHRA refutes ECJ influence in UK herbal supplements policing. William Reed Business Media; 2011. Published on the internet.
[89] Strother T. European biopharmaceutical review: quality control, analytical methods. Samedan Ltd; 2012. Published on the internet.
[90] Bate R, Porter K. The problems and potential of China's pharmaceutical industry. AEI Online; 2009. Published on the internet.
[91] Kumar A, Nair AG, Reddy AV, Garg AN. Bhasmas: unique ayurvedic metallic-herbal preparations, chemical characterization. Biol Trace Elem Res 2006;109:231–54.
[92] Kumar S, Kumar R, Khan A. Medicinal plant resources: manifestation and prospects of life-sustaining healthcare system. C J Biol Sci 2011;4:19–29.
[93] Schlag E, McIntosh M. Ginsenoside content and variation among and within American ginseng (*Panax quinquefolius L.*) populations. Phytochemistry 2006;67:1510–9.

LIST OF ABBREVIATIONS

CBD Convention on Biodiversity
CFDA China Food and Drug Administration
CITES Convention on International Trade of Endangered Species
FRLHT Foundation for the Revitalization of Local Health Traditions
GAP Good agricultural practice
GCP Good collection practice
GMP Good manufacturing practice
GVC Global value chain
HMP Herbal medicinal product
HPTLC High performance thin layer chromatography
IPR Intellectual property rights
LEDC Less economically developed country
MPVC Medicinal plant value chain
MS Mass spectrometry
NGO Nongovernmental organization
NMR Nuclear magnetic resonance
OCP Organochlorine pesticide
POE Privately owned enterprise
POP Persistent organic pollutant
TAM Traditional Asian medicine
TCM Traditional Chinese medicine
TIM Traditional Indian medicine
THMPD Traditional Herbal Medicinal Products Directive
VIVC Vertically integrated value chain
WHO World Health Organization

CHAPTER

3

Traditional Herbal Medicine, Pharmacognosy, and Pharmacopoeial Standards: A Discussion at the Crossroads

Roy Upton R.H.

American Herbal Pharmacopoeia, Scotts Valley, CA, USA

OUTLINE

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00003-9

3.1 INTRODUCTION

Since the dawn of human kind, humans have relied on plant-based medicines for the majority of their health and disease needs. The rapid and increasing rise in the worldwide use of herbal medicines in recent decades suggests botanical medicines, both traditional and modern, fulfill a therapeutic niche not adequately addressed in modern health care delivery systems. At the same time, there is a propensity, predominantly in developed, but also developing nations, to develop herbal medicines in the same manner as modern chemically characterized drugs. This trend is driven partly by a belief that the same regulatory requirements that are applied to modern pharmaceuticals should be applied to herbal medicines and partly by economics, a subject to be expounded upon later.

In the late 1800s, chemistry was advancing and the role and focus of those involved in the development of medicinal plants changed from attention to detail of the source, identity, and quality of the plant material itself to the compounds contained within the plant. Similarly, in the same time period, the use of herbal medicines was rapidly declining as use of chemical medicines was on the rise. In developed nations, the responsibility of herb quality passed from herbalists to medical doctors who became among the first modern teachers of materia medica, and then to pharmacognosists—pharmacists specializing in the development of drugs from natural products. Subsequently, this pharmacognostic knowledge was codified into national pharmacopoeias, which became the quality standard for drugs within national health care approval systems. Thus, modern drug approval systems, and subsequently, modern pharmacopoeias, were modeled for the needs of modern drugs, not traditional herbal medicines or traditional healing systems. In parallel to this, the economics of medicine changed with greater focus on research, specialization, technology, high-cost drug approval processes, and a medical system more driven by research findings and profits than practitioner or patient need. This is a key premise in the evolution of modern medicine that greatly influenced the way in which the practice of medicine and approval of medicinal agents evolved, including creating significant positive bias in pharmaceutical research and substantial negative bias against traditional herbal medicines.

The World Health Organization (WHO) in their Declaration of Alma-Ata considers among some of the more salient benefits of herbal medicines and traditional healing systems as their accessibility and affordability [162,165]. The WHO, however, does not explicitly recognize other very unique benefits of traditional medicines. For example, specifically in the United States, arguably among the most technologically advanced of countries, 58% of consumers who use traditional healing modalities do so not for the treatment of disease, but rather to "prevent future illness from occurring or to maintain health and vitality" [37]. Similar findings were reported in a survey of dietary supplement users in the US in which "preservation of health was by far the most predictive indicator for use of herbal products and dietary supplements" [94] and in Australia where prevention of sickness was the primary reason for herbal medicine use [91]. Another unique characteristic of traditional healing systems, especially, Ayurveda, traditional Chinese medicine (TCM), Unani, Tibetan, etc., is the utilization of a completely different diagnostic system than applied in the West, a system designed specifically to be applied to the individual needs of the patient. The diagnostic framework used in traditional healing systems allows for a unique understanding of health and pathology, and subsequently unique therapeutic strategies, than those in the West, thus broadening patient options. Additionally, amongst these healing traditions is an inherent belief of the relationship between human and environmental health, a belief completely integrated into the diagnostic construct of most herbal traditions and completely ignored in modern medicine.

These traditional healing strategies are in stark contrast to the prevalence of Western conventional medical strategies, which are predominantly focused on disease management more than the promotion or restoration of health. The pharmacological specificity inherent in western medical therapies gives those therapies a targeted focus, often to the neglect of the general health status of the individual. Generally speaking, in western conventional medicine, if tissue or organ structures are not functioning properly the treatment is to basically attack it: the removal of tonsils (a first line of defense against infectious disease), gallbladders (integral to digestive and eliminative processes), hips, cataracts; transplants of hearts, livers, and kidneys; radiation or poison (in cancers); or interfering with normal processes, such as pharmacological agents designed to either upregulate, downregulate, or otherwise completely shut down normal physiological processes. A good example of this is the widespread use of anti-inflammatories, inflammation actually reflecting a biological healing response of the body to an inflammatory mediator. Rather than seeking to identify and remove the inflammatory trigger, the conventional western medical strategy is to shut down, what ultimately is a healing response. From a natural health care perspective, such a strategy is the antithesis of what is needed to support human health. This does not mean there is no value in such a strategy. Militaristic search and destroy therapies are uniquely suited for crisis intervention

and emergency medicine. Severed limbs, acute poisonings, intractable severe pain, ruptured organs, etc. respond well to a militaristic approach; in contrast, pregnancy does not, a fact reflected in the appalling high rate of infant mortality in the US due to high-tech interventions for what should be a normal process. To have a medical system entirely based on militaristic therapies is inherently limited; a fact equally evidenced by the high rates of iatrogenesis, deaths due to pharmaceuticals, and chronic illness associated with modern health care systems. Perhaps of greatest consequence is that modern conventional medical strategies focus on the treatment of diseases not the treatment of people.

Thus, conventional and traditional healing practices represent two very different paradigms of medicine. Traditional healing practices offer a different paradigm and different therapeutic solutions than conventional medical care. This is precisely why traditional healing systems and herbal medicines are growing in popularity. If the two systems can be harmonized there is a greater potential for healing than either system can deliver on its own. However, regulating chamomile tea, valerian root, and hawthorn berry syrup, with their hundreds of compounds in the same way as Prilosec, Ambien, and artificial hearts, disregards the fact that each botanical medicine has several hundred years of historical use, has been in the public domain for those several hundred years, and threatens to undermine the potential herbal medicines possess.

Throughout, much of the world herbal medicine is in transition from the collection and processing of crude herbal materials of varying strengths and potencies, such as are used as powders, teas, simple syrups, and extracts by local healers and communities to well-characterized preparations made according to pharmacopoeial standards predominantly supplied through highly regulated markets. The former more crude preparations are characteristic of undeveloped and developing nations, while the latter are typical of developed nations. Forcing herbal medicines into a regulatory category that was designed for modern drugs greatly decreases accessibility while greatly increasing cost of herbal drugs, thus negating two of the primary benefits offered by herbal medicines, as articulated by WHO. Rather a new paradigm that takes into consideration the unique characters of herbal medicines is needed with a focus on the quality and sourcing of raw material based on the myriad of constituents found in single herbs and formulas and applying modern techniques to the investigation of traditional healing practices.

Ironically, and perhaps most saliently, practitioners of traditional healing systems are seldom part of traditional health care policy decisions. Rather, commercial interests by traditional medicine producers often drive national and international health care policies, frequently leading to a restriction in materia medica and impeded access to herbal drugs. Thus, it is questionable whether traditional herbal medicine will thrive, and in many cases, survive within national health care systems. If policy makers, regulatory bodies, medicinal plant researchers, and pharmacopoeias can once again place emphasis on the quality of the plant material itself rather than viewing herbal drugs as cocktails of chemicals to be pulled apart, isolated, and manipulated, there is a chance. Similarly, medicinal plant research should reorient itself to investigate the traditional use of herbal medicines within the traditional healing system from whence they came, investigating the physiological and pharmacological subtleties that give the medicine its unique healing quality, rather than simply looking at the application of herbal drugs for Western disease patterns. Truly honoring traditional healing systems and herbal medicines and maximizing their healing potential is an international challenge that minimally should be discussed as part of national and international health care policy if humankind is to continue to reap the benefits of medicinal plants based on the systems in which they were traditionally used.

3.2 HISTORICAL PERSPECTIVES ON TRADITIONAL HERBAL MEDICINE

3.2.1 Healing in Nature

"Esam bhutanam prthivi rasha, prthivya apo rasopam osadhayo rasa, osadhinam puruso rasah"—"The essence of all beings is Earth. The essence of the Earth is Water. The essence of Water is Plants. The Essence of Plants is the Human Being…." Chandogya Upanishad, CE sixth century Vedic text.

Thus, are the philosophical underpinnings of human existence recorded in the *Chandogya Upanishad*, a Vedic text dating to the *Brahmana* period sometime before the CE sixth century that clearly articulates the dynamic relationship that exists between humans, plants, and the environment overall. Similarly, the philosophy of *Vis Medicatrix Naturae* (the healing power of nature), as set down by Hippocrates, summarizes the basic principles of healing, namely that the body as a living biological organism is always trying to achieve a state of health and balance and that an observed malady is the body's attempt to reestablish equilibrium [60,68]. This connection between human health and nature is a philosophical underpinning of many traditional healing systems worldwide [115], but is a perspective considered completely irrelevant in modern medicine and most often overshadowed or completely lost in attempts to

push herbal medicine into a level of acceptance in modernity. Ancient healers recognized the human organism was made of the same substances that existed in nature and thus is an extension of nature. Therefore, so it was reasoned that healing was facilitated through nature and substances of nature, namely fresh air, pure water, sunshine, good nutrition, exercise, and herbal medicines, and, including in shamanistic or religious healing practices, invocation of ancestors, saints, spirits, and God(s).

The practice of herbal medicine historically followed two paths. The first of these was rooted in relative simplicity—an herbalist gathering plant medicines and attending to the needs of the sick in a system that can be defined as community-based health care. These were, and in many countries today, are, the folk healers, grandmothers, and grandfathers learned in the use of medicinal plants. The second path was that of the professional practitioners. In ancient times, some of these were among the most learned of their generation and are well represented from the CE first to fifteenth centuries in works such as *De Materia Medica* of Dioscorides (Figure 3.1) and the *Hippocratic Corpus* of Hippocrates, both of Greece, the *Charka Samhita* of India, and the *Shen Nong Ben Cao* of China, followed a few centuries later by the *Canon of Medicine* by Ibn Sīnā (Avicenna) of Persia and several centuries later by the Renaissance herbal writers such as Fuchs, Gerard, Mattiolis, Parkinsons, and Salmon, to name only a few.

The use of herbs within these formal medical traditions was integrated with very sophisticated theories of anatomy, physiology, and pathology. Conversely, folk-healing traditions predominantly represented an empirical use of herbs passed down through families, community knowledge, and fragments of the more formalized medical practice. While the humoral system of Hippocrates, Galen, and to a great degree, Avicenna was the ancestor of today's medical theories, today's conventional Western medical practitioners have no relationship to these past theories, obviously with a belief of the scientific superiority of current theories. In contrast, the medical theories of Ayurvedic and Chinese herbal medicine remain intact, continue to evolve, and continue to be practiced according to their same historical foundational principles. Inherent in both Ayurveda and Chinese medicine is the belief that humans are an extension of nature, and therefore, the theoretical principles developed were inherently designed to mimic nature and facilitate healing by adhering to vitalistic principles reflected in nature. These principles include

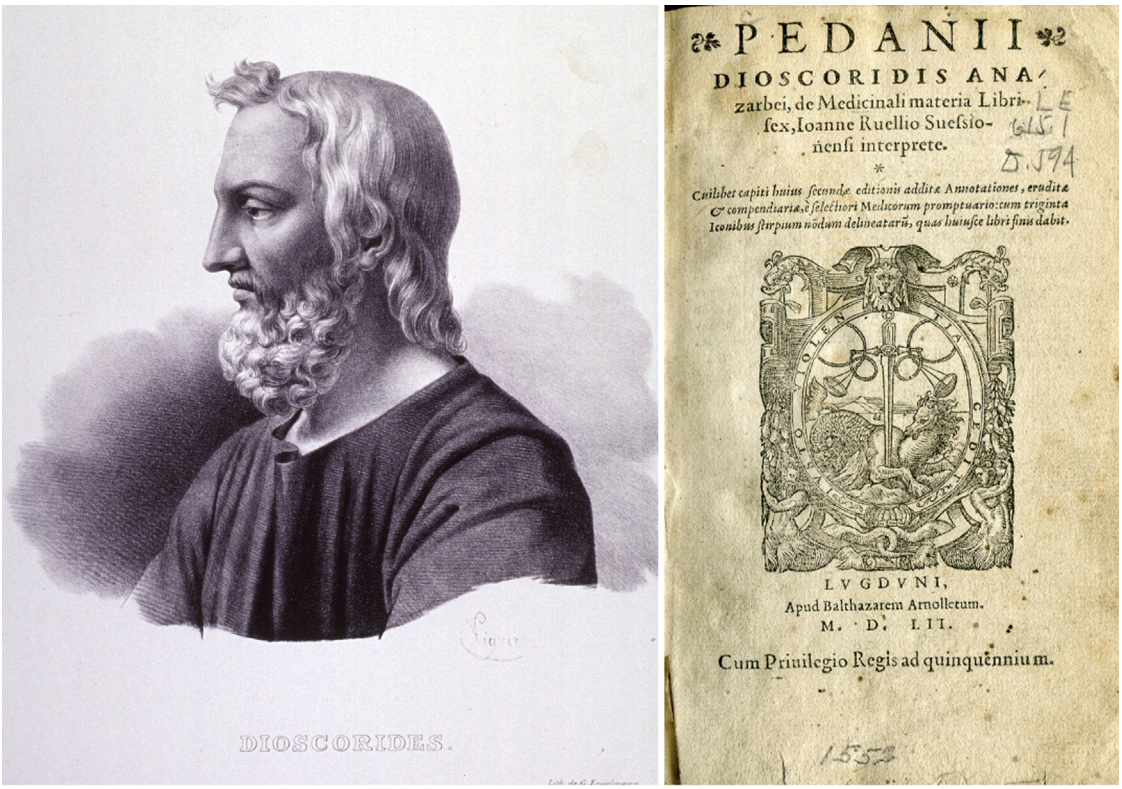


FIGURE 3.1 *De Materia Medica* of Pedanius Dioscorides (CE first century), Greece. One of the earliest formal materia medicas of Western civilization originally written CE 50–70. It remained the primary authority on medicinal substances for 1500 years, replaced by Renaissance herbals that largely continued to derive their primary information from Dioscorides. *Courtesy of the Lloyd Library and Museum, Cincinnati, OH.*

the tri-doshic and five-element (*wu xing*) systems of Ayurvedic and TCM, respectively, both of which relate human anatomy, physiology, pathology, and dietary and herbal therapies to, in Ayurveda, *vata* (air), *pitta* (fire), and *kapha* (water) (which themselves consist of earth, water, fire, wind, and ether) and in Chinese medicine to *huo* (fire), *tu* (earth), *jin* (metal), *shui* (water), and *mu* (wood). In contrast, current western medical traditions possess no principles that inherently link human health with the living biological principles of nature and only tangentially as an afterthought attempt to link health to behavior or lifestyle.

3.2.2 The Vitalistic-Mechanistic Schism of Traditional Healing and Conventional Medical Philosophies

Traditional healing systems are commonly regarded as vitalistic or holistic, while Western medical philosophy is often described as mechanistic, reductionist, or rationalistic [29]. The former believes in the innate ability of the human organism to heal itself, and, using philosophical constructs, diagnostics, and naturally oriented therapies as described above, seek to restore homeostasis. These vitalistic traditions are most commonly embodied in Ayurveda, TCM, Unani, and Tibetan traditions, and, in the West, are also reflected in the work and writings of Galenic adherents (Figure 3.2).

The latter rationalistic perspective is based on the perception that development of modern medical theory and drug development was founded on rational scientific principles, with an inherent implication that traditional herbal medicine knowledge lacked a rational basis, a premise disputed by noted medical historian John Riddle [124].

Philippus Aureolus Theophrastus Bombastus von Hohenheim, otherwise known as Paracelsus (1493–1541) (Figure 3.3), the fifteenth century, the Swiss-German physician who challenged what at the time was a relatively strict adherence to the medical philosophies of ancient texts, including those of Galen,

FIGURE 3.2 Galen of Pergamos, Greece. The medical theories of ancient Greece viewed humans as a microcosm of nature and utilized diagnostic systems and therapies based in a natural understanding of physiology designed to re-establish the health of the host in relationship to their environment. *Courtesy of the Lloyd Library and Museum, Cincinnati, OH.*

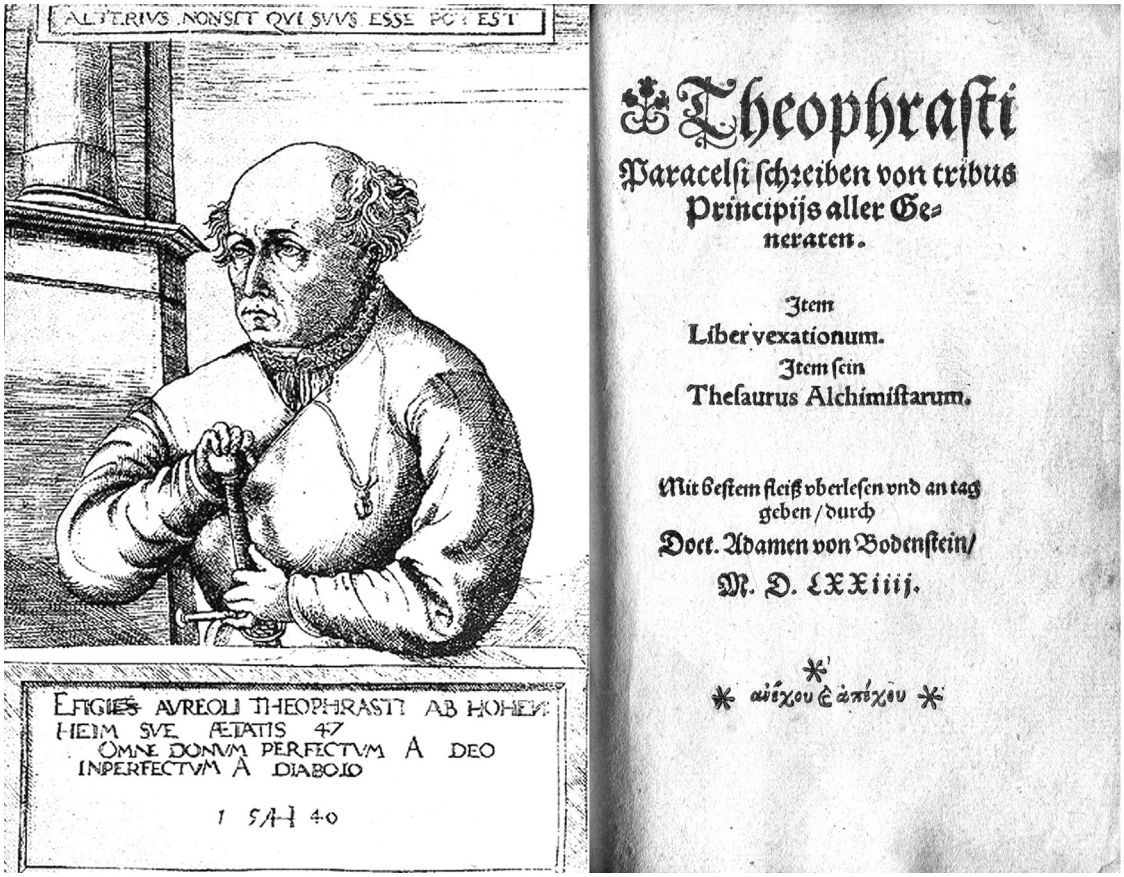


FIGURE 3.3 Swiss-German physician Philippus Aureolus Theophrastus Bombastus von Hohenheim (Paracelsus; 1493–1541). Paracelsus ardently opposed the medical philosophies of Galen and his humoral system shifting medical thought from physiology to pharmacology. Paracelsus further ushered in the use of powerful mineral-based drugs (e.g., mercury) in contrast to the relatively gentle plant-based medicines (Galenic preparations) that occupied early materia medicas and pharmacopoeias. *Courtesy of the Lloyd Library and Museum, Cincinnati, OH.*

Avicenna, and others, the books of whom he actually burned at the University of Basel, Switzerland on St. John's Day in 1527.

The earlier teachings of Galen posited there were four physiological humors (blood, phlegm, yellow bile, and black bile; originally taught by the Greek philosopher Pythagoras (570 BC–c. 495 BC)) and that when in balance, there was health. The humoral theory persisted as a prominent medical theory until the middle 1800s [39]. Paracelsus argued there were three humors: salt representing stability, sulfur representing combustibility, and mercury representing fluidity. Whereas the humoral theory of Galen was rooted in human physiology that sought to restore homeostasis naturally, the philosophy of Paracelsus was more materialistic with a greater emphasis placed on the action of compounds that assert a pronounced pharmacological effect.

Further driving Western medical thought was Harvey's (1578–1657) articulation of the circulatory system in the seventeenth century, which provided fertile soil for new mechanistic theories that challenged old humoral theory (though it is interesting to note that the concept of circulation was described in the foundational text of TCM, the *Neijing* and by Galen centuries before).

Yet further setting the stage for a separation of human and environmental health was French physiologist Claude Bernard (1813–1878) who posited that the human environment (milieu intérieur) was separate from the external environment. Others, such as the noted German physician Rudolf Virchow (1821–1902) stated the cell was the basic unit of the body that had to be studied in order to understand disease. While traditional health practitioners gave focus to the macrocosm of all that makes up an individual's life and health status (constitution, diet, lifestyle, environment, physical activity, stress, sleep, etc.) [125], allopathic physicians or "mineral doctors," as they came to be known, were bloodletting with lancets and purging with Paracelsun-type mercury compounds [125]. Contrastly, in Ayurveda and TCM patient therapies are very much individualized and have remained physician and patient centered, the practitioner predominantly making their assessment through observational means reflected in the eight

methods of diagnosis of ayurveda (*astavidha pariksa*) and four examinations (*si zhen*) of TCM. Traditional practitioners further seek to correct the underlying causative factors while providing therapeutic options to address both cause and symptoms, codified in TCM as *biao* and *ben* or treating the "root and branch."

The visualization of microbes with the advent of the microscope by Holland's Anton Van Leeuwenhoek (1632–1723), Pasteur's (1822–1895) confirmation of the potential pathogenicity of microbes, and Fleming's (1881–1955) discovery of antibiotics further concretized the material cause and cure of disease, which in turn solidified the reductionist, militaristic, search and destroy approach typical of the microcosm of cellular biology reflected in western medical thought today. Furthermore, Western medical practices develop "standards of care" for diseases, in contrast to individualized healing protocols for patients. Western diagnostics are not guided by any understanding of the individual's lifestyle that initially led to the disease state, nor do they take into consideration a patient's environment, but rather rely on automated tests of specific biomarkers that reflect the disease process not the cause. This results in an almost exclusive focus on the symptom management and an almost complete neglect of the cause and promotion of health. This perhaps represents the primary differentiation between traditional and conventional healing philosophies.

3.2.3 The Primary Schism between Traditional and Modern Medicines

Prior to the fifteenth century, the majority of medicinal agents were plant based. While numerous medicinal agents of mineral and animal origin were also used, the overwhelming majority of medicines, as reflected in the *De Materia Medica* of Dioscorides, were plants [12,112,123]. Paracelsus (1493–1541) pioneered the use of concentrated and relatively pure (for the time) chemicals in medicine. Paracelsus, interestingly, also ascribed to earlier principles of materia medica, such as the *doctrine of signatures*, the belief in which physical characteristics of a plant portend the plant's medicinal action. However, the primary effect of the teachings of Paracelsus was to almost completely digress from earlier medical philosophy and chart a new path. Another seminal change credited to Paracelsus was that while Galenists believed that a disease of a certain character or nature would be cured by a remedy possessing the opposite effect, a precept similarly espoused by the famed Chinese Tang Dynasty physician and alchemist Sun Simiao (CE 581–682), Paracelsus and his followers argued that "a poison in the body would be cured by a similar poison." While many contemporaries of Paracelsus felt the substances he was recommending were too toxic to be used safely, Paracelsus defended his position with his oft quoted: *Alle Ding sind Gift, und nichts ohn Gift; allein die Dosis macht, daß ein Ding kein Gift ist.* "All things are poison, and nothing is without poison; only the dose permits something not to be poisonous." His challenging of the medical authority of the day forced Paracelsus to leave Basel and the revolutionary physician died at the young age of 48 [17]. Nevertheless, the teachings and writings of Paracelsus took on many adherents and charted a path in medicine that was alternative to current medical thinking and can be characterized as the progenitor of the modern, chemically refined, and highly toxic pharmaceutical medicines of today.

Other seminal events in medical history contributed to the course that led to the development of the type of medicines that predominate today. In 1805, a German apothecary apprentice named Friedrich Wilhelm Adam Sertürner isolated what was to become the first pure, presumably "active" compound of a plant the alkaloid morphine from the opium poppy (*Papaver somniferum*) (Figure 3.4) [76].

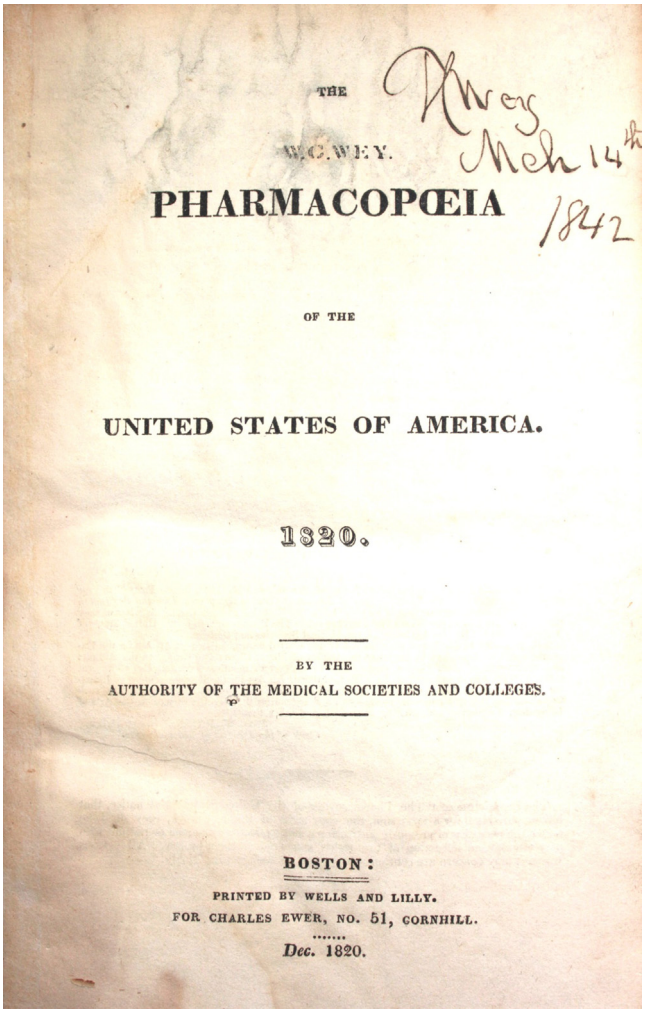
FIGURE 3.4 *Papaver somnifera* from the *Anicia Juliana Codex (Codex Vindoboensis)* a sixth century illustrated work *of De Materia Medica* of Dioscorides. The first "active" constituent discovered in a plant was morphine isolated from the opium poppy in 1805 by Friedrich Wilhelm Adam Sertürner (1783–1841). *With permission of the Austrian National Library, Vienna, Austria.*

With the isolation of morphine, an alkaloid making up approximately 10% of the total alkaloids of the plant [76], medical researchers recognized that the whole plant need not be used as a medicinal preparation but rather that one could extract an active constituent. Sertürner referred to his new compound as *principium somniferum* or "sleep-making principle," later naming it *morphium*, after *Morpheus* the Greek God of dreams or sleep [16]. Subsequent decades witnessed the isolation and eventual synthesization of pure compounds and the race for "active" constituents began changing medicine from whole plant-based to individual chemical-based entities. Traditional herbalists wonder what effects the other 90% of alkaloids in poppy contribute to either the safety or efficacy of the drug, and traditional herbalists and the WHO continue to regard the whole plant as representing the activity of a traditional medicine.

During the same time period, the medical and pharmacy professions were undergoing rapid changes. Originally, it was physicians, often referred to as "medical botanists," who were the primary teachers of materia medica and founders of early pharmacopoeias. As the pharmacy profession evolved, pharmacists began assuming an oversight role on the quality control of medicines. Later, pharmacognosy evolved as a subdiscipline of pharmacy and, initially, focused their attention and scientific investigation in the quality of crude drug materials. In modern times, this focus changed to searching for compounds in plants that were candidates for modern drugs. Subsequently, physicians became the sole prescribers of medicines, pharmacists became the primary and formal dispensers of medicines, and pharmacists and pharmacognosists assumed the primary role of drug development and quality control, as represented in the development of early pharmacopoeias.

The switch from botanical- to chemical-based medicine is clearly evidenced in the evolution of the *United States Pharmacopoeia* (USP), which in the first edition of [153] (Figure 3.5) included approximately 150 herbal drugs, 50% of the approximately 300 listed drugs at the time [153].

By United States Pharmacopeia [154], herbal drug monographs in the USP numbered approximately 50 of the more than 1100 preparation entries (a little more than 4%) [18,154]. This lack of representation of herbal drugs is in sharp contrast to the almost exclusive inclusion of herbal medicines that comprised the sixteenth century *Pharmacopoeia Augustana* [71].


THE

W.C.WEY.

PHARMACOPŒIA

OF THE

UNITED STATES OF AMERICA.

1820.

BY THE

AUTHORITY OF THE MEDICAL SOCIETIES AND COLLEGES.

BOSTON:

PRINTED BY WELLS AND LILLY.

FOR CHARLES EWER, NO. 51, CORNHILL.

Dec. 1820.

FIGURE 3.5 First edition of the *United States Pharmacopoeia* (USP) of 1820. Early editions of most all pharmacopoeias were dominated by plant-based medicines. The *United States Pharmacopoeia* [153] contained approximately 300 medications, approximately 50% of which were botanically based. By 1950, less than 5% of listed drugs were of plant origin. *Roy Upton Soquel, CA.*

3.2.4 Economic Influences in the Evolution of Medicine

Modern regulatory requirements dramatically change the accessibility and cost of herbal drugs. This is an important consideration in health care as among the most important of founding principles of the *Declaration of Alma-Ata* [166] regarding herbal medicines was accessibility and affordability, principles that have persisted to the current day more than 36 years later [165]. Generally speaking, herbal raw materials were historically gathered by local herbalists, who either prescribed them directly to patients or delivered them to practitioners. Often the price of an herbal consult and herbal formula was eggs, milk, a chicken, or the consult and treatment were free due to the practitioner being provided for by the larger community, similar to socialized systems of medicine. While such compensation practices seem out of place or inapplicable in developed nations, such practices still exist in many undeveloped and developing nations (e.g., rural China, Vietnam)

and play a significant role in the affordability factor of traditional herbal medicine. This is no better expressed than in the teachings of China's *Sun Simiao*.

Sun left a legacy of some of the most important and poignant lessons of TCM as well as medical ethics. In his *Bei Ji Qian Jin Yao Fang* (*Essential Prescriptions for Every Emergency Worth a 1000 in Gold*) Sun wrote: "physicians should respond to whoever asks for help, not distinguishing between poor and rich, young and old, ugly and elegant, friend or foe, foreigner or native, wise or fool, but should treat them equally as though they were your own relatives, without discrimination" [87]. Sun further noted: "a human life is extremely precious; more valuable than one thousand liang of gold."

A philosophy similar to Sun's was espoused in the ancient Ayurvedic literature in which payment for services was to be primarily provided by the wealthy and included formal lists of those who should receive free services [170]. This teaches that providing health care to those in needs is considered in these traditions to be more of a duty than a business. This is yet another stark contrast to, most notably, the US health care system, which can be characterized as being more of a business than a service.

At the same time that medicines were on the path of chemical development, medical professions in the US, and somewhat worldwide, were on a similar trajectory. The path taken would eventually lead to restrictions and obstacles to the acceptance of traditional herbal medicines. In the US, prior to the development of the American Medical Association (AMA) in 1847, there were multiple schools of medical thought. These disciplines included the Eclectics, Physiomedicalists, and Thomsonian practitioners (Figure 3.6), all of whom specialized in the use of botanical medicines, homeopaths, and allopaths.

The allopaths followed the path laid down by Paracelsus, and as noted, at the time, used bleeding (scarification) and calomel (mercury chloride) preparations as among their primary therapies. The AMA, an organization founded exclusively by the mineral doctors, in their first set of bylaws, adopted specific language that excluded from the professional organization, any "irregular" physicians whose "practice is based on exclusive dogma…," a clause, which at the time was specifically directed at homeopathic physicians who were at the time successfully competing with AMA physicians [9].

In the US, the Carnegie Foundation commissioned a lawyer named Abraham Flexner to report on the status of medical education in America. The result of the report published in 1910 was that schools were given specific ratings of A, B, or C according to facilities, laboratories, teaching resources, personnel, etc. Johns Hopkins, a decidedly allopathic oriented school, was considered the model by which all other schools were judged. According to Pulitzer Prize winning author Starr P. [142] in his *The Social Transformation of American Medicine: The Rise of a Sovereign Profession and the Making of a Vast Industry*, while the educational funding boards claimed neutrality in their pursuit to "improve medical education," they "sought to impose a model of medical education more wedded to research than to medical practice." According to Starr, while the policies that ensued from the Flexner report did not determine which institutions would survive, they did greatly influence which would dominate and what ideals would prevail. This greatly ushered in a change that may be described as decidedly un-Paracelsun, in that research-driven findings, not patient outcomes or physician observation, would dictate medical treatments. This was occurring at the same time as the chemical revolution was resulting in the rise of the pharmaceutical manufacturing industry whose lobbying power led to the integration of their products into the nonnationalized, largely profit-driven medical delivery system of the US today.

Economics in the international herbal medicines industry today is similarly driving health care policies, resulting in either greater or lesser degrees of access or restrictions to herbal medicines. For example, the cost of getting a traditional herbal medicine approved in the UK is between £30,000 and £120,000 (as of 2014). To have the same UK-approved herbal drug approved in other European Union (EU) countries costs an additional £10,000–£15,000 per country registration.

In the EU, the cost of getting a drug approved is approximately 100,000 euros (~$137,000 in 2014). To date, 1015 botanical drugs have been approved through the EU model, most of which are single-ingredient preparations. As most of the drug registrations are duplicative, these represent only a very small proportion of the traditional materia medica.

Another regulatory framework that impedes access and affordability to herbal medicines is the Botanical Drug Model established in the US (2004) [23], a country that does not recognize traditional herbal medicines as a distinct category, and has a modern drug approval process that results in a cost of approximately $750 million (2008 estimates) per drug and several years to bring a drug to market. If no commercial entity has an interest in a particular herbal ingredient then the cost required to meet the regulatory requirements for drug approval will not be pursued, and nonapproved herbal drugs will either be completely restricted or at least not supported within the regulatory framework. Few companies would consider astragalus, hawthorn berry syrup, or the 1000-year-old Ayurvedic compound triphala candidates for this level of investment for items that have been in the public domain for centuries.

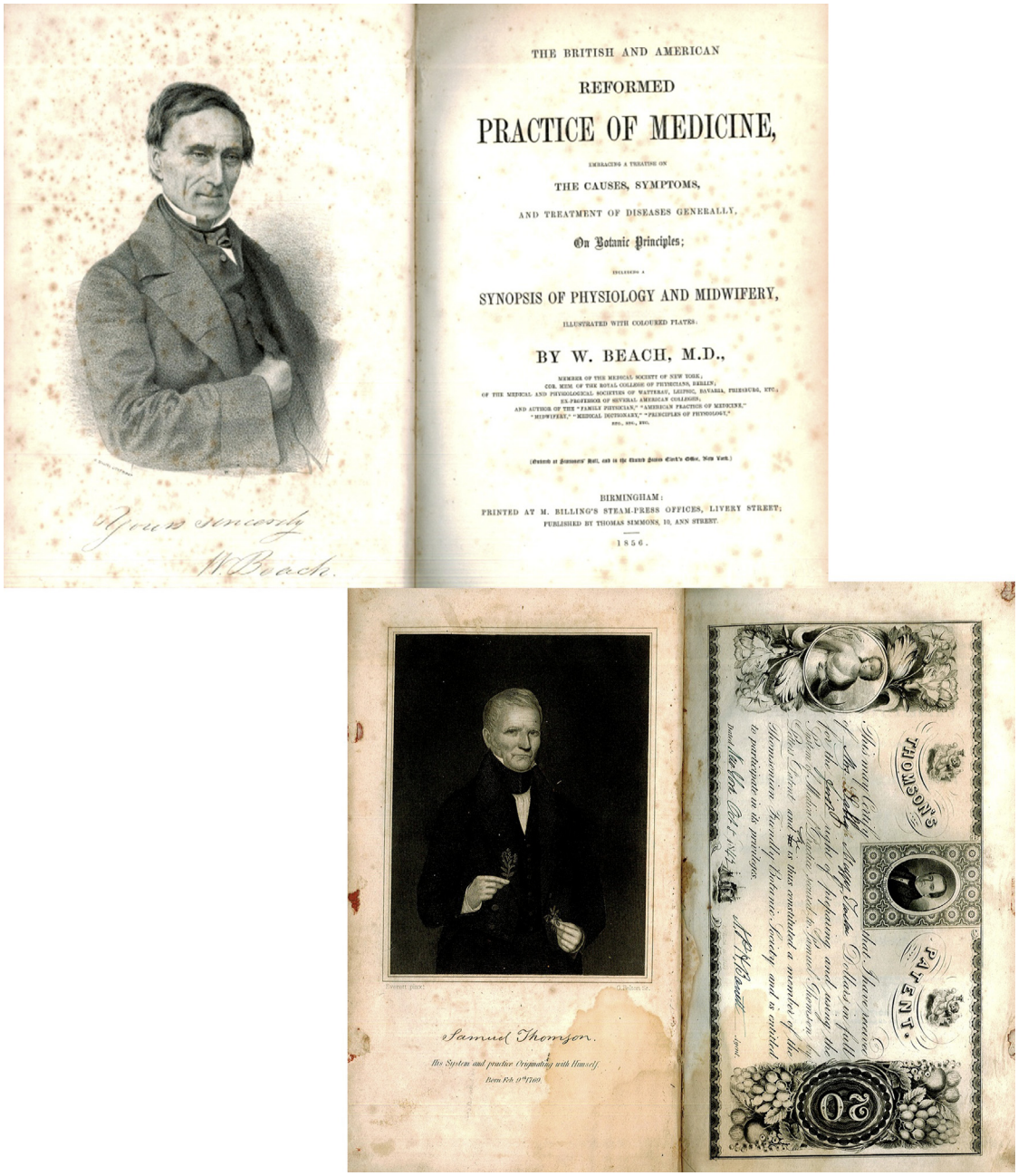


FIGURE 3.6 Examples of medical sects prominent in the late 1800s in the United States. In the late 1800s, a myriad of schools of differing medical thought were prevalent in the US and were partially represented by the Eclectics, Homeopaths, Thomsonians, and Physiomedicalists. *Roy Upton Soquel, CA.*

More poignantly, in the US, there is no regulatory model for the approval of traditional herbal medicines and the prescribing or dispensing of herbs as "medicines" is technically illegal despite recognition of some traditional healing traditions (e.g., traditional Chinese medicine and naturopathic practitioners), as primary care providers. Similarly, traditional herbal medicines are not reimbursed under any American insurance program. Thus, with regards to either traditional or conventional medicine, in a nonnationalized health care system, economics, more than medical or health needs, drives medical research and practice.

Modern regulatory policies greatly limit the approval of herbal medicine to those with an economic interest to do so. This in turn greatly restricts access to traditional herbal medicines and limits a practitioner's ability to access the full breadth of their materia medica. This is perhaps best evidenced by the fact that since the

enactment of the EU directive in 2004, not a single traditional Ayurvedic or Chinese herbal formula has been approved throughout the EU and only a single Chinese herb, *Dioscorea nipponica*, has been approved in Holland. This is highly significant to the practice of traditional herbal medicine as, in the EU, the presence of nonregistered herbal medicines on the market is technically illegal despite the fact that they have been in the public domain for literally hundreds or thousands of years. Similarly, in the US, only two botanical drugs have been approved through the botanical drug process since the regulations were promulgated; Veregen, a patented ointment prepared from green tea (*Camellia sinensis*) used for the treatment of genital warts and dragon's blood (derived from *Croton lechleri*) as an antidiarrheal drug for HIV/AIDS patients. These 2 latter botanical drugs represent novel innovations not traditional uses. The cost associated with their approval has not been reported.

Another factor in these discussions that is of particular interest to the US market is the fact that most nations, with the exception of the US, have a long history of socialized health care. When profit is the primary motivating factor for drug development this dramatically changes the dynamics of a health care system, and most specifically, the practice of medicine. Today, reimbursement or coverage of herbal medicines is an integral part of the health care delivery system of many developed countries (e.g., China, France, Germany, Japan, Netherlands, Republic of Korea) to greater or lesser degrees [178] and is key to keeping herbal medicines affordable. Yet, in most Western countries, the herbs and formulas that make up the bulk of the traditional materia medica are not recognized, approved, and in some cases, as in the US, not allowed as medicines.

Today's quest for modern medicines and the desire to fit traditional herbal medicines into a modern pharmaceutical drug regulatory model is more about the creation of a new highly profitable blockbuster drug than relief of suffering of humankind, a model that affects health care policies worldwide.

3.3 MODERN MEDICINE—AN AMERICAN CASE HISTORY

Evidence for the failings of relying strictly on a research- and profit-driven versus a practitioner-driven medical system is no better demonstrated than in the health care statistics of the US. Schuster et al. [136] reported that in almost all aspects of health care, the US is failing. These failings encompass the spectrum of services from preventive, acute, and chronic care; from infants to the elderly; and the health care delivery system of a single city or nation overall. Patients are either not receiving the care they should be receiving or they are receiving inappropriate care that is related to both under treatment and over treatment, as well as the inappropriate use of pharmaceutical medications. Similar findings were reported by numerous other authors (e.g., McGlynn et al. [96]) and organizations over the last decade.

By all measures of individual and social health, America is ranked among the worst of all similarly developed nations [168]. In infant mortality, a general marker of overall societal health, the US ranks an appalling 31 (based on 2008 data) [65], in the world behind virtually all similarly industrialized nations. In 2000, the WHO ranked the US a dismal 37th in overall health care systems [160]. In a similar ranking by the Commonwealth Fund [25], the US was far behind other nations in terms of healthy lives, quality, access, efficiency, and equity of health care delivery services and 19th of 23 nations surveyed in healthy life expectancy at age 60 years [24]. Perhaps most importantly, the report noted a "strikingly consistent and pervasive pattern of higher mortality and inferior health in the United States, beginning at birth," and that this pattern of poorer health was evident at all stages of life, from infancy to childhood, to adolescence to young adulthood, to middle and old age, and could not be explained by disparities in social demographics. Those who could afford health care and were financially well off fared little better than those less fortunate. More unfortunate is that some of these findings have been reported for decades (such as the persistent high rates of infant mortality) and yet no meaningful change in the system has occurred. Primary care pioneer Barbara Starfield (1932–2011) highlights that the majority of those countries with the best health statistics have a strong primary care infrastructure and that better outcomes are correlated with primary care services. Primary care providers can be described as being patient centric. This in contrast to the predominant reliance on specialists in the US, which can be described as more research centric. The WHO has a long history of recognizing traditional health care practitioners as primary health care providers, but many countries, like the US, lack acceptance or full integration of traditional healing practitioners into their national health care systems.

More importantly, no improvements in health statistics can occur without a fundamental change in the philosophical basis by which health and healing are approached. As long as the militaristic approach of search and destroy is employed as the primary therapeutic paradigm, big guns represented in powerful pharmaceutical and high-tech interventions will continue to yield the extremely poor health statistics reflected in the American system (Table 3.1).

TABLE 3.1 US Health Care Statistics Compared to 13 Other Countries

Outcome	Ranking of 13 countries[a]
Average for all health indicators surveyed	12
Low birth-weight %	13
Neonatal and infant mortality	13
Years of potential life lost	13
Post-neonatal mortality and life expectancy at 1-yr-old for females	11
Post-neonatal mortality and life expectancy at 1-yr-old for males	12
Life expectancy at 15 years of age	10
Life expectancy at 40 years of age (males)	9
Life expectancy at 40 years of age (females)	10
Life expectancy at 65 years of age (females/males)	7
Life expectancy at 80 years of age (females/males)	3
Age adjusted mortality	10

[a]*Japan, Sweden, Canada, France, Australia, Spain, Finland, the Netherlands, the United Kingdom, Denmark, Belgium, United States, Germany [141].*

3.3.1 Over Diagnosis—Over Treatment—Iatrogenesis

Medical diagnostic technology that leads to a cascade of subsequent treatments is partly responsible for the relatively high rate of iatrogenesis in the US [141]. Such diagnostics are predominantly research driven and represent how lack of a patient-centered focus results in negative health outcomes. This is no more evidenced than in policies regarding prostate-specific antigen (PSA) screening and mammograms. For almost two decades in the US, annual PSA screening was recommended for healthy men over 50 years of age despite the lack of robust scientific evidence for a benefit (see Ref. [3]). Literally millions of men were screened annually at an annual cost of $447 million to America's Medicare program alone (2009) [90]. False positives and detection of slow growing (indolent) tumors resulted in aggressive surgical or radiation treatments that caused serious harm including urinary incontinence, erectile dysfunction, pain, infections, hospital readmission, and death [101]. It has only been more recently that a number of studies specifically looking at the benefit-risk assessment of routine PSA testing over the last 20 years determined that the moderate to high risks of PSA screening and the ensuing cascade of treatments as described by Starfield [141] far outweighed the low to moderate benefits [21]. Annual screening resulted in high levels of false positives that led to unnecessary biopsies that carry a risk of bleeding, infection, pain, urinary symptoms, and hospitalization [104]. Because of this, PSAs are no longer recommended for most of the demographics for which it was previously used. Routine mammograms followed the same trajectory and were, and among many, remain, research-driven and profit-motivated technology [38]. While early detection with mammograms benefits a small subset of the population, the very act of compressing breasts between two plates and subjecting breast tissue to the cumulative DNA-damaging effects of radiation increases women's risk of breast cancer, some estimates accounting for up to 20% of breast cancers in the US (Ref. [38] and references therein).

Thus, despite mounting evidence of questionable benefit (see Ref. [98]) in recent decades regarding the substantial risk of mammograms, as of February 2014, 75% of American women reported having received a mammogram in the past year. In the United States alone, approximately more than 37 million mammograms are performed annually at a cost of about $100 per mammogram. Among the interventions chosen by women after diagnosis is bilateral mastectomy. In the largest study to date [84] there was no difference in mortality of women receiving bilateral mastectomies compared with women receiving unilateral mastectomy or lumpectomy and standard follow-up radiation. Interestingly, unilateral mastectomy was associated with a higher mortality than either lumpectomy or bilateral mastectomy.

It is now recognized that PSA and mammography screening are almost identical in their overdiagnosis, overtreatment, marginal benefit, enormous cost, and substantial risk. Ironically, according to a study by Fenton et al. [45], patients who reported they were most satisfied with their health care had greater chances of being admitted to the hospital and had an approximately 9% higher total health care cost as well as 9% higher prescription drug expenditures. Additionally, death rates among those most satisfied with care were more than 25% higher than death rates in those reporting the least satisfaction with health care services received. This is particularly poignant as it illustrates a marked differentiation between patients seeking conventional versus traditional health care services. The Fenton et al. [45] study suggests that patients seeking conventional care services equate satisfaction with increased treatments and interventions to their detriment that is paid for by the entire society.

Such practices represent medicine at its worst and is a complete antithesis of the common medical dictum of *Primum non nocere* (*first do no harm*), often attributed to Hippocrates and still integral, in theory only, to modern medical ethics. More significantly, these examples of research-driven technologies represent an insidious

aspect of the modern health care system as every major cancer association as well as local and national government agencies strongly supported the use of the aforementioned screening tools for the majority of the adult population despite a lack of clinical evidence of benefit and despite significant mechanistic and observed evidence for risk. The negative impact of such research and economic-driven policies is clear. However, more importantly, this is a poignant example of how much of what is considered technologically advanced medicine is not based in sound clinical science, but rather research-based science. A primary criticism levied against herbal medicines, and other traditional healing practices, is their lack of scientific proof of efficacy based in modern pharmacological research. However, much of traditional herbal medicines and traditional healing practices in general are precisely based on human clinical outcomes codified in human experience for hundreds or thousands of years. This is not to say that traditional healing knowledge is complete, safe, effective, and appropriate for all people all of the time or does not have limitations and knowledge gaps. This merely reflects a different knowledge base that has been a consistently evolving foundation of human healing since the beginning of human existence, and at the very least, should be given the same respect, honor, and use as technologically advanced therapies that only have decades of largely experimental use.

3.3.2 Pharmaceutical Medications—A Huge Price to Pay

In addition to the philosophical differences between traditional and conventional medical practitioners and the economics that drive medical practice is the problem of having a medical system that relies predominantly on pharmaceutical medications. There is no doubt that pharmaceuticals save lives, especially in acute crisis. The advent of antibiotics, while solidifying a search and destroying militaristic philosophy as a primary approach in health care, has been responsible for saving tens of millions of lives. Morphine, while representing an inherent split between whole plant-based medicines and active constituents, is an incredibly effective painkiller that, due to its potential for diversion to opium and heroin, is no longer allowed in the public domain as a plant-based medicine as it once was. And the story of pharmaceutical development can easily be written to focus on the positive attributes modern drugs have contributed to human health (e.g., *The Inside Story of Medicines*) [67]. But despite some great successes in the world of pharmaceuticals, many ironically that were derived from plants (e.g., aspirin, morphine, and vinca alkaloids), there is a tremendous price that current and future societies pay for the adverse effects inherent in such powerful medications.

Firstly, pharmaceutical medications are represented to the public and health care professionals as proven to be safe and effective according to rigorous scientific methodology. This is not entirely true. In 2009, the pharmaceutical giant Pfizer plead guilty to a felony charge and agreed to pay $2.3 billion in fines for fraudulently marketing select drugs. This was the fourth fine levied against Pfizer since 2002. In recent decades, a large number of the most recognized pharmaceutical companies have paid similar fines, most for fraudulent marketing practices and some for violations of good manufacturing practices (GMPs). Such practices reflect relatively gross violations of federal law. The research and development side of pharmaceutical development is much more nuanced and insidious as has been highlighted in recent writings such as '*Deadly Medicines and Organised Crime: How Big Pharma Has Corrupted Healthcare*.' This expose was written by Peter Gøtzsche, a co-founder of the highly prestigious independent evidenced-based Cochrane Collaboration. Another, '*The Truth About Drug Companies: How They Deceive Us and What To Do About It*,' was written by medical doctor Marcia Angell, former editor-in-chief of the *New England Journal of Medicine*, arguably one of the most prestigious medical journals in the world. Both works provide clear and evidence-based examples of broad-based bias and fraud in the world of medical literature. These authors demonstrate clearly that published clinical research is positively biased to the sponsoring companies, provide examples of gross fraud by medical writers claiming to conduct trials that were never conducted (reviewed in Ref. [62]), and report that negative findings are often not publicized, allowing for a drug to be released on the market with the belief that it has been scientifically demonstrated to be safe and effective.

The consequence of poor science in pharmaceutical drug development is significant. There are numerous examples of heavily marketed drugs that were removed from the market due to serious, sometimes fatal, adverse effects including Oraflex, Propulsid, Rezulin, and perhaps the crowning jewel of this collection, Vioxx, a widely prescribed arthritis drug that was withdrawn from the market after disclosures that the pharmaceutical manufacturer Merck withheld information from patients and physicians about the potential for Vioxx to increase the risk of heart attacks and stroke. Vioxx use resulted in between 88,000 and 140,000 cases of serious heart disease. Most significantly, cautions regarding the safety of Vioxx were put forth by epidemiologist David Graham, who at the time was the Associate Director of the Food and Drug Administration's (FDA) Office of Drug Safety. In formal testimony given before the US Senate

Committee on Finance, Dr. Graham provided poignant testimony stating that policies within FDA were insufficient to protect the public from drugs, which carry unacceptable risks, stating;

> I would argue that the FDA, as currently configured, is incapable of protecting America against another Vioxx. We are virtually defenseless… Finally, the scientific standards CDER applies to drug safety guarantee that unsafe and deadly drugs will remain on the US market. [57]

While such testimony can be viewed as representing only an individual opinion, similar findings were reflected in a US Government Accountability Office [54] report that stated:

> FDA lacks a clear and effective process for making decisions about, and providing management oversight of, postmarket drug safety issues.

In a follow-up interview with Dr. Graham, congressional representatives asked specifically what within FDA needed to be fixed. Dr. Graham replied:

> FDA is inherently biased in favor of the pharmaceutical industry. It views industry as its client, whose interests it must represent and advance. It views its primary mission as approving as many drugs it can, regardless of whether the drugs are safe or needed. [20]

Graham's testimony in 2005 was largely echoed by Leonard Paulozzi, a medical epidemiologist with the US Centers for Disease Control and Prevention, in other congressional testimony, in Ref. [114], in which Dr. Paulozzi stated;

> We cannot lose sight of the pressing realities of public health issues that we face every day, such as unintentional poisonings, which are now the second leading cause of unintentional injury death…. Most unintentional drug poisoning deaths are not 'accidents' caused by toddlers or the elderly taking too much medication… These deaths are largely due to the misuse and abuse of prescription drugs.

In actuality, in the 35- to 54-year-old age group, poisonings due to conventional medications were the leading cause of death, exceeding that of motor vehicle accidents. In 2009, deaths due to pharmaceutical medications overtook motor vehicle accidents as the primary cause of preventable death in the US [66], the US Department of Health and Humans Services (HHS) stating: "The United States is in the midst of an unprecedented drug overdose epidemic," specifically referring to pharmaceutical medications, a five-fold increase over 1980 pharmaceutical-related deaths.

Regarding efficacy, or specifically, lack thereof, a review of studies and meta analyses of studies of approved antidepressant medications found that "antidepressants are only marginally efficacious compared to placebos" and further document profound publication bias that "inflates their apparent efficacy" [118]. The relative lack of efficacy of approved antidepressant medications was underscored by the National Institute for Health and Clinical Excellence (UK) who concluded: "Given these data, there seems little evidence to support the prescription of antidepressant medication to any but the most severely depressed patients, unless alternative treatments have failed to provide benefit" [79]. Concern regarding publication bias specifically regarding antidepressants was further raised by the US FDA who stated: "By altering the apparent risk/benefit ratio of drugs, selective publication can lead doctors to make inappropriate prescribing decisions that may not be in the best interest of their patients and, thus, the public health" [44]. Approximately 11% of Americans take antidepressants and between 2005 and 2008, this class of drugs was the third most common prescription drug taken by Americans of all ages [120]. According to a study of Mojtabai and Olson [100], approximately 62% of these subjects are misdiagnosed and do not meet appropriate diagnostic criteria warranting medication.

The fallacies of the scientific validity of the US drug approval process are further hidden from the American public in equally infactual ways. In a 2013 article in the *Journal of General Internal Medicine*, authors Faerber and Kreling reported that 60% of prescription drug ads and 80% of over the counter drug ads were misleading or false. The study found 43% of the claims in direct to consumer (DTC) drug ads were "objectively true" while 55% were "potentially misleading" and 2% were "false." Moreover, 84% of regulatory letters sent by the FDA from 1997 to 2006 cited ads for minimizing risks and/or exaggerating effectiveness of drugs. For example, one study found that although 19% of DTC ads mentioned lifestyle changes as an adjunct to medication, none mentioned them as an alternative to drug treatment [49]. Thus, promoting a drug in consumer advertisements as the solution to a specific health problem serves to disincentivize viewers from making healthy lifestyle changes and fostering the belief that such changes are ineffective or unnecessary [137]. DTC drug advertisement, which is illegal in most all countries except the US and New Zealand, rarely focuses on public health messages about diet, exercise, addictions, social issues, and other treatments that may be more cogent to wellness than pharmaceutical interventions [4]. Such behavioral changes can result in fundamental improvements in an individual's health status, is free of side effects, and is free of cost. It makes sense for product marketing to focus on the intervention and not the lifestyle, as, from a marketing perspective, promoting

healthy lifestyle practices would be a disincentive to using the medication.

3.3.3 Safety of Herbal versus Pharmaceutical Medications

The consequences of the adverse effects associated with conventional medications go far beyond those associated with the numerous drugs removed from the market due to their danger and from the lack of scientific rigor in establishing their safety prior to releasing them into the hands of physicians who know little of their dangers. There is a gross misconception by physicians and consumers that drugs that have been subjected to formal approval can be relied upon as being safe for their intended indications within the parameters as proscribed and disclosed in required prescribing information. However, this is not the case and is well illustrated in the history of adverse drug effects in the US. In 1990, the US General Accounting Office (GAO) reviewed 198 FDA-approved drugs and reported that of these approximately 102 (51.5%) had "serious post-approval side effects" that were not known at time of approval. The additional side effects reported included anaphylaxis, cardiac failure, hepatic and renal failure, birth defects, blindness, and death [53]. At the time of the report, all but two of the medications remained on the market. All the others remained as they were or with additional warning language.

Leape et al. [86] in a comprehensive review of adverse drug events in hospitalized patients, reported that drug complications were the most common source of adverse events. Bates et al. [11] reported that 1% of adverse drug events were fatal, 12% life threatening, 30% serious, and 57% significant. In the same year, Cullen et al. [30] noted significant underreporting of adverse drug events in hospitalized patients including more than 10% of events that were serious or life-threatening and went unreported. In 1996, it was reported that among 1000 elderly patients admitted to the hospital from the emergency room, 538 were exposed to 1087 drug-drug interactions [33]. In a review of hospital surveillance reports of adverse effects associated with approved medications, Lazarou et al. [85] reported that 2,216,000 patients experienced serious adverse effects resulting in 60,000–140,000 fatalities annually due to the proper use of conventional drugs. Not included in this figure was the number of deaths that occurred due to misuse of medications, which accounted for another 200,000 patients annually. A variety of studies report varied incidences of adverse drug events ranging from 20–25% of patients experiencing such an event, 13–38% of which are considered serious [52,63]. Zhan et al. [177] reported 4.3 million adverse drug events in 2001, showing an upward trend from 1995–2001. In total, adverse drug effects have been estimated to cost $77 billion in extra health care costs annually in the US alone [74,141]. A 2008 memo of the European Commission [40] shows that dangers associated with pharmaceutical medications are not isolated to the US. In acknowledging the need for legislative changes to improve drug safety, the European Commission estimated that 5% of all hospital admissions in the EU are due to an adverse drug reaction, that adverse drug reactions are the fifth most common cause of hospital death, and that adverse drug reactions are responsible for approximately 197,000 deaths annually. Some may argue that the benefits of the drugs outweigh the risk, a general premise that is widely accepted. However, the bare facts suggest that a medical system so reliant on conventional pharmaceutical medications with their inherent toxicity is fundamentally flawed, echoing back to the warnings of pre-Paracelsun pharmacopoeias that cautioned against the use of such toxic medications.

The relevance of all this to traditional healing practices, especially herbal medicine, is two-fold; first is that research, economic, and regulatory agendas are heavily biased to pharmaceutical development based on research findings that are often flawed at best and fraudulent at worst versus being patient centric and outcomes oriented. Second, no single herbal medicine or collective of herbal medicines in the history of the world has resulted in the magnitude of public harm as a single drug such as thalidomide, Vioxx, or Rezulin or the collective of pharmaceutical drugs. Yet, despite hundreds or thousands of years of clinical and empirical data with herbal medicines, their use is often restricted or limited based on claims of their "unknown dangers" simply because modes of action have not been fully articulated according to modern conventional drug standards, which, in actuality, fail to accurately disclose the dangers of modern drugs.

3.4 TRADITIONAL HERBAL MEDICINES: CENTURIES OF EMPIRICISM

One of the points of demarcation between the basis of evidence by which traditional and modern drugs are differentiated is empirical observation of the former and modern clinical trials of the latter. Interestingly, as in the examples of the toxic pharmaceutical discussed above, it is only when the drugs get into empirical use is the actual toxicity and limited efficacy revealed. Additionally, in the US, 20% of prescription drugs are used for off-label uses, uses for which they were not specifically approved, including use in pregnancy and in children, uses for which were determined by practitioners empirically and are legally allowed. Moreover, a 2006

study reported that most all of these off-label uses (73%) had little or no scientific support [121]. This is most troubling as one of the primary reasons given worldwide that dissuades physicians and insurance carriers from integrating herbal medicine into national health care plans is a perceived lack of scientific evidence; yet most of what is practiced in Western medicine, including the use of pharmaceuticals and medical procedures (e.g., cesarean sections, hysterectomies, mammography and PSA screening) are not evidenced based or are based on very poor evidence.

3.4.1 St. John's Wort—A Herbal Case History

St. John's wort (*Hypericum perforatum*) (Figure 3.7) is one of the most ancient of herbal remedies in Western civilization and continues to be used today in much the same ways as in ancient times.

Dioscorides wrote: a decoction of the fruit (taken as a drink with a pint of honey water) is available for sciatica.

It expels much bilious excrement… Smeared on, it is good for burns [112]. Today, St. John's wort oil is routinely used topically by modern medical herbalists for burns, scrapes, and neuralgic pain and internally for the treatment of depression. While St. John's wort was introduced into modern western medical practice by homeopaths, who regarded it as "arnica for the nerves," a dictum attributed to Paracelsus [73], and arnica being a highly regarded healing agent for tissue damage, the herb became widely accepted by nonhomeopaths. St. John's wort was historically one of the most relied upon botanicals for the treatment of wounds. Part of this activity is due to *Hypericum's* antimicrobial activity, which modern research attributes to the essential oil, phloroglucinols, and flavonoids. In a relatively modern study, a St. John's wort oil, one of the oldest of Galenical St. John's wort preparations, was shown to facilitate the healing of second and third degree burns [129] and successfully treat infection with *Staphylococcus aureus* [105] to a degree greater than standard treatment with sulfonilamide [2]. In another study of St. John's wort, a tincture (1:10) of the herb was studied for its wound-healing properties and compared with *Calendula*. The effect of orally administered tincture of St. John's wort was greater than topical application of *Calendula* tincture in the healing of incision, excision, and dead space wounds as evidenced by an increase in epithelization and wound-breaking strength [122]. In recognition of the ancient use of St. John's wort as a healing balm, St. John's wort oil was listed in the *Pharmacopoeia Augustana* (1622) and in the first [116]. Gerard [55] wrote that St. John's wort's use as a balm for wounds, burns, ulcers, and bites was without equal, stating: "for I dare undertake to cure any such Wound as absolutely in each respect, if not sooner and better, as any Man whatsoever shall or may with Natural Balsam." Linnaeus [89] in his *Materia Medica* reported on the use of the flowers and herb of St. John's wort (*Hypericum vulgare*) to stop bleeding of the lungs and urinary tract and as an anthelmintic. The Latin name *Hypericum* was derived from the earlier Greek *eikon* as recorded in the second century BCE by Nikander (Alexipharmaca V, line 603) and then later, according to Ref. [69] by the noted Greek physician Euryphon (*upereikon*; fifth century BC) as *yperikon*, which meant "over an apparition" [69].

Paracelsus also recommended St. John's wort for *phantasmata*, which referred to psychoses and hallucinations, and for "healing of the soul." In early times, depression was undoubtedly considered a form of possession, and, from a humanistic perspective if not a medical diagnostic perspective, depression can very

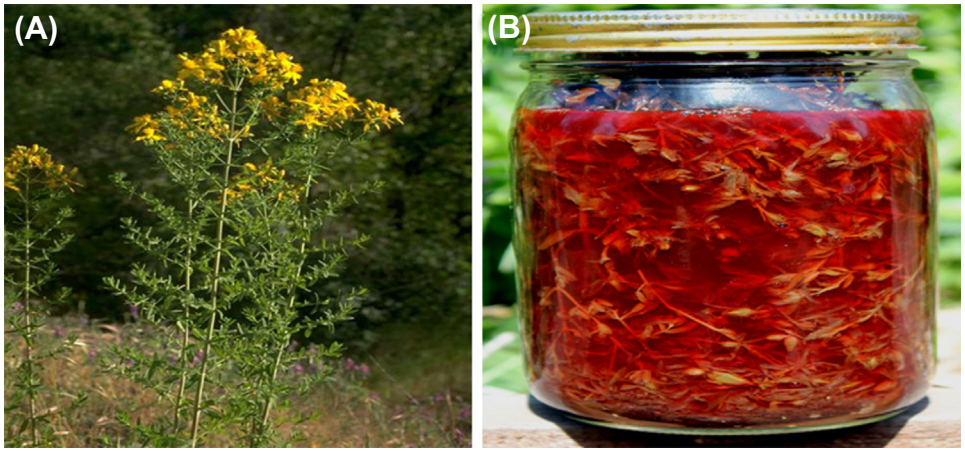


FIGURE 3.7 (A): St. John's wort (*Hypericum perforatum*); (B): Oil maceration of St. John's wort (*Hypericum perforatum*) flowering tops. The oil of St. John's wort flowers is one of the most ancient of vulnerary (wound healing) oils used. Modern research attributes wound-healing effects due to phloroglucinols contained in the flowers and seeds. *Roy Upton, Soquel, CA.*

much be described as a "disease of the soul." In more modern times, formal clinical trials including meta-analyses, demonstrate St. John's wort's efficacy in the treatment of mild to moderate depression [5,88]. In one of a few trials where significant benefit was not demonstrated with either St. John's wort or the approved antidepressant imipramine, the herb performed better than the pharmaceutical both in patient outcome and side-effect profile [31]. Thus, even in a study where the herb presumably was not statistically significantly better than placebo, it nevertheless showed greater efficacy than an approved pharmaceutical agent and was safer.

3.4.2 Triphala and Si Wu Tang

Ayurvedic medicine provides us with a particularly poignant example of a traditional herbal drug that has similarly stood the test of time and modern research—the three-fruit combination *triphala*. Triphala was first written about and referenced extensively more than 1000 years ago in the seminal text of Ayurveda the *Caraka Samhita* (~100 BCE). This formula is a mainstay of the practice of virtually every modern Ayurvedic practitioner today and, for many, it is the first therapy given before any others can be considered effective. Additionally, triphala is regarded as a *rasayana*, a rejuvenative tonifier used to preserve life [119]. Much of the modern research of the individual herbs in triphala, as well as the combination itself, focuses on cytoprotective, immunomodulating, and antioxidant activity [103,140], three pharmacological actions that can be considered to be associated with preserving life.

A last example is provided by a classic Chinese herbal formula known as *si wu tang* (*four substance decoction*). The formula was first written about in the *Xian Shou Li Shang Xu Duan Mi Fang* (*Secret Formulas to Manage Trauma and Reconnect Fractures Received from an Immortal*) approximately CE 846 [133]. The formula was originally described for its use in menstruation, among other indications. Regarding menstrual difficulties, Western conventional medicine has few therapeutic options for promoting gynecological health and fewer good therapeutic options. In 2003 in the US alone, there were approximately 602,457 hysterectomies performed, 538,722 (~89%) of which were for benign conditions [169]. Some studies show that many hysterectomies are deemed unnecessary by second opinion or when alternative treatments are offered [15,144]. Some experts suggest that up to 90% of hysterectomies are not medically necessary [108]. Rates of hysterectomies have differed between countries over the years, for example, ranging from a high of 5.4 per 1000 women in the US [43]; intermediate rates of 3.7 per 1000 in Italy [95]; and a low of 1.2 per 1000 in Norway [97]. The conditions for which hysterectomies are performed, and are often accompanied by removal of the ovaries (oophorectomy) as prophylaxis against ovarian cancer, are predominantly to relieve symptoms of difficult menstruation that generally do not threaten lives. Current scientific evidence suggests that elective oophorectomy is not advisable for the majority of women as it may lead to a higher risk of death from cardiovascular disease and hip fracture and a higher incidence of dementia and Parkinson disease [127]. Recently, it was concluded that preserving ovaries until at least the age of 65 years was associated with higher survival rates. Conversely, women who have a bilateral oophorectomy have a higher incidence of risk of all-cause death, fatal and nonfatal coronary heart disease, stroke, and lung cancer. Markedly, none of the groups in which oophorectomies were performed showed any correlation with increased survival [113] suggesting that botanical alternatives offer a completely different therapeutic strategy with tremendous potential for benefit. Similarly, bioprospecting in the traditional herbal medical literature for potential medicinal compounds has been demonstrated to yield more positive findings than random screening of plant compounds [26,145]. Moreover, noted medicinal plant researcher Cordell [27] underscores the need for humans to pay close attention to the preservation and investigation of traditional knowledge and natural resources that provide traditional and modern medicines for future generations, a message that should be taken to heart.

3.5 TRADITIONAL MEDICINE: THERAPEUTICS, DEFINITIONS, AND ORIENTATIONS

Historically there were two primary arms to the study of traditional herbal healing modalities, *therapeutics* and *materia medica*. The first provides the theoretical framework by which health and disease is understood, the second the medicinal agents used. Therapeutics provides the diagnostic principles that form the basis for which materia medica is to be applied. Today, the majority of herbal medicines are researched in a way that takes little or no consideration into the therapeutic framework in which herbal medicines were traditionally used. Rather, individual (relatively rarely) formulas are investigated from a western pharmacological perspective for Western disease endpoints, out of cultural diagnostic and therapeutic context. The primary reason that Chinese medicine offers potentially effective treatments for functional gynecological problems, as discussed above, is due to the theoretical basis of understanding the physiology and pathology of the gynecological system. In Western medicine there is little understanding of the cause of functional gynecological imbalances. Western medicine employs metrics to identify hormonal imbalances, growths, and

abnormalities, but has little to no understanding of the cause. Conversely, Chinese medicine offers unique perspectives on promoting the health of the gynecological system through a myriad of theories of the causative factors of gynecological imbalances that correspond directly to therapies to address those imbalances. Whereas Western medicine offers hormonal replacement therapy (that itself increases mortality), palliative medications, and surgical and ablation therapies, TCM offers a host of therapies that include dietetics, baths, exercise, moxibustion, acupuncture, and a myriad of diverse herbal prescriptions, such as *si wu tang*, some of which have been in continued use for several hundred years. Many of these therapies have been demonstrated to have high rates of efficacy relative to controls for a wide range of gynecological imbalances including infertility, endometriosis, polycystic ovarian syndrome, dysmenorrhea, and pelvic inflammation, to name only a few (Refs [75,126]; among others), though most of the non-Asian world remains unaware that TCM offers a completely different healing paradigm that could render a large percentage of hysterectomies unnecessary.

Traditional herbal medicines are generally and specifically defined within numerous WHO documents and are categorized into four main groups as follows [161]:

1. *Herbs*: crude plant material such as leaves, flowers, fruit, seed, stems, wood, bark, roots, rhizomes, or other plant parts, which may be entire, fragmented, or powdered.
2. *Herbal materials*: in addition to herbs, fresh juices, gums, fixed oils, essential oils, resins, and dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting, or stir-baking with honey, alcoholic beverages, or other materials.
3. *Herbal preparations*: the basis for finished herbal products and may include comminuted or powdered herbal materials, or extracts, tinctures, and fatty oils of herbal materials. They are produced by extraction, fractionation, purification, concentration, or other physical or biological processes. They also include preparations made by steeping or heating herbal materials in alcoholic beverages and/or honey or in other materials.
4. *Finished herbal products*: herbal preparations made from one or more herbs. If more than one herb is used, the term mixture herbal product can also be used. Finished herbal products and mixture herbal products may contain excipients in addition to the active ingredients. However, finished products or mixture products to which chemically defined active substances have been added, including synthetic compounds and/or isolated constituents from herbal materials, are not considered to be herbal.

The WHO more specifically defines herbal medicines as follows:

"For the purpose of these guidelines, the Consultation agreed that Herbal Medicines should be regarded as: Finished, labeled medicinal products that contain as active ingredients aerial or underground parts of plants, or other plant material, or combination thereof, whether in the crude state or as plant preparations. Plant material also includes juices, gums, fatty oils, essential oils, and any other substances of this nature. Herbal medicines may contain excipients in addition to the active ingredients. Medicines containing plant material combined with chemically-defined active substances, including isolated constituents of plants, are not considered to be herbal medicines."

These categories draw clear distinctions between a traditionally prepared herbal medicine and a modern chemical isolate. This is poignant as an herbal substance such as St. John's wort with several centuries of accumulated data regarding indications, efficacy, and safety, and numerous modern studies documenting relative safety and efficacy, must be considered differently than the newly developed drug of the year that has no history of existence let alone a sufficient body of data regarding safety and efficacy.

While some countries (e.g., Japan, India, People's Republic of China) have worked diligently to represent traditional healing practices, including herbal medicine, as part of their national health care systems, as of 2005, approximately 60% of WHO member countries had no policy on traditional healing practices [164]. Moreover, while it is largely public interest that is driving the desire for greater access to herbal medicines, and thus driving policies, oftentimes, national policies are established with little or no input from the traditional medicine practitioner community. For example, neither the EU traditional medicines Directive 2001/83/EC nor the Dietary Supplement Health and Education Act, the latter that constitutes the primary access by which traditional herbal agents are allowed in the US, had formal input from the most well established of the traditional medicine practitioners, namely, Ayurvedic, Chinese medicine, or naturopathic practitioners. Thus, in both the US and the EU, it is industry with vested interests in specific herbal remedies that drive both health care policy and herbal medicine approval. This is a significant impediment to traditional healing practices as the availability of materia medica is often dependent upon the availability of crude herbs or herbal preparations through industry. The 2006 banning of the Chinese herb ephedra (*Ephedra sinica*) (Figure 3.8) in the US is a clear example of how the misuse of the herb by industry, and subsequently by consumers, in stimulant and weight loss products led to significant adverse events.

FIGURE 3.8 Ma huang (*Ephedra sinica*). Ma huang is an herbal drug used since ancient times in China. It is a source of the sympathomimetic amine ephedrine, which is used as a common ingredient in decongestants. Its inappropriate use in herbal stimulant and weight loss products led to the complete ban of the herb in the US in 2004, though over-the-counter ephedrine drug products remain on the US market. *Roy Upton Soquel, CA.*

This resulted in both perceived and real concerns regarding the herb's safety that resulted in a complete restriction from public and practitioner use, losing one of the most important articles of both Chinese and Western herbal materia medica and losing access to an herb that had been used with a relatively high degree of safety (when properly used) for more than 1000 years. A similar ban of kava root (*Piper methysticum*) (Figure 3.9) occurred, almost worldwide due to concerns over its potential for hepatotoxicity in certain individuals.

Kava has a long history of use throughout Polynesia, where it is used as a ritualistic calming beverage. It was developed as a modern phytomedicine and its safety and efficacy as an anxiolytic has been reported in individual studies and meta-analyses [130,131]. Prior to its ban, the root preparation was available worldwide. In the early 2000s, case reports of alleged hepatotoxicity associated with kava use were published (see Ref. [41], among others). In subsequent years, a total of a few hundred case reports were made. These were systematically reviewed by a variety of regulatory authorities and authors and resulted in an almost complete ban of the herb worldwide. According to most reviews, only a small percentage of the total number of the reports could be attributed to kava itself as many users were also using potentially hepatotoxic drugs such as acetaminophen, chemotherapeutic drugs, or alcohol, and some were not using kava at all. In their formal assessment at the time, Swiss regulatory authorities estimated that 8 cases occurred in a total of 40 million daily doses or 1 case per 170,000 courses of treatments of 30 days duration [143]. In a formal review of the case reports at the time, noted toxicologist Donald Waller concluded: "In summary, there are only a few of these cases in which kava might be directly associated with liver damage, although speculation about the cause of liver injury in even these few cases is not scientifically supportable in the absence of more complete information. Each of these possible cases appear to have been hypersensitivity or idiosyncratic based responses. There are several other reports which have weak associations with kava consumption and many reports which have inadequate information to make any rational assessments." [155]. Some attributed potential hepatotoxicity to poor sourcing practices; i.e., purchase of the least expensive but less desirable variety of kava or manufacture of the extract from the stems rather than the root. Regardless, the incidence of alleged negative events is extremely small relative to other anxiolytics, at best such an effect is idiosyncratic and rare, and at worse, poor sourcing practices may have contributed to the case reports and is correctable. Rather, despite very weak evidence of harm, two of the most important botanicals in the materia medica, whose efficacy is well documented, were removed from the market nationally or internationally.

In contrast, the common over-the-counter drug acetaminophen (paracetamol) is one of the most common drugs associated with acute liver failure, an effect that has been known since at least 1966, yet it has remained on the market despite it causing approximately 46% of the approximately 2800 cases of acute liver failure that occur in the US annually, 20% of which results in death [47]. In 1993, 47.8% of all overdoses involved paracetamol or paracetamol-containing drugs, making it a primary cause of liver failure in the UK [110], though restrictions in prescribing practices there reduced this incidence [70]. National policies regarding herbal medicines do not usually involve formal benefit to risk assessments. Rather, even small risks are often not tolerated, such as in the case of kava and ephedra, while it is acceptable for a drug such as acetaminophen to be associated with 50% of the world's liver failures and for pharmaceutical medications to be the leading cause of death in many countries.

FIGURE 3.9 Kava (*Piper methysticum*) the legendary anxiolytic herb of Polynesia. Anxiolytic activity of kava is supported by both the traditional and scientific literature. Concerns over alleged hepatotoxicity lead to the international ban of this plant despite numerous formal toxicity reviews and studies attesting to its safety when used appropriately, eliminating a safe and effective botanical medicine from the modern herbal materia medica. *Kava plant and kava bowl: Roy Upton, Soquel, CA; Kava roots: Lynette Casper, American Herbal Pharmacopoeia, Scotts Valley, CA.*

In addition to the loss of an effective anxiolytic, there are other far-reaching consequences to cultures and economies when such a ban occurs. Prior to the kava ban, annual production of kava throughout the Pacific Island islands was approximately $200 million annually. After the ban in 2002, revenues from the sale of kava in Fiji, Vanuatu, Tonga, and Samoa decreased between 75 and 98%. At the time, kava production was one of the few profitable industries throughout Polynesia [130,131]. This illustrates the larger role that plants play in human health, ecology, culture, and economies.

Policies regarding herbal medicines tend to focus predominantly on supply and manufacturing issues, with relatively little resources expended on human clinical trials, and fewer resources expended to investigate botanicals medicines in the manner in which they were traditionally used, as part of a multifaceted healing program that included dietary, stress, and behavioral modification. Too much emphasis is placed on studying

herbal drugs in the same way that conventional drugs are studied, thus, often investigating the herbal drug out of the cultural and medical context in which it was historically used.

3.5.1 Pharmacognosy's Separation from Traditional Herbal Medicine

"To talk about pharmacognosy is to follow the evolution of man's knowledge during the various civilizations, i.e. the evolution of mankind from the dawn of time to the present." [32]

Historically and until relatively recent times, pharmacognosy, the study of drugs derived from natural products, represented an intersect between traditional herbal medicine, ethnobotany (the traditional and cultural use of plant medicines and textiles), and scientific inquiry. In ancient times, the predecessors of modern pharmacognosists, represented by early medical scholars such as the Greek physician Dioscorides (CE first century), were more closely linked with the practice of medicine than their descendents of the twentieth century, many of whom were influenced by the chemical revolution taking place at the time and became more aligned with the business of drug development rather than the practice of medicine.

With its formal beginnings in the writings of the Austrian professor of medicine Johann Adam Schmidt (1759–1809), the original focus of pharmacognosy was on ensuring the identity, quality, and purity of plant-based drugs [135]. A principle focus at the time was on the source and quality of the raw material used in the manufacture of herbal drugs. This was prior to the advent of modern analytical chemistry and so the morphological and organoleptic characters of the crude plant drug, and later, the cellular structures of the plant as observed with a compound microscope, were the primary analytical tools used. As analytical chemistry and the manufacture of synthetic drugs advanced, the science of pharmacognosy evolved into the field of pharmaceutical biology with an emphasis on natural products chemistry, molecular biology, and biotechnology. The modern pharmacognosist, rather than applying the skills of classical botanical pharmacognosy to ensure the identity, quality, purity, and integrity of plant drugs, which were being replaced by chemical drugs, was employed to search for novel compounds that could be exploited as drugs for commercial purposes. This change in scientific focus paralleled the changes in the medical profession described previously. According to a 1941 article in the magazine *Science*,

"The desertion of the study of vegetable drugs soon became almost complete…at the present time researchers dealing with plant medicinals are relatively rare and are becoming more so. Today is the heyday for organic synthetic chemicals." [59].

3.5.2 The Warp and Woof of Ancient and Modern Medical Thought

The focus on the germ theory in Western medicine as the primary cause of pathology paralleled earlier medical philosophies of China's Zhang Ji (Zhang Zhong Jing; ~CE 150–210) in his *Shang Han Za Bing Lun* (*Shang Han Lun; Treatise on Cold Damage Disorders*) that recognized pathogenic factors as a cause of epidemics. Germs and their destruction and the subsequent advent of antibiotics, an incredible life-saving but one-sided facet of disease treatment, became the primary focus of twentieth century medicine that employed a militaristic strategy to search and destroy disease that has persisted. This is in stark contrast to the vitalistic principles of Chinese and Ayurvedic medicine, whose primary focus was and is on the health of the host, and on the earliest doctrines of Western medical practice of *first do no harm*. The earliest text of TCM, the *Neijing Suwen* (475 BCE–CE 221) records:

> Uneducated practitioners behave very aggressively, believing they can launch an attack. Before the old disease has ended, a new disease emerges in addition. [150].

The myriad of serious negative side effects that are an inherent part of modern pharmaceutical therapies, as reflected in increasing rates of iatrogenesis, provide ample evidence of the consequences of aggressive treatments. According to traditional systems of healing, a healthy host, it is reasoned, is less susceptible to external pathogenic influences and has the greatest chance of health restoration, for example, from chronic degenerative diseases, for which modern medical constructs have little to offer. This realization was articulated in the historical TCM literature of Chinese herbalist and acupuncturist Li Dongyuan (CE 1180–1252). Li broke from the earlier traditions of the *Shang Han Lun* that focused on external pathogenic factors as the cause of disease and noted that if a person's core health, as embodied in the health of the spleen and stomach (*bu tu pai: earth engendering school*), is strong they will have resistance from external invasion no matter how strong the pathogen [174]. This was a revolutionary concept in medical history that has yet to be practically applied in Western medicine. Most importantly, these two concepts, the *shang han* and *di sheng* traditions, along with several other principle philosophies (e.g., *yin-yang*, *wei qi ying xue bian zheng* (4 levels), *qi qing* (7 emotions), and *ba gang* (8 principles), among others) reflect a system that embraces a myriad of medical philosophies that are applied for an individual patient. This is in stark

contrast to the single-minded focus of western medicine on a pathogen and pathology.

As modern pharmaceuticals increased in isolation, potency, and pharmacological exactness, herbal medicines largely vanished from use in Western countries. Conversely, Ayurvedic and traditional Chinese medicine remained relatively strong up until the twentieth century. There were periods in the history of China and India (e.g., in India while under British rule) when traditional medical practices were formally discouraged in favor of European medicine. However, for example, after decades of influence of western medical theories in China, traditional Chinese medicine practitioners began to identify deficiencies in western theories and argued that traditional Chinese medicine had much to offer the totality of medical care and began a process of integrating Western and traditional Chinese medical theories [22] that continues today. Subsequently, as of a 2005 survey conducted by the WHO, 1242 herbal medicines were listed on China's essential drug list [164]. Similarly, after the end of British rule in India, Ayurveda once again regained its popularity and today, there are more hospitals of Ayurvedic care than there are conventional Western medical hospitals and the government actively supports the development of Ayurveda, as does the Chinese government for TCM. There are approximately 960 plants species used by the Indian herbal industry [128].

Herbal medicine in Europe experienced resurgence for more practical reasons; after World War I, a number of drug patents, such as aspirin and heroin, were lost to the Allied Forces under the Treaty of Versailles [72]. Similarly, during World War II, much of the infrastructure for drug development was destroyed in major cities throughout Europe. This forced many, most notably Germans, to once again look at traditional herbal medicines and began a trend of cultural use and research that has persisted to the present day.

While herbal medicine in the US survived in pockets of minority groups (African Americans and Caucasians from the rural south, certain Christian sects, Mormons, Native Americans, etc.), a true resurgence did not occur in the US until the 1960s with the back to nature "hippy" movements occurring then. This movement was overlaid with experimental use of a variety of hallucinogens, a number of which were of plant origin (e.g., *Psilocybe, Datura, Lophora, Cannabis*). At the same time, America was introduced to all the *isms* of the rest of the world such as Buddhism, Confucianism, Daoism, Hinduism, shamanism, all of which emphasize an individual's relationship within the context of all else that lives, ancestors, and future generations. This was in contrast to the dominant Christian constructs that suggest that "man has dominion over the earth," a philosophy rejected as new thoughts from the east and from Native Americans were introduced. This partially established in modern youths the philosophical foundation of nature being the primary healer and the recognition or belief that humans and plants have coevolved and, because of that, plants were the optimal form of medicine for human health; a philosophy firmly held by many herbalists and natural health care practitioners today.

3.6 STANDARD OF HERBAL DRUGS IN EARLY PHARMACOPOEIAS

The term pharmacopoeia is derived from the Greek *pharmakon* (meaning medicine or charm) and *poien* (meaning to make). Thus, any of the earlier works of materia medica, which attempted to codify the preparation of medicinal ingredients or formulae, can be considered the antecedents of today's pharmacopoeias. In this regard, China's *Shen Nong Ben Cao,* Egyptian papyri (e.g., *Eber's Papyrus*), the *Caraka Samhita* of India, and the early Greek and Roman works of Theophrastus, Dioscorides, and Galen all represent such works. However, over time, pharmacopoeias came to encompass consensus documents generated by groups of physicians or pharmacists, versus individual authors, and were given formal authority by governmental bodies. Sonnedecker [139] defines the modern concept of pharmacopeias as containing "pharmaceutical specifications that are intended to secure uniformity in the composition, quality, and therapeutic activity of medicines and that are made obligatory within a political unit by legally effective authority."

Historians differ as to the earliest of formal works of medical authority, but perhaps the first such consensus standard was *Nuovo Receptario Composito*, a book of medical ingredient standards commissioned by the guild of physicians of Florence and made official in Florence in 1498 [50]. Fifty years later in 1548, the actual word *pharmacopoeae* appeared in *Pharmacopoeae Jacobi Sylvii libri tres* of French physician Jacques [34], but the term was used only in its generic sense. The first officially sanctioned work with the word pharmacopoeia was the *Pharmacopoeia Augustana* of 1601 [139]. Other pharmacopoeias soon followed establishing the model for official pharmacopoeias.

Early pharmacopoeias (e.g., *Pharmacopoeia Augustana* 1622 (Figure 3.10); [116]) were predominantly books of recipes for extracts, syrups, and oils and provided guidance for relative standardization of medicinal preparations, many of which were Galenical, relatively crudely extracted, herbal preparations.

Over time, pharmacopoeias (e.g., British Pharmacopoeia [19]) introduced standards of identity and quality for individual herbal medicines. Many of these reflected

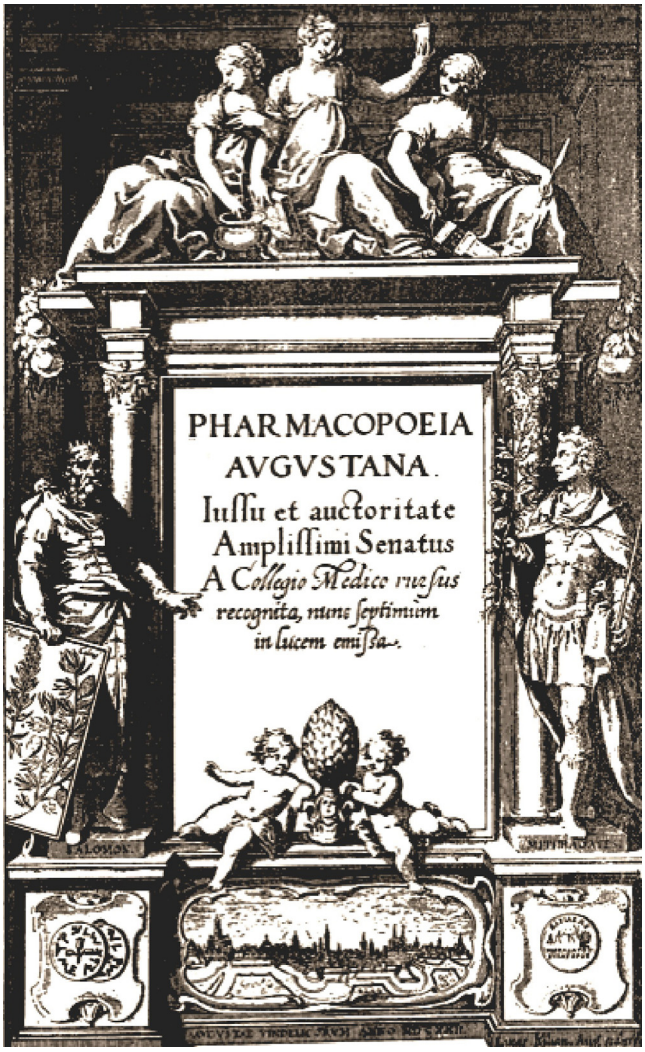


FIGURE 3.10 *Pharmacopoeia Augustana* (1618). The *Pharmacopoeia Augustana* was the first officinal pharmacopoeia in which the word Pharmacopoeia was used in the title. Many of today's botanical medicines were included in this and early pharmacopoeias. *Roy Upton, Soquel, CA.*

relative generality, sometimes not specifying plant species (e.g., acacia) but notably included information regarding the identifying morphological characters, origin, collection practices, and drying conditions of the drug material, clearly the primary considerations of early herbalists and plant collectors. Another early pharmacopoeia, the *Dispensatorium Lippiacum* [134], introduced organoleptic characterizations and the use of magnifiers as part of the assessment criteria for plant medicines. Interestingly, the *Dispensatorium Lippiacum* (1792) was described as "predominantly addressing handcraft savvy pharmacists in small towns" [48], a description that could readily be applied to numerous traditional herbal extract (tincture) manufacturers in the US today.

In addition to being predominantly recipe books of relatively crude herbal preparations, some of the earliest of formal pharmacopoeias (e.g., *Pharmacopoea Augustana*) attempted to resist the ever-growing movement to use Paracelsun "chemical" medicines. According to history of pharmacy scholar George Urdang (1882–1960), in a decree of the Augsburg Senate in 1582, the apothecaries (druggists) were "admonished" not to prepare or offer for sale "substances which are known to be detrimental or poisonous, such as *Labdanum minerale*, the so-called antimony, also *Turpethum minerale* and other purging mecurials." Many of the precepts of the Paracelsun use of powerfully acting drugs were in direct opposition to the empirical lineages of Galenists and Hippocratists. However, soon after, in 1589, the London College of Physicians proposed that the chemicals, salts, extracts, and metals of Paracelsus be included in the *London Pharmacopoeia*. This was eventually realized in 1618 with the inclusion of calomel (mercury chloride) among the medications for internal use. While the juxtaposition of Paracelsun ideologies and those of the Galenists were in great part an attempt of the Paracelsuns to not blindly follow the empirical philosophies of the ancients, and were very much attempting to apply scientific experimentation in medicine, Galenists were alarmed at what they saw as the introduction of highly poisonous substances that had no basis of safety or efficacy into medical practice. As noted, this trend laid the groundwork for modern drug development that persists today.

Another development marking the distinction between traditional and modern drugs was the introduction of chemical testing methods into pharmacopoeial monographs. Prior to analytical chemistry, the focus of pharmacopoeias and materia medicas was on morphology as the primary identity test and proper harvesting conditions and organoleptic characters as the primary criteria by which herb quality was assessed. The *Pharmacopoea Wirtenbergica* (1741; Ref. [48]) was the first pharmacopoeia to introduce chemical testing of drugs, a trend that continued as analytical chemistry advanced and chemically characterized drugs became dominant. Whereas the earliest pharmacopoeias and materia medicas reflected a relatively herbal-botanical approach to the assessment of herbal medicines, later Western pharmacopoeias, persisting to the current day, established chemistry as the primary marker of herb quality.

3.6.1 Identity, Purity, Quality, and Testing—The Foundation of Pharmacopoeial Standards

Pharmacopoeias codify the standards of identity, purity, quality, and testing for herbal drugs, traditional and

modern. Generally speaking, if a pharmacopoeia does not provide a monograph for a specific drug then the drug cannot be traded commercially. Most pharmacopoeias worldwide are relatively consistent in the fields of information they provide. The primary principle of pharmacopoeial monographs is to ensure the proper identity of the herbal ingredient to be used in a drug and develop minimal standards of quality and purity so that when a pharmacopoeial-grade drug ingredient is used it will deliver the intended activity. Some pharmacopoeial monograph standards are consistent with the herbal assessment practices employed by traditional herbalists. Others are a result of the ever modernization of herbal medicines including the requirement for chemical testing that often overshadow traditional assessment techniques as discussed below. Most pharmacopoeias lack information that is valuable in procuring a high-quality herbal medicine. While most monographs create a minimum standard of acceptance, they fall short in helping to establish an optimal standard. Most importantly, over-reliance on chemical technologies can result in impediments to traditional herbal medicine use. In most all cases, the interface between the medical practitioner, the classical botanical pharmacognosist, and the resultant pharmacopoeial standard has been largely lost creating a significant disassociation between the manufacture and practice of medicine, a connection that traditionally was much more integrated.

3.6.2 Pharmacopoeial Standards

As noted, pharmacopoeial botanical drug monographs outline the standards of identity, purity, quality, and testing required to ensure the consistency of botanical drug ingredients. Additionally, these standards form the qualitative basis of the regulatory framework for botanical drug approval and oftentimes, availability of herbal drugs to the public through public health care systems. Pharmacopoeial drug monographs are generally officially accepted by the regulatory officials of a specific country. However, approximately 74% of countries do not have their own pharmacopoeias. Of countries lacking their own pharmacopoeia, approximately 56% utilize the monographs of the European Pharmacopoeia or USP; approximately 30% utilize no pharmacopoeia; and, perhaps most telling, 78% of countries do not include herbal medicines in their national drug lists [164]. In both developed and developing nations, unless provisions for access to nonofficial plant drugs are in place, or at the very least not restricted, availability to traditional herbal drugs can be impeded, a situation currently in place in the US where there is no formal regulatory allowance of traditional herbal medicines (there is a level of regulatory discretion applied to TCM and naturopathic practitioners).

The following fields of information are contained in the majority of pharmacopoeial monographs worldwide. Different pharmacopoeias give greater or lesser emphasis to certain fields of information over others. Compliance with monographs in developed countries, as most represented by the pharmacopoeias of the European Union and the United States, requires adherence to all aspects of the monograph, a requirement that fosters redundancy and, in some cases, unnecessary testing, all of which drive the cost of traditional herbal medicines up.

3.6.3 Pharmacopoeial Definition

Most monographs begin by defining the medicinal substance. This definition establishes the identity of the material being used and oftentimes, the minimum quality of the ingredient to be used, as determined by the presence of a specific quantity of a chemical compound as noted in Table 3.2.

The pharmacopoeial definition is often the first body of information that establishes that a chemical test is required for acceptance of the herbal drug. Chemical testing is especially relevant when most of the activity of the botanical can be assigned to a specific constituent, when there is a narrow therapeutic to safety window of the drug, or when the medicinal qualities of the plant cannot be discerned through sensory analysis. Generally speaking and specifically regarding relatively safe herbal drugs, chemical testing has great value as a complement to organoleptic assessment. However, analytical chemistry does not take the place of ensuring optimal times of harvest and proper drying, processing, and storage conditions. Chemistry can more fully inform these practices, but in itself is not sufficient to render analytical techniques such as botany, microscopy, and sensory evaluation obsolete (see *Macroscopic and Sensory (Organoleptic) Evaluation* below).

TABLE 3.2 Pharmacopoeial Definitions of St. John's Wort (*Hypericum perforatum*)

European Pharmacopoeia (EP)	Whole or cut, dried flowering tops of *Hypericum perforatum* L., harvested during flowering time. Content: minimum 0.08 per cent of total hypericins, expressed as hypericin ($C_{30}H_{16}O_8$; Mr 504.4) (dried drug).
United States pharmacopeia (USP)	St. John's wort consists of the dried flowering tops or aerial parts of *Hypericum perforatum* Linne' (fam. *Hypericaceae*), gathered shortly before or during flowering. It contains not less than 0.04 percent of the combined total of hypericin ($C_{30}H_{16}O_8$) and pseudohypericin ($C_{30}H_{16}O_9$) and not less than 0.6 percent of hyperforin ($C_{35}H_{52}O_4$).

3.6.4 Macroscopic and Sensory (Organoleptic) Evaluation

All pharmacopoeia monographs include a morphological description of the plant drug under various descriptive headings such as: *Identification in the European Pharmacopoeia; Botanic Characteristics* in the *USP*; and *Description* in the *Ayurvedic Pharmacopoeia of India and Pharmacopoeia of the People's Republic of China*. These sections primarily give the relative form, size, shape, and physical features of the crude plant drug. These were the primary criteria used by traditional herbalists and early pharmacognosists in discerning the authenticity of the plant drug material (see Figure 3.11).

Great emphasis was similarly placed on plant morphology in the numerous pharmacognosy works of the nineteenth and early twentieth centuries (e.g., Refs [46,92,132]; etc.). Detailed instruction was provided of how an appropriate macroscopic/morphological assessment was to be performed on various plant parts. Today there is a plethora of references (e.g., Refs [6,180,181]) on the morphological assessment of crude plant drugs that provide a scientifically valid means for identifying a large number of medicinal plants to determine conformity with pharmacopoeial identification specifications.

From an evolutionary perspective, tastes are either aversive or desirable and inform human decisions about what and what not to eat. At a very basic level, the sweet flavor typically identifies high-energy foods; strongly bitter and or strongly acrid tastes are undesirous and may indicate a poison. In herbal medicine, the tastes represent specific medicinal properties that have been codified and integrated into a unified system of therapeutics and materia medica.

Organoleptic assessment was, historically, and among herbalists today, the most important testing modality for determining the relative quality of a crude plant drug. Organoleptic or sensory evaluation of plant material includes an assessment of the taste, aroma, texture, and sensation experienced by the assessor and requires a specialized skill set akin to those required by food scientists and sommeliers, but to a much lesser degree. From a botanical medicine sense, taste is broadened to include the sensation of texture and mouth feel. Taste arises when chemicals in foods (and herbs) react chemically with receptors on taste buds (gustatory calyculi) on the tongue, mouth, and throat. The tongue is covered with 2000–5000 taste buds, each one containing 50–100 taste receptor cells. In modern food sensory evaluation, five basic tastes are recognized: sweet, sour, salty, bitter, and umami. This latter umami taste was defined by the Japanese to describe the "savory" flavor of the commonly used food flavoring monosodium glutamate. There are relatively few herbs that possess this flavor and so its relevance to organoleptic assessment of botanicals is limited. More relevant are the tastes recognized in traditional herbal medicine systems, namely, sweet, sour, bitter, salty, pungent, and in Ayurveda the addition of astringent, which is more accurately a mouth feel, but nevertheless, regarded as a taste for purposes of describing herbal actions.

Prior to the advent of chemistry, traditional herbal systems developed a very sophisticated system of

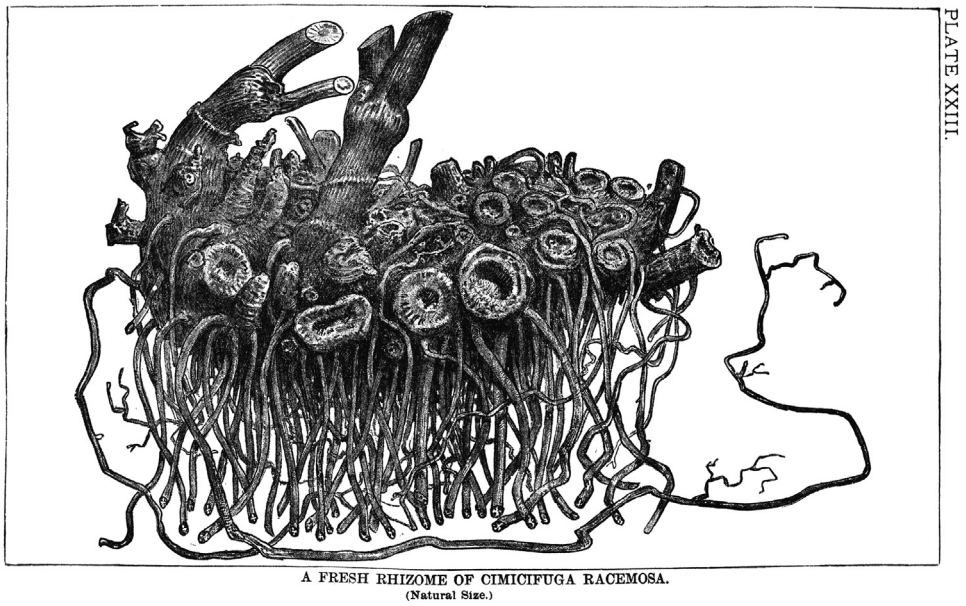


FIGURE 3.11 Macromorphological images of crude medicinal plant parts. Root and rhizome of black cohosh (*Actaea racemosa*). Lloyd JU, Lloyd CG. 1931. Cimicifuga racemosa. *Bulletin of the Lloyd Library of Botany, Pharmacy and Materia Medica 9 (30 Pt 2):224–88.*

sensory assessment that formed the didactic basis of their understanding of the pharmacology of the herbs. These systems linked sensory qualities found in nature with sensory qualities of pathology and health (hot, cold, moist, dry, etc.) and then correlated those with the sensory quality of plant drugs (cool of menthol, heat of cayenne, moist of slippery elm, etc.).

The *Neijing* (Inner Classic), the foundational text of Chinese medicine (dated variously as originating 475–221 BCE or 206 BCE–CE 220), for example, writes:

> *Xin neng san, neng xing (Acrid can disperse, can move);*
> *Gan neng bu, neng huan, neng he (Sweet can build, can slow, can harmonize);*
> *Ku neng xie, neng zao, neng jian (Bitter can drain, can dry, can make firm);*
> *Suan neng shou, neng se (Sour can gather, can astringe);*
> *Xian neng xia, neng ruan (Salty can descend, can soften);*
> *Dan neng shen, neng li (Bland can leech, can benefit).*

The *Neijing* further states:

> These five tastes, respectively…each possess their respective benefits. …depending on the four seasons and five zang organs, diseases are to be treated through the indicated five tastes…acrid enters the lungs; salty enters the kidneys; sour enters the liver; bitter enters the heart; sweet enters the spleen. [8].

Flavor as the basis of traditional Chinese pharmacology was underscored by later Chinese physicians such as the Ming Dynasty physician *Zhang Jingyue* (1563–1640) who in his *Jingyue Quanshu (Complete Works of Jingyue)* wrote:

> The way of using herbs, there is one way, there is no other. That is to master the herb's nature and flavor, and to understand its yin and yang. Only with this understanding, even if the herbs are many, can you achieve desired results. [109].

Over time, herb compatibility (mixing of more than one herb) was studied and codified, and after this, incompatibilities, and herbal formulas, a clinically based study of medicine and pharmacology that evolved over two millennia, and continues to evolve using the same foundational principles.

Tastes contribute part of the sensation of foods and are complemented by aroma detected by olfactory epithelium receptors; texture detected mechanically such as through nerves in muscles; temperature through thermoreceptors; and sensations of coolness (peppermint leaves or oil), heat (cayenne, black pepper), and pungency (cloves, ginger root) through chemical sensations detected by skin and mucous membranes (chemesthesis) (in TCM referred to as the "nature" of the herb). In some cases, the tastes directly correspond to medicinally active constituents as established in western science, such as menthol from peppermint; capsaicin and piperine from cayenne and black pepper, respectively; and eugenol and gingerol from cloves and ginger root, respectively. For example, in addition to the oral cavity, bitter taste receptors are expressed in neuroendocrine cells of the large intestine, chemosensory cells of nasal epithelium, human airway cells, and even the brain (brainstem, cerebellum, cortex, and nucleus accumbens) [138]. Menthol is an active constituent in topical analgesics and acts by triggering cold-sensitive TRPM8 receptors in the skin. It is through this mechanism that the familiar cooling sensation when inhaled, ingested, or applied topically are experienced [35], while menthol's analgesic properties are mediated through a selective activation of κ-opioid receptors [51]. The yellow color and bitter taste of the Chinese herb *huang lian* (*Coptis* spp.) [181] and the North American goldenseal root (*Hydrastis canadensis*) are correlated with berberine content, the herbs' putative primary antimicrobial constituent; the mucilaginous content of herbs such as slippery elm (*Ulmus rubra*), flax seed (*Linum usitatissimum*), and psyllium seed (*Plantago ovata*), as determined by the swelling index included in most pharmacopoeias, is correlated with the soothing and moistening nature of the herbs; the bitter flavor of *Ganoderma* species is correlated with immunomodulating triterpenes [83,106]; the numbing effect of *Echinacea angustifolia* root experienced when chewed is correlated with isobutylamides, among the most biologically and pharmacokinetically relevant of echinacea's immunomodulatory constituents [167]. These are only a few examples where there is a clear association between flavors and known active constituents and pharmacological effects.

Most of all traditional herbal healing systems use taste and the organoleptic qualities of plant drugs as the foundational principle in understanding the plant's physiological effects. In Ayurveda, this is codified as *dravyaguna* (pharmacology) comprised of the six *rasas* (primarily regarded as tastes), *guna* (physical properties), *veerya* (temperature or potency), *vipaka* (post-digestive effect), and *prabhav* (specific or unique action). Western classical herbal traditions utilized similar nature-plant-physiology-pathology correspondences. Avicenna in the introduction of his *Canon* states that "taste is the most precise indication of the nature of the drug" and is part of a larger system, as in TCM, where the tastes, actions, and the nature or "temperament" of a drug are directly correlated with the primary elements of nature, namely air, fire, earth, and water, and are further correlated with physiological states of health and disease [10]. Indigenous healing systems similarly used taste and the physical nature of the herb to guide prescriptions.

While the aforementioned basic taste-chemistry correspondences are simplistic and not specific to disease complexes from a Western medical approach, they

form the basis of pharmacology that is completely unified in the diagnostic system of traditional systems of healing. Similarly, while herbalists historically did not specifically know what compounds were correlated with what activity, like the WHO, they recognized that it was the whole herb that was considered the active ingredient and that the quality and action of the herbal drug were discerned, and often directly correlated to, its sensory characteristics. These relationships were then either proven or disproven through experience and refined over centuries. In Ayurveda and TCM, this system of sensory relationship between nature, humans, and medicines is still actively applied by almost all herbal practitioners. It is this very system that uniquely correlates qualities of nature with qualities possessed in plants; qualities reflected in anatomy, physiology, and pathology; and qualities of an individual's environment, creating a unified system based on natural principles of human health and plant evolution that is, itself, a microcosm of nature. This is another stark differentiator between traditional and modern systems whose diagnostic systems seldom look at the nature of the person but rather defer the understanding of human health and pathology to mechanical diagnostic equipment that then generates, often, arbitrary values that represent only the symptoms experienced by a human but not the human experience. Consumers experience benefits from traditional healing systems precisely because those systems offer a paradigm that is different from conventional medicine and addresses a universal need to restore the human aspects to human health.

3.6.5 Can We Integrate Traditional Knowledge and Modern Science?

For quality control purposes, sensory assessment of crude drugs was codified into the historical herbal, materia medica, and pharmacognosy literature and is a critical part of the herbal medicine assessment process that has been almost completely discarded in modern times, far overshadowed by chemistry first, and now by metabolomics and genetics. To the modern pharmacognosist or regulator, relying on organoleptic assessments for determining the quality of an herbal drug may appear to be outdated considering modern analytical techniques that can quantify to parts per million or billion. From a modern pharmacognosy perspective the correlation of medicinal effects with sensory characters of plant drugs is a potentially large area of interest for future research in helping to correlate traditional herbal pharmacology with western pharmacology.

Despite the importance of organoleptic characteristics as a basis of traditional herbal medicine, these characters are seldom given in Western pharmacopoeias (e.g., *European Pharmacopoeia, USP-NF*) but do persist in Ayurvedic and Asian pharmacopoeias (e.g., *Ayurvedic Pharmacopoeia, Pharmacopoeia of the People's Republic of China, and Japanese Pharmacopoeia*). Consistent with historical practices, conformity with organoleptic characteristics in these references is considered part of the acceptance criteria for herbal drugs. Like the morphological assessment of crude drugs, texts that focus on classical botanical pharmacognosy provide detailed guidance on how to conduct an appropriate organoleptic assessment and also provide the morphological and organoleptic characteristics of hundreds of plants. The importance of developing the skills needed for assessing crude drug quality was underscored by the highly respected Eclectic pharmacist John Uri Lloyd who stated:

> The crude drug is the foundation of the pharmaceutical preparation…. The [pharmacognosist] must be able to judge of the intrinsic qualities of drugs. This last is the most important part of the art of pharmacognosy, for while it is easy to learn to identify different drugs it is difficult to obtain the experience necessary to judge of quality shades…. The study of crude drugs is most important.

The importance of macroscopic assessment of plant parts is equally underscored by the WHO in their *Guidelines for the Assessment of Herbal Medicines*, which states:

> Visual inspection provides the simplest and quickest means by which to establish identity, purity and, possibly, quality. If a sample is found to be significantly different, in terms of color, consistency, odor or taste, from the specifications, it is considered as not fulfilling in the requirements. [159]

Applying western standards of either chemical or pharmacological assessment to traditional herbal medicines can inform and increase our knowledge base of the botanical, its optimal time of harvest and processing conditions, pharmacology, purity, and safety. However, the majority of the herbs used today have been in continued use for several hundred years, often used in exactly the same manner as herbalists and physicians did historically, before the advent of modern pharmacology and chemistry. Attempting to regulate or restrict traditional herbal healing systems because they do not fit into the typical western paradigm is an antithesis to the healing system itself. From a traditional herbal medicine perspective, preservation, development, and acceptance of morphological and organoleptic assessment skills are critical in preserving traditional medicine practices and are worthy of modern scientific investigation within the context of the medical system in which those qualities exist and the attributes assigned to those qualities.

Regarding traditional medicine practice, there is some protection of traditional herbal medicine principles internationally. In most countries that regulate herbal

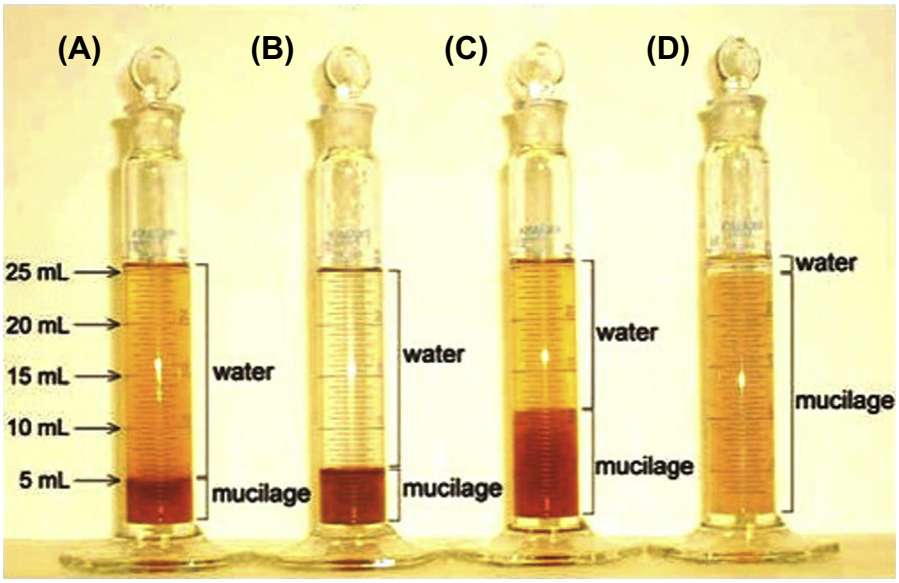


FIGURE 3.12 Slippery Elm (*Ulmus rubra*) mucilage swelling index. Simple tests such as the *swelling index* are used for quantifying mucilage, the mucilage historically correlated to the nature or physical quality of a botanical drug. *Upton R, editor. Slippery Elm monograph. Am Herbal Pharmacopoeia 2013. Scotts Valley, CA.*

medicines (e.g., much of Asia, the European Union, and India), herbal medicine practitioners are exempt from standard manufacturing GMPs. This is not the case in the US, where FDA maintains the authority to require practitioners to be in full compliance with standard dietary supplement manufacturing GMPs, an authority that threatens the perpetuation of traditional herbal healing in that country. As recognized by the WHO and European Union, the whole crude plant part is considered to be the active ingredient, not a specific amount of a compound(s) that can be quantified. Macroscopic and sensory evaluation of herbal ingredients, followed by therapeutic experience, is the primary means by which traditional herbal practitioners can continue practicing their traditional healing systems as outlined in the classical medical literature. Not recognizing traditional herbal assessment principles in formal pharmacopoeia limits the expression and evolution of traditional herbal medicine, and instead, pushes herbal medicines solely into a western pharmaceutical paradigm.

3.6.6 Histology and Identification (Microscopic Characterization)

Just prior to the advent of modern analytical chemistry, microscopic examination of crude herbal drugs, along with gross morphological and organoleptic assessment as described above, were the primary tools used for crude herbal drug assessment.

European and American pharmacognosists put tremendous value in the ability to identify plants to species microscopically (see Figure 3.13), including those that are closely related, detect adulterations, and, in some cases, even assess relative quality.

According to noted American pharmacognosist Henry Kramer [80],

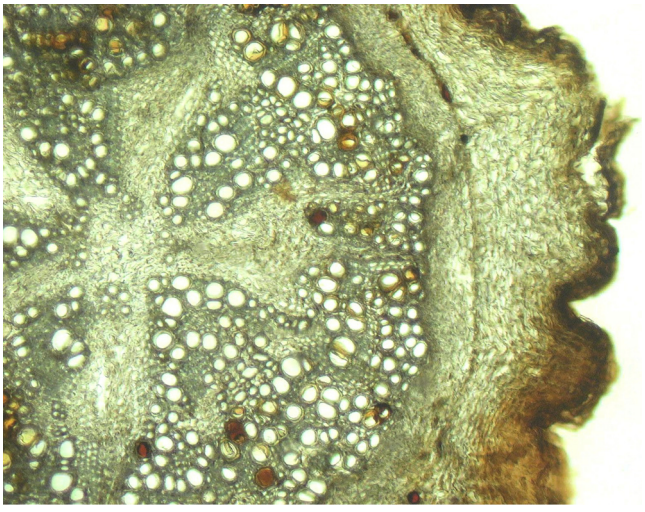


FIGURE 3.13 Image showing the identifying features of a cross-section of *Echinacea purpurea* root as viewed under a compound microscope. Transverse section of the root of *Echinacea purpurea* showing cork, parenchyma, secondary phloem, vascular cambium, secondary xylem, and primary xylem poles just exterior to the pith with secretory ducts filled with orange-brown secretions occurring in a ring along the endodermis between the parenchyma and secondary phloem, all identifying features of the crude plant drug. *Prof. Dr. Reinhard Laenger, AGES PharmMed, Vienna, Austria.*

> The microscope furnishes the surest means of determining the identity of a powdered drug at our command…the microscope also furnishes the most reliable means for detecting and determining adulterants in powdered drugs… [and] detecting the presence of worm-eaten drugs or powders of certain classes of drugs which have been exhausted in whole or in part...

Kraemer went so far as to say that even the time of gathering, method of drying, and length of time for which a botanical had been stored could "be judged in many instances by the use of the microscope." Additionally, microscopy offers a highly sensitive tool in being able to detect adulterating species. For example, many years ago there were numerous reports of the Chinese herb *fang feng* (*Stephania tetrandra*) being mixed up with a different species of plant (*Aristolochia fangchi*), which contains the renal toxin and potential carcinogen aristolochic acid. This adulteration was responsible for several hundred to a few thousand deaths, predominantly in Belgium and France. With microscopic examination, the addition of as little as 0.3% of *Aristolochia fangchi* in a stephania sample can be detected by observation of the differing oxalate crystals that occur within the species [151]. Discerning crude drug species microscopically reached a very high level of refinement and is reflected in numerous seminal texts on the subject in the mid-nineteenth to twentieth centuries, most notably [13] of Germany; Moeller of Austria [99]; Tschirch and Oesterle of Switzerland [146]; and microscopists of the UK and US such as Refs [58,81,93,132,175].

With advancements and evolution of analytical chemical techniques, first with paper chromatography followed by thin-layer chromatography (TLC) and then more quantitative techniques (liquid chromatography, gas chromatography), and finally molecular technologies (e.g., DNA), microscopy, like morphology and sensory assessment, as an analytical tool was regarded as outdated in the face of these more recent techniques. However, seldom is one analytical technique inherently superior to another. The superiority or applicability of one analytical method over another is dependent upon the desired analytical endpoint. From this perspective, microscopy is as scientifically valid of an analytical tool as any other technique and is currently enjoying a resurgence in the US where it had fallen into almost complete neglect in recent decades.

3.6.7 Chromatographic Technologies—Quantitation and Pattern Recognition Identification: Thin-Layer Chromatography

The first of the chemical assessment techniques in most pharmacopoeias, Eastern and Western, is TLC, and more recently, High performance thin-layer chromatography (HPTLC) (Figure 3.14), and is primarily used as an identification assay.

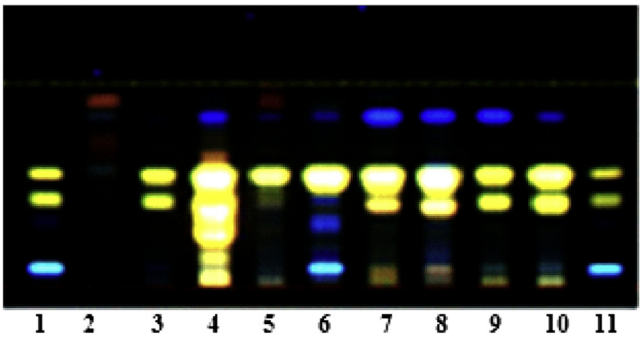


FIGURE 3.14 HPTLC chromatogram of goldenseal (*Hydrastis canadensis*) and its potential adulterants. TLC was one of the earlier techniques of modern analytical chemistry. *Upton R, editor. Goldenseal Root monograph, Am Herbal Pharmacopoeia 2001. Scotts Valley, CA.*

TLC and HPTLC analyses provide a snapshot of the constituent profile of crude plant drugs and have been a standard entry in pharmacopoeias for decades. In previous decades, TLC as an analytical technique was very crude, had a limited degree of reproducibility, was cumbersome, and not very compliant with good manufacturing practices. In more recent decades, the introduction of HPTLC has addressed many of these deficiencies. As a chemical analytical tool, HPTLC is extremely versatile and sensitive for the identification of many crude herbal drugs and is especially useful for the detection of adulterations, often with a very high degree of sensitivity.

However, it must be recognized that any chemical testing is a surrogate for identification. When identifying plant material botanically, macroscopically, organoleptically, or microscopically, it is the actual plant material that is being assessed. With chemistry, it is the constituent profile of the plant being assessed, not the plant material itself. Different herb parts (e.g., goldenseal *Hydrastis candadensis*) may possess a similar chemical fingerprint that is not clearly distinguished through chemical testing. Goldenseal root that has been properly harvested and dried can yield up to 6% berberine [152]. Pharmacopoeial monographs (e.g., American Herbal Pharmacopoeia, USP) require a minimum of 2.5% berberine, the typical minimum concentration of berberine found in commercial goldenseal. Roots yielding relatively high concentrations of berberine can be mixed with either goldenseal leaf material, which contains much lower concentrations of berberine, or other berberine-containing plants and pass most all chemical tests. Identification of plant materials chemically is therefore limited and is most accurate when coupled with appropriate physical tests. For compliance with pharmacopoeia monographs, TLC/HPTLC or

other quantitative assays are conducted in tandem with the physical tests outlined above.

Critical to any chemical testing methodology of medicinal plants is to look at the suite of constituents from the perspective of chromatographic fingerprinting and not only individual markers. The concept of chromatographic fingerprinting was developed primarily by Chinese phytochemists who recognized that the activity of a Chinese herb is in its collection of compounds not just the single active constituent approach typical of modern drugs. Numerous authors provide guidance and perspectives on appropriate ways to apply modern chemistry to the analysis of traditional herbal drugs (Refs [42,171]; among others).

A critical starting point to chromatographic fingerprinting is to obtain multiple samples of the desired botanical that is grown, harvested, and dried in a manner that is optimal for the plant part. Such criteria are traditionally determined organoleptically by evaluating the color, smell, taste, texture, relative purity, and other physical characteristics of the plant. Optimal picking times can be informed by traditional literature, such as harvesting the flowering tops of St. John's wort on the eve of St. John's day (June 24). While the specificity of a single day may not be required, in most growing areas, St. John's day reflects a harvest time when the plant is in full bloom, the traditional time to harvest most flowers; roots and barks are typically harvested in the Spring or Fall; and leaves, prior to flowering before the energy of the plant goes to flower, seed and fruit production, etc. In Chinese herbal medicine, specific herbs must be gathered at a specific age, such as the roots of *dang gui* (*Angelica sinensis*) at a minimum of 4 years of age or Chinese ginseng (*Panax ginseng*) at a minimum of 6 years of age. Optimal harvest times can also be informed by chemical analysis. Specifically regarding St. John's wort, the highest concentration of the suite of St. John's wort constituents including flavonoids, and naphthodianthrones are yielded during the budding and flowering stage (around St. John's day), the concentrations dropping dramatically after flowering [147], thus confirming traditional harvesting practices of herbalists.

Another critical aspect in developing chromatographic fingerprinting methodologies for medicinal plants is to obtain samples of closely related and potentially adulterating species that may be inadvertently traded for the authentic material. Having multiple species of the same appropriate quality material, ideally from multiple regions for multiple years, allows the analyst to observe the variation inherent in natural products due to changing environmental conditions. Having closely related or adulterating species helps the analyst know if the chromatographic fingerprint is robust enough to identify the authentic from substitute species. In China, chromatographic fingerprinting techniques are coupled with chemometric programs (e.g., Ref. [178]). These programs are designed to store and analyze multiple data points from multiple samples and provide statistically relevant comparative fingerprints that allow for developing relatively objective criteria for what constitutes an acceptable chromatographic fingerprint. Similar chemometrics have been more recently applied by analysts at the United States Department of Agriculture (USDA) (e.g., Ref. [64]). Ideally, such determinations should be made based on the available clinical data that establishes the efficacy of the herbal drug being analyzed.

It is important to recognize that much of the basic work in developing appropriate chromatographic fingerprints is best done in academic settings and is a very expensive and time-consuming process that does not allow for the high throughput testing often needed in industry. Once done in academia, such testing criteria can then be codified into pharmacopoeia as a way to impose a consistent standard that is then relevant to the herbal industry at large and herbal practitioner.

3.6.8 Quantitative Assays

In addition to identity tests, most Western pharmacopoeial monographs require quantitative assays, many of which will correlate directly with the pharmacopoeial definition of the botanical drug ingredient and may or may not directly correlate with activity. Quantitative assays are often lacking in Eastern pharmacopoeias (e.g., *Ayurvedic Pharmacopoeia; Pharmacopoeia of the People's Republic of China*). In most cases, quantitative assays will quantify a specific compound that is correlated with a known active constituent or class of constituents. Other compounds assayed are considered surrogate marker compounds for activity, other chosen compounds may reflect a constituent or class of constituents that provide a baseline of "quality" based on harvest or processing practices, which may or may not correlate with activity, and yet others will reflect other measures of "quality," such as an organoleptic bitterness value for a particular botanical such as gentian root (*Gentiana* spp.) or a swelling index (Figure 3.12) as discussed previously regarding mucilage content of slippery elm bark or flax seed.

As repeatedly noted, very seldom is a single constituent correlated with the total activity of a particular botanical. Rather, the total extract or profile of the crude drug is considered the active ingredient or substance. This is reflected in WHO documents [158] and acknowledged by renowned medicinal plant researchers (e.g., see Refs [102,173]). For example, valerian root (*Valeriana officinalis*) is a sedative herb used at least since the first century [117]. The essential oil was long considered to

represent the active fraction. However, early pharmacological work demonstrated that the essential oil fraction was only associated with approximately 1/3 of the total activity of the extract [61]. Later, research demonstrated depressant activity of the dichloromethane extract but activity could not be attributed to either the valepotriates, valerenic acid, valeranone, or the essential oil [82]. To date, the total activity of valerian has not been fully articulated. Today, Western pharmacopoeias require quantitation of the essential oil fraction of valerian as the primary marker of quality. St. John's wort (*Hypericum perforatum*) provides another example of a plant whose efficacy as an antidepressant has been documented over a period of more than 2000 years, but for which the pharmacological mechanisms in humans have yet to be determined [28]. Thus, quantification of a single marker or group of markers should be viewed as representing only a portion of the activity of the whole plant or extract made therefrom.

Regarding quantitative assays, varying pharmacopoeias take different approaches in determining what compound(s) are to be assayed. For example, the pharmacopoeias of Germany and the US tend towards specificity of compounds favoring relatively sophisticated quantitative technologies such as HPLC (Figure 3.15) in contrast to more generic technologies such as spectrophotometry.

Whereas HPLC has greater accuracy in quantifying individual compounds, spectrophotometry groups similar compounds together, often resulting in higher quantitative values as compared to HPLC. Both technologies have strengths and weaknesses. HPLC offers greater accuracy in quantitation; this is its primary advantage. If used appropriately, HPLC can also be used for purposes of identification if the total chromatographic fingerprint of the plant is considered and thus has a greater chance of detecting adulterants. Spectrophotometric techniques, in grouping like compounds, often gives a more accurate representation of the totality of the plant or extract, but has the distinct disadvantage of being easily fooled by admixtures of adulterants. In the US, there are no requirements to follow any monograph standards for herbal supplements. For practical and economic reasons, analysts in botanical dietary supplement manufacturers often attempt to perform a single test for all or most identity and quality testing, often times choosing the most economically feasible assay, but not always the most appropriate. Such testing may or may not provide scientifically valid results for the desired analytical endpoint. In the EU, where there is a legal requirement to follow all aspects of the European Pharmacopoeia monographs, there appears to be a greater use of spectrophotometric methods, which are only employed after appropriate identity of the botanical ingredient has been confirmed and the presence of potential adulterants, through morphological and microscopic analyses, has been ruled out. In the USP, there appears to be greater utilization of specific methods, requiring multiple analytes, greater time, and greater expense. In contrast, quantitative assays are not typically a criterion of Asian pharmacopoeial monographs, where the crude botanical drug is considered the medicinally relevant material and requires conformity with the morphological and organoleptic

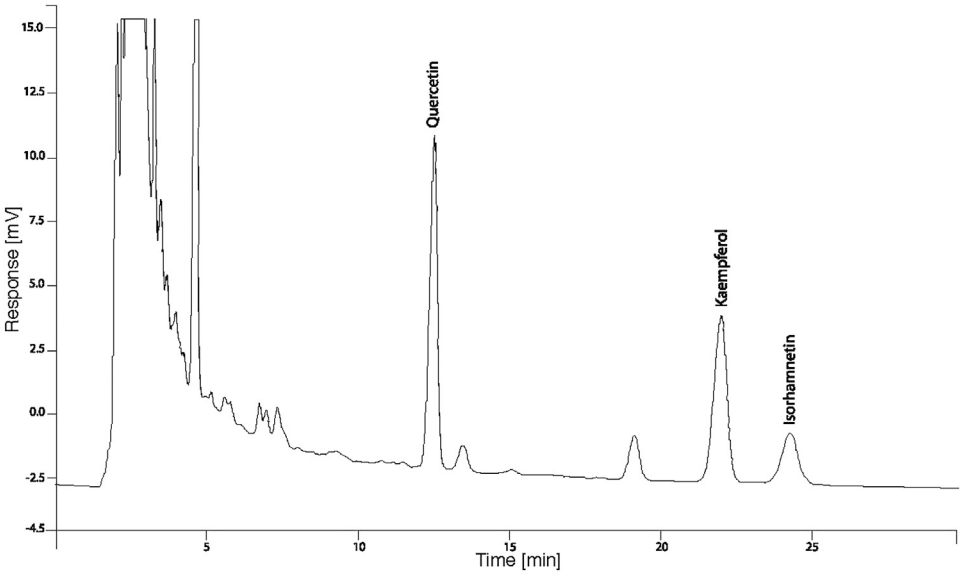


FIGURE 3.15 HPLC chromatogram of *Ginkgo biloba* flavonoids. HPLC is the primary analytical technique used in developed nations for quantification of specific compounds.

characteristics historically attributed to the plant material. The Easter pharmacopeias reflect a more traditional approach to herbal ingredient assessment.

Some Chinese researchers propose a tiered approach to traditional Chinese herbal drug quality control development, recommending "elementary, intensive, and advanced" levels of testing. The "elementary" level reflects current standard requirements of pharmacopoeias that focus on basic identity, qualitative tests, and analysis of a single marker compound. The "intensive" level includes multiple component analysis, including differentiation between "high-" and "low"-quality materials based on quantitative assays of a particular compound(s). The "advanced" level proposes that the multiple component herbal medicine be subjected to formal pharmacological study but acknowledges the inherent lack of Western pharmacological models for assessing traditional Chinese actions of herbs such as to "expel wind" and "nourish kidney" [172]. Similarly, it has been proposed that the comparison of an herbal drug to a properly made well-characterized extract is a more appropriate way to assess the quality of a Chinese herbal drug than to simply assay a single constituent [173]. While these represent more appropriate ways to ensure the integrity of multicomponent ingredients and formulas than the typical Western pharmacopoeial approach, they continue to depend on chemical analysis versus traditional herbal assessment techniques and therefore, do not take into consideration the full suite of traditional assessment techniques.

3.7 OTHER QUALITATIVE FACTORS NOT CONSIDERED IN PHARMACOPOEIAS

3.7.1 Botanical Identification

Botanical identification remains the primary technique for the identification of plants worldwide. While chemotaxonomy and DNA analysis have made great strides in altering the classifications of traditional taxonomy, neither technology has supplanted botany as the primary means of identifying medicinal plants. However, botanical identification is not included as a required test with which to conform in virtually all official pharmacopoeias, the reason being that oftentimes the botanically unique characters of a specific plant are not intact in the crude plant drug.

Historically, different species of a specific genus of plant, and sometimes, different genera of plant, were used as a specific botanical drug. Rather than being referred to by its generic and specific name, e.g., *Salix purpurea*, medicinal plants were described according to their Galenic names, e.g., *Cortex salicis*, the Galenic name referring to the bark of varieties of willow that were historically used interchangeably without differentiation of species. Such a practice is represented in most pharmacopoeias but is increasingly moving towards greater speciation. For example, in the *European Pharmacopoeia*, the bark of several species of *Salix* is allowed as long as the barks conform to the identity tests given and a minimum quantity of salicin (1.5%), the primary putative active constituent and precursor to the development of acetyl salicylic acid (aspirin). Conversely, in the US, where herbs are predominantly traded as "dietary supplements" not "medicines," for purposes of supplement labeling, the names of botanical ingredients must conform to "Herbs of Commerce" of the American Herbal Products Association (AHPA). This text, rather than recommending use of Galenic or more common names (e.g., "willow bark"), requires more precise speciation such as crack willow (*Salix fragilis*), purple willow (*Salix purpurea*), white willow (*Salix alba*), etc. Unfortunately, while conformity to the identification tests of pharmacopoeias for these barks can be assured, most of these barks cannot be identified to species when in their crude form as they are morphologically, organoleptically, chemically, and medicinally very similar, the very reason why the multiple species are used interchangeably. Thus, greater levels of specificity are often required for either academic or arbitrary regulatory reasons creating unnecessary impediments, to continued use of traditional herbal medicines.

3.7.2 Daodi (Region Specificity)

Daodi is a philosophy that is exclusively applied to the development of medicinal substances in Chinese medicine. *Dao* is an ancient Chinese unit of measure, which became applied to the development of districts. *Di* means earth or land and is applied to specific geographical regions. According to Zhao et al. [179], *daodi* medicinal materials are generally defined as medicinal materials produced in specific geographic regions under natural ecological and environmental conditions that are optimum for the growing of the plant, with particular attention to cultivation, harvesting, and processing techniques. *Daodi* is believed to lead to quality and clinical effects that are considered to surpass those of the same botanical produced in a different region. The concept of *daodi* was used by early Chinese herbalists and codified by Sun Simiao in his *Qian Jin Yi Fang* (*Supplement to the Formulas of a Thousand Gold Worth*). According to the venerable Sun:

> When ancient doctors used medicinals they depended on the earth, therefore when they treated ten people they achieved results in nine. Although contemporary doctors understand the pulse and prescriptions, they discard the timing for harvesting

> medicinals. They are not familiar with the originating land or the freshness, age, nature, emptiness or fullness; therefore they only achieve results in five or six cases out of ten.

Sun's teachings underscore the importance of organoleptic assessment and a quality control system that integrates the proper harvest time and source of the originating plant material. Furthermore, in the *Ben Cao Pin Hui Jing Yao (Essentials of Chinese Materia Medica*) of China's Liu Wentai (1488–1505), specific attention was given to medicinal plant development that encompassed the sprouting of the plant, the land on which it was grown, the timing of planting and harvest, and potential substitutes. Thus, prior to the development of formalized good manufacturing practices and the adherence to mandatory pharmacopoeial standards, Chinese physicians recognized the need for strict adherence to quality control in ways that ensured the quality of the medicinal plant. This care is reflected in the many grades of herbs that can be observed in any Chinese herbal pharmacy today.

Many of these principles reflect common sense in ensuring the quality of herbal drugs. Similar principles are being applied internationally and in a myriad of Good Agriculture and Collection Practices (GACP) developed by varying national and international bodies. The People's Republic of China is perhaps most active in establishing good agricultural practices for medicinal herbs.

Similar to Sun's observation in the Tang Dynasty, modern herbal practitioners are often not aware of the quality control needs of herbal medicines, and rather rely on industry to produce quality medicines, when much of the industry lacks the skills necessary to do so. *Daodi* is an important concept to include in the development and selection of herbal medicines and there may be wisdom in codifying such principles in modern pharmacopoeias. According to Zhao et al. [179], among the 500 most commonly used Chinese medicinal materials, approximately 200 are recognized as having *daodi* correspondences. These 200 medicinal materials account for 80% of the total consumption of medicinal materials in China, making *daodi* a very important concept in modern herbal medicine GMPs.

3.7.3 Molecular Identification

Molecular techniques of crude plant identification are not currently included in any pharmacopoeia. Like all analytical technologies, molecular analysis has much strength and some weaknesses in crude plant identification. Among the technique's strengths is its great sensitivity. If the unique identifying primers for the particular target species has been appropriately developed, and the technique used is robust enough to filter DNA from nontarget organisms (e.g., insect fragments, other plant parts, etc.), or prevent rejection due to the presence of acceptable levels of foreign matter (which may also contain DNA) then it is an exceptionally powerful tool for identifying plant material to species and beyond, including discerning the same species that have different genetic lineage, such as American ginseng (*Panax quinquefolium*) grown in the wild in Kentucky, versus the same species from cultivated root stock in Wisconsin, versus that grown in Canada. Another advantage of the technique is that it uses very small amounts of material for analysis. Among its weaknesses is also the technique's great sensitivity. Much of the time, identifying plants to species is sufficient and, when the plant is properly grown, there is little reason to know whether one's chamomile (*Matricaria recutita*) was grown in Romania, Bulgaria, or Poland, unless there is evidence to suggest that specific growing regions are not appropriate for specific botanicals. It is more important from a traditional assessment perspective to know if a given sample possesses the traditional morphological and organoleptic characters of material that has been properly harvested and dried.

3.7.4 Environmental Sustainability of Medicinal Plants

At the heart of most all herbal medicine traditions is a relationship with earth and the natural world. This stems from the inalienable fact that humans are an extension of the natural world, are dependent upon the natural world for their survival, and that what affects the natural world affects human health (e.g., diet, pollution, etc.). This relationship also extends to cultures, some of whom for generations have either cultivated medicinal plant crops or harvested them from the wild and has formed the basis of countless economies worldwide. While excessive harvesting of medicinal plants can have negative environmental effects, cultivation or wild harvesting of medicinal plants when done according to GACP, international standards for which exist (e.g., Refs [1,163]; among others), maintains and fosters environmental stewardship of natural resources, thus ensuring future supplies of medicinal plants.

Juxtaposed against the largely positive environmental impact of medicinal plant trade is the largely negative environmental impact of pharmaceutical medications, which have significant consequences on both human and environmental health. There are two primary sources of pharmaceutical pollution: pharmaceutical production and the pollution generated therefrom through effluence and emissions and the excretion of medications and their metabolites ingested by humans and farm animals.

In the US, a report from the US Geological Survey detected pharmaceutical contaminants in 80% of 139 streams sampled. Contaminants included antibiotics, hypertensive medication, antidepressants, analgesics, reproductive hormones, and other prescription drugs that also affected municipal water [56,107]. The most stark environmental impact of pharmaceuticals has been from endocrine disruptors, partially due to metabolites excreted in urine from oral contraceptive use. A 2007 study reported that 75% of male smallmouth bass in certain areas of the Potomac River basin (US) had ovarian tissue in their gonads resulting in a feminization of the fish [14]. Other studies have shown similar negative effects directly associated with oral contraceptives delivered through municipal wastewaters affecting fish populations (e.g., Ref. [78]). In another study, fish exposed to effluent from a cattle feedlot in the US state of Nebraska experienced reproductive abnormalities, including reduced testes size in male fish and a lower level of estrogen in female fish [111]. Other studies have linked such xenoestrogen exposure to testicular and breast cancer in humans (Ref. [156] and references therein). The US Geological Survey [56] also reported finding nonprescription pharmaceuticals in more than 40% of the municipal water samples tested and prescription and nonantibiotic pharmaceuticals in more than 30% of samples. Moreover, the ubiquitous over use of antibiotics, especially in farm animals, bioaccumulate in soil, potentially giving rise to antibiotic resistance pathogens [107], an event that has contributed to a serious international health care crisis.

Such events are not limited to the US. In Pakistan, the nonsteroidal antiinflammatory diclofenac was linked to widespread die off of vulture populations leading to the listing of three vulture species as endangered (Ref. [7] and references therein). Moreover, the same drug increases the risk of heart attack in humans [77]. A number of surveys in Germany report that 95% of the pharmaceuticals studied are not readily biodegradable, 15% are persistent in surface water, approximately 50% of veterinary pharmaceuticals studied are persistent in soil, and a large number of pharmaceutical substances and metabolites can be found in wastewater and surface water throughout Europe. Standard long-term tests conducted with fish, daphnia, and algae as test organisms revealed effects at pharmaceutical concentrations of less than 1 mg/L, while one study described changes in aquatic organisms at concentrations of less than 0.001 mg/L [36]. There currently exists little health risk assessment data regarding the potential adverse effects from chronic exposure of the myriad of pharmaceuticals that accumulate in the environment. From a traditional herbal medicine perspective, it is incongruent to have a healing system that causes so much toxicity to the environment and contributes so much to human disease as do pharmaceutical medications. Conversely, medicinal plants have coevolved with the environment for millennia and such negative consequences do not exist in healthy ecosystems.

3.7.5 Spiritual Well-Being

Lastly, an often neglected or completely shunned aspect of modern health care including traditional herbal medicine is the role medicinal plants can play in spiritual health. This is largely due to modern medicine's attempt to be scientific, a paradigm that, when applied in a typical manner, tends to divorce human and material existence from any precept of spirit, whether spirit is defined from a creationist or evolutionary perspective, or from a perspective of humanity's relationship with each other, and the relation of humans to the larger world or ecosystem.

In many systems of traditional healing, spiritual well-being is the highest ideal to achieve. In a practical sense, this means an attainment of a sense of peace and harmony with self and one's surrounding world. In Ayurveda, for example, significant emphasis is placed on mental and emotional well-being through the incorporation of meditative practices, prayer (*puja*), and therapies such as *pancha karma* and *shirodara*, designed to promote a long life of physical, mental, and emotional well-being. Numerous herbs figure into a variety of spiritual practices. In India, the herb tulsi (*Ocimum sanctum*) is highly revered and by some is considered the wife of god *Vishnu* (*Vishnupriya*). Tulsi is central in *Tulsi Vivah*, a ceremony marking the end of the monsoon season and beginning of the Hindu wedding season, the rites of which are described in Hindu scripture. Among some regions of India, households will grow tulsi plants and perform ceremonies by circling the plant reciting mantra morning and evening. Also in India, the Hindi avatar *Dhanvantari* is depicted as Lord Vishnu with four hands, holding medical herbs in one hand and a pot containing rejuvenating nectar called *amrita* in another. *Dhanvantari* is also considered the God of Ayurveda. In Western herbal traditions stemming from ancient Greece, the botanical genus *Asclepias* (commonly known as milkweed) is named after *Asclepias*, the Greek God of medicine. Thus, in most all medical traditions, including those in the West, there was an innate relationship between physical and spiritual health that was connected through plant-based medicines.

In TCM, especially in Taoist practices, there was and continues to be great emphasis of aligning one's life with the spiritual will of heaven. In the five-phases (*wu xing*) theory of TCM, each of the five major organ systems is said to be correlated with a spirit that both serves to guide one to one's authentic self and is meant to be cultivated through various spiritual practices. In this regard,

the use of herbs serve to facilitate this cultivation. In TCM principles, the *shen* (spirit or mind) is housed in the heart; the *hun* (ethereal soul) in the liver; the *po* (corporeal soul) in the lungs; *yi* (intellect or ideation) in the spleen, and *zhi* (will) in the kidneys. In turn, the physical manifestations of each of these organ systems affect these various "spirits" (*wu shen*). According to Chinese medical philosophy, the *hun* governs one's nature. It drives and has the ability to nourish one towards their destiny. When in disharmony, the *hun* wanders aimlessly or perhaps violently, and in either case, cannot direct one to their greater good or expression. The *yi* or intellect represents our ability to discern ideas and experiences and to form intent, intent of thought and action. When in disharmony, the judgment of the *yi* is clouded or misguided. The *po* most closely reflects human instinct, our inner knowing despite having all the facts, our gut senses, and is inherently focused on expressing our greatest self in one lifetime. When in disharmony, the *po* can cause us to act on gross human desires without considering the larger consequences of such actions. The *zhi* or will provides (or not) the motivation to accomplish and act on intent. When in disharmony, the ability to act according to what is in the best interest of the five spirits is impaired. Lastly the *shen* or mind (*xin*) is consciousness itself. On a physical level it is our level of peace of mind, while on a more esoteric level it governs our ability to mobilize all the other *shen* to their higher purposes, imparting benevolence, compassion, and love when healthy, and muddled, erratic, or violent thinking and lack of the aforementioned positive qualities when in disharmony. The *shen, yi, po, zhi,* and *hun* are said to be "housed" in the heart, spleen, lungs, kidneys, and liver, respectively and are therefore influenced either positively or negatively by the physical health or disease of these organ systems. As such, herbs and formula that support the health of these systems support the higher goals of spiritual self, compassion, and love for humanity for which humans are capable.

While some of these concepts may seem archaic and religious, possibly not worthy of inclusion in modern medical thought, in the context of human and social health, the attainment of mental, emotional, and social well-being is a central doctrine to most concepts of health as articulated by the World Health Organization [157] as:

> Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity.

Interestingly, America, a nation with the worst health care statistics of all developed nations (and several developing nations) also exceeds similarly developed nations in violent deaths, predominantly due to homicide, despite being among the most affluent of nations. A quick look at health statistics data across the US record Vermont and New Hampshire as the two healthiest states in the nation [148]. More interesting is that these two states have the two lowest rates of homicide in the nation. Conversely, the two unhealthiest states, Louisiana and Mississippi, have the highest rates of homicide and highest rates of infant mortality in the nation [149] demonstrating a strong correlation between physical health statistics, mental and emotional health, social welfare, and spiritual wellbeing. Thus, any complete healing system must take into account how an individual can attain, or a practitioner can facilitate, the attainment of higher states of health and healing than simply the removal of symptoms and treatment of disease. This is the highest ideal of traditional healing systems that has no foundation or construct in the current medical delivery system, pharmaceutical industry, or modern pharmacognosy. It is only through traditional healing systems that such healing is even considered. Whether one is willing to apply what many consider to be spiritual aspects of healing to modern medicine is a matter of choice. In a more material sense, as succinctly stated by DePasquale [32]:

> Pharmacognosy is the science of drugs that originate from living beings and are studied to help other living beings.

3.8 PREVENTIVE CARE AND SELF-RESPONSIBILITY

Inherent in a different approach to health and healing is the need to instill in society, and to create incentives for, a high degree of individual responsibility for one's own health. No medication, pharmaceutical, or herbal can change the lifestyles that are the cause of the majority of human ills. Obesity, smoking, poor nutrition choices, lack of exercise, and exposure to the myriad of environmental and human toxins that we allow in our foods and homes are at the root of many modern sicknesses and are relatively easily remedied, first with education about proper choices and then integration and promotion of healthier lifestyles that lead to healthier outcomes. First and foremost in the realm of medical care is to acknowledge the gross limitations and dangers associated with modern pharmaceutical drugs and an over-reliance on specialization.

3.8.1 Proposal for a New Paradigm

The benefits obtainable through the proper use of herbal medicines are inherently different than those attained through standard use of pharmaceutical medications and Western disease care paradigms. Because of this, a different paradigm for ensuring the integrity of traditional herbal medicines is to apply scientific

investigation in a way that fully honors the unique characteristics of traditional healing systems. First and foremost is, through human investigation, understanding the scientific basis of the theoretical healing principles of our varying healing traditions, pharmacologically, physiologically, and with an emphasis on patient outcomes. All other experimentation is abstract and of limited utility. Second, and most importantly regarding herbal medicine, is intensive investigation of what constitutes a "quality" herbal medicine that would then drive the quality control guidelines and requirements of the supply chain so as to ensure appropriate quality of herbal medicines. Such investigation could include a myriad of testing parameters. First of these would be morphological and organoleptic profiling of botanicals growing in their natural habitat and then to codify these characters in pharmacopoeias, GACP guidance documents, and in regulation or practice, as was done historically. Chemical profiling of the botanicals can allow for making determinations of optimal harvest times, drying, processing, extracting, and storage conditions but should not supplant traditional assessment skills. Morphological, organoleptic, and chemical characterizations can also help determine what species of plants can be used interchangeably, again, in manner that was similarly done historically. In the world of medicinal plant research, focus can be given, not only to Western pharmacological mechanisms but also on how those mechanisms correspond, or not, to traditional characteristics such as taste, smell, and sensation of the botanical, or determination of what it really means for an herb to be classified as a "yin tonic" or to "pacify vata." Clinical investigation should look at the application of the traditional medicine within the context of the lifestyle recommendations that usually accompany such prescriptions. Lastly, recognizing the inherent connection that exists between humans, plants, and their environment, fostering this connection through true healing practices, and bringing this consciousness into health care paradigms can contribute greatly to individual and societal health. Such investigation can open a world of new possibilities for health care that is greater than what either traditional or modern systems offer alone. Such inquiry could then more fully inform the practice of both paradigms of medicine and create new opportunities for research and practitioner training, product development, and environmentally sustainable economies.

3.9 CONCLUSION

While developing nations continue to rely on traditional healing systems for their primary health care needs, there is ever increasing recognition in developing countries of the value these traditional healing systems have in human health and as a part of modern health care systems. This is a phenomenon occurring internationally. Herbal medicines serve an integral role in traditional healing systems and there is a need to preserve, promote, and continue to develop the knowledge base needed for ensuring the identity, purity, and quality of botanical ingredients. Pharmacopoeias play a key role in these processes. However, in most Western countries, pharmacopoeial monograph systems developed as chemistry was overtaking crude botanical medicine and drugs were evolving into pure, isolated, and then synthetic compounds, in contrast to the multiple compounds and pharmacological activities inherent in herbal medicines. Because of this, modern pharmacopoeias, and subsequently regulatory systems, attempt to characterize botanical medicines in the same way they do pharmaceutical medications. Lacking in this system is a realization that traditional herbal medicine systems have a distinct way of understanding their materia medica that is fundamentally different than western pharmacological approaches. Over several millennia, knowledge of plant-based medicines evolved based on the properties inherent in the plant, which includes a plant's chemistry, and is reflected in the taste, smell, and qualities as ascertained through sensory evaluation. More importantly, in traditional healing systems, botanicals are applied in a manner that is similarly distinctly different from the typical western model whose focus is on disease pathology and amelioration of symptoms in contrast to human health and the elimination of the cause. In traditional medical systems, herbs are used to promote health, vitality, and longevity, in addition to treating disease and ameliorating symptoms. In conventional western medical practice, there is a single focus on pathology and almost no emphasis on health. Whereas there are hundreds of botanical medicines that are used to promote health, there is not a single pharmaceutical medication designed to promote health and vitality.

Driving the popularity in traditional herbal medicine is a desire on the part of consumers to be healthier. Another significant factor are the many failings of modern medicine in dealing with many diseases, most specifically, chronic degenerative diseases for which pharmaceuticals offer only palliative care and the significant dangers posed by pharmaceutical medications, which are widely recognized as one of the leading causes of preventable deaths. Yet a last, but little recognized driving factor for the popularity of herbal medicine is its potential for environmental sustainability. Preserving herbal medicine preserves cultural knowledge, cultural integrity, environmental integrity, and the integrity of human health. If herbal medicine is to survive and thrive for future generations it cannot simply be approached using the same models as those used for pharmaceutical medications. Similarly, pharmacopoeias must evolve to take

into consideration the unique philosophies and traditional systems in which herbal medicines are applied, most importantly, by not judging herb quality simply by assessing botanical ingredients chemically. Rather, all aspects of the source of medicinal ingredients from the field to the pharmacy must be taken into consideration as was done historically by herbalists and early pharmacognosists. If modern pharmacopoeias and regulatory systems can evolve to integrate traditional healing knowledge and practices, herbal medicine can thrive and reach its full potential in serving humanity. If not, it will continue to survive and be utilized by those who are more committed to the promotion of health and minimization of disease, but will continue to be marginalized in modernity that maintains the status quo of disease management to the detriment of individual and societal health.

References

[1] AHPA-AHP. AHPA-AHP Good agricultural and collection practice for herbal raw materials. Silver Spring, MD: American Herbal Products Association; December 2006. 32 pp.

[2] Aizenman BE. Antibiotic preparations from *Hypericum perforatum*. Mikrobiol Zh (Kiev) 1969;31:128–33. CA 70: 118006e.

[3] Albertsen PC. Screening for prostate cancer is neither appropriate nor cost-effective. Urol Clin North Am November 1996; 23(4):521–30.

[4] Almasi EA, Stafford RS, Kravitz RL, Mansfield PR. What are the public health effects of direct-to-consumer advertising? PLoS Med 2006;3(3):e145.

[5] Andreescu C, Mulsant BH, Emanuel JE. Complementary and alternative medicine in the treatment of bipolar disorder - a review of the evidence. J Affect Disord September 2008; 110(1–2):16–26. Epub 2008 May 5.

[6] Applequist W. The identification of medicinal plants: a handbook of the morphology of botanicals in commerce. Austin, TX: American Botanical Council; 2006. 209 pp.

[7] Arnold KE, Boxall AB, Brown AR, Cuthbert RJ, Gaw S, Hutchinson TH, et al. Assessing the exposure risk and impacts of pharmaceuticals in the environment on individuals and ecosystems. Biol Lett June 26 , 2013;9(4):20130492. http://dx.doi.org/10.1098/rsbl.2013.0492. Print 2013 Aug 23. PMID: 23804293.

[8] Atkins J, Versluys A. The classical energetics of the five tastes. J Chin Med 2006;80:261–9.

[9] Baker RB, Washington HA, Olakanmi O, Savitt TL, Jacobs EA, Hoover E, et al. Creating a segregated medical profession: writing group on the history of African Americans and the Medical Profession, African American physicians and organized medicine, 1846–1910. J Natl Med Assoc June 2009;101(6):501–12.

[10] Bakhtiar L. In: Nasr SH, editor. The canon of medicine, vol. 2. Chicago, IL: Great Books of the Islam World, Inc; 2012. p. 1322.

[11] Bates DW, Cullen DJ, Laird N, Petersen LA, Small SD, Servi D, et al. Incidence of adverse drug events and potential adverse drug events. Implications for prevention. ADE Prevention Study Group. JAMA July 5, 1995;274(1):29–34.

[12] Beck L. Pedanius dioscorides of Anazarbus: de materia medica. Hildesheim: Olms-Weidmann; 2005. 540 pp.

[13] Berg O. Anatomischer Atlas zur Pharmazeutischen Waarenkunde. Berlin: Verlag von Rudolph Gaertner; 1865. 103 pp.

[14] Blazer VS, Iwanowicz LR, Iwanowicz DD, Smith DR, Young JA, Hedrick JD, et al. Intersex (testicular oocytes) in smallmouth bass from the Potomac river and selected nearby drainages. J Aquat Anim Health 2007;19(4):242–53.

[15] [British Medical Journal] BMJ. Second opinion reduces hysterectomies for uterine fibroids, study shows. BMJ 2014;348: g2765.

[16] Booth M. Opium: a history. NY: St. Martin's Griffin; 1996. 400 pp.

[17] Borzelleca JF. Profiles in toxicology. Paracelsus: Herald of modern toxicology. Toxicol Sci 2000;53:2–4.

[18] Boyle W. Official herbs: botanical substances in the United States pharmacopoeias 1820–1990. East Palestine, OH: Buckeye Naturopathic Press; 1991. 77 pp.

[19] British Pharmacopoeia. London: Spottiswoode & Co.; 1864. 444 pp.

[20] Carozza D. FDA incapable of protecting US, scientist alleges. Fraud Mag 2005 [September/October]. http://www.fraudmagazine.com/article.aspx?id=4294967770 (accessed 11.19.14).

[21] Carter HB, Albertsen PC, Barry MJ, Etzioni R, Freedland SJ, Greene KL, et al. Early detection of prostate cancer: AUA Guideline. J Urol August 2013;190(2):419–26. http://dx.doi.org/10.1016/j.juro.2013.04.119. Epub 2013 May 6.

[22] Chen P, editor. History and development of traditional Chinese medicine. Beijing: Science Press; 1999. p. 287.

[23] Guidance for Industry: Botanical Drug Products. US Department of Health and Human Services, Food and Drug Adminstration, Center for Drug Evaluation and Research (CDER). June. 48 pages.

[24] Commonwealth Fund. Why not the best? Results from the national scorecard on us health system performance. 2008. The Commonwealth Fund Commission on a High Performance Health System. 81 pp.

[25] Commonwealth Fund. Why not the best? Results from the national Scorecard on us health system performance. 2011. The Commonwealth Fund Commission on a High Performance Health System. 81 pp.

[26] Cordell GA, Colvard MD. Some thoughts on the future of ethnopharmacology. J Ethnopharmacol August 22, 2005; 100(1–2):5–14.

[27] Cordell GA. Natural products in drug discovery—creating a new vision. Phytochem Rev 2002;1:261–73.

[28] Cott J. Encyclopedia of dietary supplements. NY: Marcel Dekker; 2005. http://dx.doi.org/10.1081/E-EDS-120022129. 840.

[29] Coulter H. Divide legacy. In: A history of the schism in medical thought. Hippocrates to paracelsus, vol. 1. Wash DC: Weshauken Press; 1975. p. 537.

[30] Cullen DJ, Bates DW, Small SD, Cooper JB, Nemeskal AR, Leape LL. The incident reporting system does not detect adverse drug events: a problem for quality improvement. Jt Comm J Qual Improv October 1995;21(10):541–8.

[31] Davidson JRT. Effect of *Hypericum perforatum* (St. John's wort) in major depressive disorder: a randomized controlled trial. J Am Med Assoc 2002;287(14):1807–14.

[32] DePasquale A. Pharmacognosy: the oldest modern science. J Ethnopharmacol 1984;11:1–16.

[33] Doucet J, Chassagne P, Trivalle C, Landrin I, Pauty MD, Kadri N, et al. Drug-drug interactions related to hospital admissions in older adults: a prospective study of 1000 patients. J Am Ger Soc 1996;44:944–8.

[34] Dubois J. Pharmacopoeae jacobi sylvii libri tres. Lugduni: Apud Guliel. Rovillium; 1548. 432 pp.

[35] Eccles R. Menthol and related cooling compounds. J. Pharm. Pharmacol 1994;46(8):618–30. PMID 7529306.

[36] [European Environment Agency] EEA. Pharmaceuticals in the environment. 2010. Technical report No 1/2010, p. 4, www.eea.europa.eu/publications/.

[37] Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M, et al. Trends in alternative medicine use in the United States: 1990–1997: results of a follow-up national survey. JAMA November 11, 1998;280(18):1569–75.
[38] Epstein SS, Bertell R, Seaman B. Dangers and unreliability of mammography: breast examination is a safe, effective, and practical alternative. Int J Health Serv 2001;31(3):605–15.
[39] Estes JW. In: Higby GJ, Stroud EC, editors. The inside story of medicines: a symposium. Madison, WI: American Institute of the History of Pharmacy; publication 16; 1997. p. 304.
[40] [European Commission] EUC. Strengthening pharmacovigilance to reduce adverse effects of medicines. European Commission MEMO/08/782; 10/12/2008; 2008.
[41] Escher M, Desmeules J, Giostra E, Mentha G. Hepatitis associated with Kava, a herbal remedy for anxiety. BMJ 2001; 322:139.
[42] Fan XH, Cheng YY, Ye ZL, Lin RC, Qian ZZ. Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. Anal Chim Acta 2006;555:217–24.
[43] Farquhar C, Steiner CA. Hysterectomy rates in the United States. Obstet. Gynecol 2002;99:229–34.
[44] [Food and Drug Administration] FDA. Antidepressant efficacy questioned. 2008. 01.17.2008. FDA WebReview.
[45] Fenton JJ, Jerant AF, Bertakis KD, Franks P. The cost of satisfaction: a national study of patient satisfaction, health care utilization, expenditures, and mortality. Arch Intern Med March 12, 2012;172(5):405–11. http://dx.doi.org/10.1001/archinternmed.2011.1662. Epub 2012 Feb 13.
[46] Flückiger FA, Tschirch A. The principles of pharmacognosy. NY: William Wood & Co; 1887. 294 pp.
[47] Fontana RJ. Acute liver failure including acetaminophen overdose. Med Clin North Am 2009;92(4):761–94. http://dx.doi.org/10.1016/j.mcna.2008.03.005.
[48] Friedrich C. German pharmacopoeias. Marburg, no date unpaginated.
[49] Frosch DL. Creating demand for prescription drugs: a content analysis of television direct-to-consumer advertising. Ann Fam Med 2007;5(1):6–13.
[50] Gaddum JH. Pharmacopoeia Londinensis of 1618. Nature May 25 , 1946;157:677. http://dx.doi.org/10.1038/157677a0.
[51] Galeottia N, Mannellia LDC, Mazzantib G, Bartolinia A, Ghelardini C. Menthol: a natural analgesic compound. Neurosci Lett 2002;322(3):145–8. http://dx.doi.org/10.1016/S0304-3940(01)02527.
[52] Gandhi TK, Weingart SN, Borus J, Seger AC, Peterson J, Burdick E, et al. Adverse drug events in ambulatory care. N Engl J Med April 17, 2003;348:1556–64.
[53] GAO. FDA Drug Review: postapproval risks 1976-85.GAO/PEMB-90-15. 1990. 131 pp.
[54] GAO. DRUG SAFETY: Improvement needed in FDA's postmarket decision-making and oversight process. Government Accountability Office; March 2006. Report to Congressional Requesters (GAO-06–402). 67 pp.
[55] Gerard J. The herbal or general history of plants. New York: Dover; 1633. 1630 pp.
[56] Glassmeyer ST, Furlong ET, Kolpin DW, Cahill JD, Zaugg SD, Werner SL, et al. Transport of chemical and Microbial compounds from known wastewater Discharges: potential for Use as indicators of human Fecal Contamination. Environ Sci Technol 2005;39(14):5157–69.
[57] Graham DJ. Testimony of David J. Graham. Washington, DC: US Senate Committee on Finance; November 19, 2004. 9 pp.
[58] Greenish H, Collin E. An anatomical atlas of vegetable powders. London: J & A: Churchill; 1904. 287 pp.
[59] Griggs B. Green pharmacy: a history of herbal medicine. NY: The Viking Press; 1981. 379 pp.
[60] Grube GMA. Greek medicine and the Greek genius. Phoenix 8; 1954. 123–135.
[61] Gstirmer F, Kind HH. Chemical and physiological examination of valerian preparations. Pharmazie 1951;6:57–63.
[62] Gupta A. Fraud and misconduct in clinical research: a concern. Perspect Clin Res Apr-Jun 2013;4(2):144–7. http://dx.doi.org/10.4103/2229-3485.111800.
[63] Gurwitz JH, Field TS, Avorn J, McCormick D, Jain S, Eckler M, et al. Incidence and preventability of adverse drug events in nursing homes. Am J Med August 1, 2000;109(2):87–94.
[64] Harnly J, Chen P, Harrington Pde B. Probability of identification: adulteration of American Ginseng with Asian Ginseng. J AOAC Int 2013 Nov-Dec;96(6):1258–65.
[65] Heisler EJ. The US infant mortality rate: international comparisons, underlying factors, and federal programs. Congressional Research Services; April 4, 2012. 1–30 pp. R41378.
[66] [Department of Health and Human Services] HHS. Addressing prescription drug abuse in the United States: current activities and future opportunities. Behavioral Health Coordinating Committee. Wash, DC: Prescription Drug Abuse Subcommittee, US Department of Health and Human Services; 2013. 36 pp.
[67] Higby GJ, Stroud EC. The inside story of medicines: a symposium. Madison, WI: American Institute of the History of Pharmacy; publication 16; 1997. 304 pp.
[68] Hiroshi H. On Vis medicatrix naturae and Hippocratic idea of physis, 22. Memoirs of School of Health Sciences, Faculty of Medicine, Kanazawa University; 1998. 45–54, http://sciencelinks.jp/j-east/article/199907/000019990799A0162403.php.
[69] Hobbs C. St. John's wort: *Hypericum perforatum* L. A review. HerbalGram 1989;18/19:24–33.
[70] Hughes B, Durran A, Langford NJ, Mutimer D. Paracetamol poisoning—impact of pack size restrictions. J Clin Pharm Ther 2003;28(4):307–10. http://dx.doi.org/10.1046/j.1365-2710.2003.00497.x. PMID 12911683.
[71] Husemann T. A facsimile of the first edition of the Pharmacopoeia Augustana (1622). Madison, WI: State Historical Society of Wisconsin; 1927. 260 pp.
[72] Jeffreys D. Aspirin: the remarkable story of a wonder drug. New York: Bloomsbury Publishing; 2005. 352 pp.
[73] Jones SA. A glance at the empirical history of *Hypericum perforatum*. American Observer, April 1810. 1880. pp. 207–215.
[74] Jones CM, Mack KA, Paulozzi LJ. Pharmaceutical overdose deaths, United States, 2010. JAMA February 20, 2013;309(7):657–9. http://dx.doi.org/10.1001/jama.2013.272.
[75] Jue Z, Fan Q. Treating gynaecological disorders with traditional Chinese medicine: a review. Afr J Tradit Complement Altern Med 2009;6(4):494–517.
[76] Kapoor LD. Opium poppy: botany, chemistry, and pharmacology. New York: Haworth Press; 1997. 326 pp.
[77] Kearney P, Baigent C, Godwin J,H, Emberson J, Patrono C. Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. BMJ 2006; 332(7553):1302–8. http://dx.doi.org/10.1136/bmj.332.7553.1302. PMC 1473048. PMID 16740558.
[78] Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, et al. Collapse of a fish population after exposure to a synthetic estrogen. Proc Natl Acad Sci USA 2007;104(21):8897–901.
[79] Kirsch I, Deacon BJ, Huedo-Medina TB, Scoboria A, Moore TJ, Johnson BT. Initial severity and antidepressant benefits: a meta-analysis of data submitted to the Food and Drug Administration. PLoS Med 2008;5:e45.
[80] Kraemer H. A text-book of botany and pharmacognosy. Philadelphia and London: JB Lippincott; 1908. 850 pp.

[81] Kraemer H. Scientific and applied pharmacognosy. New York: J Wiley; 1920. 741 p.
[82] Krieglstein J, Grusla D. Central depressant constituents in Valeriana, valepotriate, valerenic acid, valeranone and volatile oil are ineffective afterall. Dtsch Apoth Ztg 1988;128:2041–6.
[83] Kubota T, Asaka Y, Miura I, Mori H. Structures of ganoderic acids A and B, two new lanostane type bitter triterpenes from *Ganoderma lucidum* (Fr) Karst. Helvet Chim Acta 1982;65(62): 611–9.
[84] Kurian AW, Lichtensztajn DY, Keegan THM, Nelson DO, Clarke CA, Gomez SL. Use of and mortality after bilateral mastectomy compared with other surgical treatments for breast Cancer in California, 1998–2011. JAMA 2014;312(9):902–14. http://dx.doi.org/10.1001/jama.2014.10707.
[85] Lazarou J, Pomeranz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. JAMA 1998;279(15):1200–5.
[86] Leape LL, Brennan TA, Laird N, Lawthers AG, Localio AR, Barnes BA, et al. The nature of adverse events in hospitalized patients — results of the Harvard medical practice study II. N Engl J Med February 7, 1991;324(6):377–84.
[87] Li JW. Sun Si Miao. J Chin Med 1983;13:3–7.
[88] Linde K. St. John's wort – an overview. Forschende Komplementarmedizin 2009;16:146–55.
[89] Linnaeus C. Materia medica. Liber I. Des plantis. HOLMIÆ; 1749. 252 pp.
[90] Ma X, Wang R, Long JB, Ross JS, Soulos PR, Yu JB, et al. The cost implications of prostate cancer screening in the medicare population. Cancer January 1, 2014;120(1):96–102. http://dx.doi.org/10.1002/cncr.28373. Epub 2013 Oct 4.
[91] MacLennan AH, Wilson DH, Taylor AW. The escalating cost and prevalence of alternative medicine. Prev Med August 2002;35(2): 166–73.
[92] Mansfield W. Mansfield's–materia medica and pharmacognosy. Albany, NY: William Mansfield; 1926. 664 pp.
[93] Mansfield W. Materia medica, toxicology and pharmacognosy. St. Louis: CV Mosby; 1937. 707 pp.
[94] Marinac JS, Buchinger CL, Godfrey LA, Wooten JM, Sun C, Willsie SK. Herbal products and dietary supplements: a survey of use, attitudes, and knowledge among older adults. J Am Osteopath Assoc January 2007;107(1):13–20.
[95] Materia E1, Rossi L, Spadea T, Cacciani L, Baglio G, Cesaroni G, et al. Hysterectomy and socioeconomic position in Rome, Italy. J Epidemiol Community Health 2002;56:461–5.
[96] McGlynn EA, Asch SM, Adams J, Keesey J, Hicks J, DeCristofaro A, et al. The quality of health care delivered to adults in the United States. N Engl J Med June 26, 2003;348(26): 2635–45.
[97] McPherson K, Wennberg JE, Hovind OB, Clifford P. Small area variations in the use of common surgical procedures. An international comparison of New England, England and Norway. N Engl J Med 1982;307:1310–4.
[98] Miller AB, Wall C, Baines CJ, Sun P, To T, Narod SA. Twenty five year follow-up for breast cancer incidence and mortality of the Canadian National Breast Screening Study: randomised screening trial. BMJ February 11, 2014;348:g366. http://dx.doi.org/10.1136/bmj.g366.
[99] Moeller J. Mikroskopie der Nahrungs- und Genussmittel aus dem Pflanzenreiche. Berlin: Verlag von Julius Springer; 1886. 394 pp.
[100] Mojtabai R, Olfson M. Proportion of antidepressants prescribed without a psychiatric diagnosis is growing. Health Aff (Millwood) August 2011;30(8):1434–42. http://dx.doi.org/10.1377/hlthaff.2010.1024.
[101] Moyer VA. US preventive services task force. Screening for prostate cancer: US preventive services task force recommendation statement. Ann Intern Med 2012;157:120–34.
[102] Mukherjee PK, Ponnusankar S, Venkatesh P. Synergy in herbal medicinal products: concept to realization. Ind J Pharm Edu Res July–September, 2011;45(3).
[103] Naik GH, Priyadarsini KI, Bhagirathi RG, Mishra B, Mishra KP, Banavalikar MM, et al. In vitro antioxidant studies and free radical reactions of triphala, an ayurvedic formulation and its constituents. Phytother Res July 2005;19(7):582–6.
[104] Nam RK, Saskin R, Lee Y, Liu Y, Law C, Klotz LH, et al. Increasing hospital admission rates for urological complications after transrectal ultrasound guided prostate biopsy. J Urol 2013; 189:S12–7.
[105] Negrash AK, Pochinok PY. Comparative study of chemotherapeutic and pharmacological properties of antimicrobial preparations from common St. John's wort. In: Fitonotsidy, Mater. Soveshch. 6th, Meeting date 1969; 1972. p. 198–200 (CA 78:66908u).
[106] Nishitoba T, Goto S, Sato H, Sakamura S. Bitter triterpenoids from the fungus. *Ganoderma applanatum*. Phytochemistry 1989; 28(1):193–7.
[107] [Natural Resources Defense Council] NRDC. Dosed without prescription: preventing pharmaceutical contamination of our nation's drinking water 3. 2009. http://docs.nrdc.org/health/files/hea_10012001a.pdf.
[108] [National Women's Health Network] NWHN. Hysterectomy in the United States: facts and figures. 2014. http://nwhn.org/hysterectomy [accessed 5.25.14].
[109] Nugent-Head JA. Returning our focus to the flavour and nature of herbs. J Chin Med June 2014;105:30–6.
[110] Hawton K, Ware C, Mistry H, Hewitt J, Kingsbury S, Roberts D, Weitzel H. Paracetamol self-poisoning: characteristics, prevention and harm reduction. Br J Psych 1996;168:43–8.
[111] Orlando EF, Kolok AS, Binzcik GA, Gates JL, Horton MK, Lambright CS, et al. Endocrine-disrupting effects of cattle feedlot effluent on an aquatic sentinel species, the fathead minnow. Environ Health Persp 2004;112(3):353–8.
[112] Osbaldeston TA, Wood RPA. The herbal of Dioscorides the Greek. Johannesburg (South Africa): Ibidis Press; 2000. 923 pp.
[113] Parker WH, Broder MS, Chang E, Reskanich D, Farquhar C, Liu Z, et al. Ovarian conservation at the time of hysterectomy and long-term health outcomes in the nurses' health study. Obstet Gynecol 2009;113:1027–37.
[114] Paulozzi LJ. Trends in unintentional drug poisoning deaths. US Congress: Energy and Commerce Committee, House of Representatives; 2007. October 24.
[115] Payyappallimana U. Role of traditional medicine in primary health care: an overview of perspectives and challenges. Yokohama J Soc Sci 2010;14(6).
[116] Pharmacopoea Londinensis. Culpeper (translator) 1720. Cornhill (UK): John Allen; 1618. 340 pp..
[117] Pickering C. Chronological history of plants. Boston: Little, Brown; 1879. 518 p.
[118] Pigott HE, Leventhal AM, Alter GS, Boren JJ. Efficacy and effectiveness of antidepressants: current status of research. Psychother Psychosom 2010;79(5):267–79. http://dx.doi.org/10.1159/000318293. Epub 2010 Jul 9.
[119] Ponnusankar S, Pandit S, Babu R, Bandyopadhyay A, Mukherjee PK. Cytochrome P450 inhibitory potential of triphala—a rasayana from ayurveda. J Ethnopharmacol January 7, 2011;133(1):120–5. http://dx.doi.org/10.1016/j.jep.2010.09.022. Epub 2010 Sep 29.
[120] Pratt LA, Brody DJ, Gu Q. Antidepressant use in persons aged 12 and over: United States, 2005–2008. NCHS Data Brief October 2011;76:1–8.
[121] Radley DC, Finkelstein SN, Stafford RS. Off-label prescribing among office-based physicians. Arch Intern Med 2006;166(9): 1021–6. http://dx.doi.org/10.1001/archinte.166.9.1021. PMID 16682577.

[122] Rao SG, Laxminarayana AU, Saraswathi LU, Padma GM, Ganesh R, Kulkarni DR. *Calendula* and *Hypericum*: two homeopathic drugs promoting wound healing in rats. Fitoterapia 1991;6:508–10.
[123] Riddle JM. Dioscorides on pharmacy and medicine. Austin: Univ Texas Pr; 1985. 298 pp.
[124] Riddle JM. Old drugs, old and new history. In: Higby GJ, Stroud EC, editors. The inside story of medicines: a symposium. Madison, WI: American Institute of the History of Pharmacy; publication 16; 1997. p. 304.
[125] Riise GB. In: Higby GJ, Stroud EC, editors. The inside story of medicines: a symposium. Madison, WI: American Institute of the History of Pharmacy; publication 16; 1997. p. 304.
[126] Robinson C, Wiczyk H. Treating common gynecologic conditions with acupuncture. Gynecology Update 2011;36:32–8.
[127] Rocca WA, Shuster LT, Grossardt BR, Maraganore DM, Gostout BS, Geda YE, et al. Long-term effects of bilateral oophorectomy on brain aging: unanswered questions from the Mayo Clinic cohort study of oophorectomy and aging. Lond Engl: Womens Health; January 2009. 5(1): 39–48.
[128] Sahoo N, Manchikanti P, Dey S. Herbal drugs: standards and regulation. Fitoterapia 2010;81:462–71. http://dx.doi.org/10.1016/jfitote.2010.02.001.
[129] Saljic J. Ointment for the treatment of burns. Ger Offen 1975;2: 406–52 (CL. A61K), 21 August 1975 (CA 83: 197797).
[130] Sarris J, Kavanagh DJ, Byrne G, Bone KM, Adams J, Deed G. The kava anxiety depression spectrum study (KADSS): a randomized, placebo-controlled crossover trial using an aqueous extract of Piper methysticum. Psychopharm May 9, 2009;205:399–407. http://dx.doi.org/10.1007/s00213-009-1549-9 [epub ahead of print].
[131] Sarris J, Adams J, Wardle JL. Time for a reassessment of the use of Kava in anxiety? Complementary Ther Med (Editorial) 2009;17: 121–2.
[132] Sayre LE. A manual of organic materia medica and pharmacognosy. Philadelphia: P Blakiston's Son; 1917. 606 pp.
[133] Scheid V, Bensky D, Ellis A, Barolet R. Chinese herbal medicine: formulas and strategies. Seattle, WA: Eastland Press; 2009. 1017 pp.
[134] Scherf JCF. Dispensatorium lippiacum. Lemgoviae: Libraria Meyeriana; 1792. 236 pp.
[135] Schmidt JA. Lehrbuch der materia medica. Vienna: Boy kupffer und Wimmer; 1811. 515 pp.
[136] Schuster MA, McGlynn EA, Brook RH. How good is the quality of health care in the United States? The Milbank Quarterly 1998; 76(4):517–63.
[137] Shaw A. Direct-to-consumer advertising of pharmaceuticals. ProQuest Discovery Guides. March 2008:1–14. Available at: www.csa.com/discoveryguides/direct/review4.php [accessed 28.07.11].
[138] Singh N, Vrontakis M, Parkinson F, Chelikani P. Functional bitter taste receptors are expressed in brain cells. Biochem Biophys Res Commun March 4, 2011;406(1):146–51. http://dx.doi.org/10.1016/j.bbrc.2011.02.016. Epub 2011 Feb 12.
[139] Sonnedecker G. Pharmacy in history. The founding period of the US Pharmacopoeia. I. European Antecedents. Am Inst Hist Pharm 1993;35(4):151–62.
[140] Srikumar R, Jeya Parthasarathy N, Sheela Devi R. Immunomodulatory activity of triphala on neutrophil functions. Biol Pharm Bull August 2005;28(8):1398–403.
[141] Starfield B. Is US health really the best in the world? JAMA 2000; 284(4):483–5.
[142] Starr P. The social transformation of American medicine: the rise of a sovereign profession and the making of a vast industry. Basic Books; 1982. 514 pp.
[143] Stoller R. Leberschadigungen unter kava-extrakten. Schweizerische Arztezeitung 2000;81(24):1335–6.
[144] Tan N, McClure TD, Tarnay C, Johnson MT, Lu DSK, Steven S. Women seeking second opinion for symptomatic uterine leiomyoma: role of comprehensive fibroid center. J Ther Ultrasound 2014;2(3). http://www.jtultrasound.com/content/2/1/3.
[145] Tempesta MS, King SR. Tropical plants as source of new pharmaceuticals. In: Barnacal PA, editor. Pharmaceutical manufacturing International: International review of pharmaceutical technology research and development. London: Sterling Publications; 1994.
[146] Tschirch A, Oesterle O. Anatomischer atlas der Pharmakognosie un Nahrungsmittelkunde. Leipzig: Chr Herm Tauchnitz; 1900. 241–244 pp.
[147] Tsitsina SI. Results of studying some medicinal plants containing flavone compounds. TR Bot Sadov Akad Nauk Kaz 1969:111–4.
[148] [United Health Foundation] UHF. America's health rankings. 2011. http://statehealthstats.americashealthrankings.org/#/country/US/2011/Overall-State-Ranking [accessed 09.02.14].
[149] [United Nations Office on Drugs and Crimes] UNODC. Global study on homicide. 2013. Vienna. 166 pp.
[150] Unschuld P, Tessenow H. Huang di nei jing su wen, vol. 1. Berkeley: Univ Cal Press; 2011. 798 pp.
[151] Upton R, editor. Characterization of selected plants that may contain or be adulterated with aristolochic acid. Santa Cruz, CA: American Herbal Pharmacopoeia; 2006. 214 pp.
[152] Upton R, editor. Goldenseal root *Hydrastis canadensis*. Monograph of the american herbal pharmacopoeia. Santa Cruz, CA: American Herbal Pharmacopoeia; 2001. p. 36.
[153] [United States Pharmacopoeia] USP. Pharmacopoeia of the United States. 1st ed. Boston: Wells and Lilly; 1820. 272 pp.
[154] [United States Pharmacopeia] USP. Pharmacopoeia of the United States. 14th revision. United states pharmacopoeial Convention, Inc. Eaton, PA: Mack Printing Co.; 1950. 1067 pp..
[155] Waller DP. Report on kava liver damage. Silver Spring, MD: American Herbal Products Association; 2002. 7 pp.
[156] Walters-Wright M, Volz C. Municipal wastewater concentrations of pharmaceutical and xeno-estrogens: wildlife and human health implications. In: Uzochukwu G, Schimmel K, Chang S-Y, Kabadi V, Luster-Teasley S, Reddy G, et al., editors. Proceedings of the 2007 national Conference on environmental science and technology. NY: Springer; 2007. p. 378.
[157] [World Health Organization] WHO. Preamble to the constitution of the World Health Organization as adopted by the international health conference. New York, June 19–22, 1946. 1946. signed on 22 July 1946 by the representatives of 61 States (Official Records of the World Health Organization, no. 2, p. 100); entered into force on 7 April 1948.
[158] [World Health Organization] WHO. Guidelines for the assessment of herbal medicines. Programme on traditional medicines. Geneva: WHO TRM_91.4; 1991.
[159] [World Health Organization] WHO. Quality control methods for medicinal plant materials. Geneva: World Health Organization; 1998 [unpaginated].
[160] [World Health Organization] WHO. World Health Report: world Health Organization assesses the world's health systems. Geneva: World Health Organization; 2000a. http://www.who.int/whr/2000/media_centre/press_release/en/ [accessed 29.08.14].
[161] [World Health Organization] WHO. General guidelines for methodologies on research and evaluation of traditional medicine. Geneva: World Health Organization; 2000b. WHO/EDM/TRM/2000.1 74 pp. http://www.who.int/medicines/areas/traditional/definitions/en/ [accessed 29.08.14].
[162] [World Health Organization] WHO. WHO traditional medicine strategy 2002–2005. Geneva: World Health Organization; 2002. 61 pp.

[163] [World Health Organization] WHO. 80 pp. Guidelines on good agricultural and collection practices (GACP) for medicinal plants. Geneva: World Health Organization; 2003. http://apps.who.int/medicinedocs/en/d/Js4928e/.

[164] [World Health Organization] WHO. National policy on traditional medicine and regulation of herbal medicines: report of a WHO global survey. Geneva: World Health Organization; May 2005. 156 pp.

[165] [World Health Organization] WHO. WHO traditional medicine strategy 2014-2023. Hong Kong: World Health Organization; 2013. 76 pp.

[166] WHO-UNICEF. Declaration of Alma-Ata. World health organization-United nations Children's Fund (UNICEF). In: International conference on primary health care, Alma-Ata, USSR, September 6–12; 1978. p. 3.

[167] Woelkart K, Koid C, Grisold A, Gangemi JD, Turner RB, Marth E, et al. Bioavailability and pharmacokinetics of alkamides from the roots of Echinacea angustifolia in humans. J Clin Pharmacol June 2005;45(6):683–9.

[168] Woolf SH, Aron L. US health in international perspective: shorter lives, poorer health. National Research Council and Institute of Medicine. US Washington, DC: The National Academies Press; 2013. 420 pp.

[169] Wu JM, Wechter ME, Geller EJ, Nguyen TV, Visco AG. Hysterectomy rates in the United States, 2003. Obstet Gynecol 2007;110(5):1091–5.

[170] Wujastyk D. Well-mannered medicine: medical ethics and etiquette in ayurvedic medicine. Oxford: Oxford University Press; 2012. 238 pp.

[171] Xie P, Chen S, Liang YZ, Wang X, Tian R, Upton R. Chromatographic fingerprint analysis—a rational approach for quality assessment of traditional Chinese herbal medicine. J Chromatogr A April 21, 2006;1112(1–2):171–80. Epub February 3.

[172] Xie PS, Li SP. Back to the future in quality control of Chinese herbal medicines. Nat Rev Drug Discovery July 2007;6:506–7. http://dx.doi.org/10.1038/nrd2350.

[173] Xie PS, Ma SC, Tu PF, Wang ZT, Stoeger E, Bensky D. The prospect of application of extractive reference substance of Chinese herbal medicines. Chinese medicine. 2013. Published Online December, http://www.scirp.org/journal/cm.

[174] Yang SZ, Li JY. Li Dong-Yuan's treatise on the spleen and stomach: a translation of the pi wei lun. Boulder, CO: Blue Poppy Press; 2002. 192 pp.

[175] Youngken HW. A Textbook of pharmacognosy. Philadelphia: P Blakiston's; 1930. 817 pp.

[176] Zeng ZD, Chau FT, Chan HY, Cheung CY, Lau TY, Wei SY, et al. Recent advances in the compound-oriented and pattern-oriented approaches to the quality control of herbal medicines. Chin Med 2008;3(9). http://dx.doi.org/10.1186/1749-8546-3-9. Published online Aug 4, 2008. PMCID: PMC2531114.

[177] Zhan C, Arispe I, Kelley E, Ding T, Burt CW, Shinogle J, et al. Ambulatory care visits for treating adverse drug effects in the United States, 1995-2001. Jt Comm J Qual Patient Saf July 2005;31(7):372–8.

[178] Zhang XR. Legal status of traditional medicine and complementary/alternative medicine: a worldwide review. Geneva: World Health Organization; 2001. 189 pp.

[179] Zhao ZZ, Guo P, Brand E. The formation of daodi medicinal materials. J Ethnopharmacol 2012;40(3):476–81. http://dx.doi.org/10.1016/j.jep.2012.01.048. Epub 2012 Feb 5.

[180] Zhao ZZ. Illustrated Chinese materia medica. Hong Kong: The Stratis Publishing and Distribution Group; 2009. 535.

[181] Zhao ZZ, Chen HB. In: Guo P, Brand E, editors. A concise introduction to Chinese medicinal identification. Seattle, WA: Paradigm Press; 2014. p. 560.

This page intentionally left blank

CHAPTER

4

Taxonomy—An Irreplaceable Tool for Validation of Herbal Medicine

Rainer W. Bussmann

William L. Brown Center, Missouri Botanical Garden, St. Louis, MO, USA

OUTLINE

4.1 INTRODUCTION

The global inventory of plant diversity consists currently of about 350,000 species, and the most current estimates expect about 420,000 plant species to exist. This tremendous diversity accounts for a wide range of phytochemicals, and a high variation of compound composition even within one single species, depending on growth conditions (soil, climate, nutrient status, etc.), and harvest practices and timing, not even taking intraspecific variation into account. Eisenmann et al. [1,2] showed this effect exemplarily for *Artemisia dracunculus*. While traditional plant use and medicine preparation normally take these details into account, they are often seen as of marginal importance in the herbal trade. In the United States, botanical supplements are supposed to be labeled, with the requirement to include the correct scientific name [3,4]. However, in practice this does not prevent accidental or deliberate adulterations [5], or can contain heavy-metal contaminations [6].

The most problematic occurrence in herbal medicine trade is, however, linked to the purchase and use, either in medication or research, of botanicals that are either accidentally or purposefully wrongly identified, or are simply collected under a vernacular name without any subsequent taxonomic treatment, and often without having any vouchered material that could later be used for the verification of plant identity.

Substitution of common, nontoxic species with toxic species has been reported frequently in the literature (e.g., Ref. [3]). Good examples for life-threatening adulterations are replacements of *Plantago major* L. with *Digitalis lanata* Ehrh. [7], *Illicium verum* Hook. F., with *Illicium*

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00004-0

anisatum L. [8,9], or *Arctium lappa* L. with *Atropa belladonna* L. [10].

A much more frequent occurrence is, however, the often deliberate adulteration of botanicals with more common and cheaper species, which, although generally not toxic, might completely lack efficacy. Bulk herbs are readily available unprocessed, which allows for the retention of material for a botanical voucher, although material in trade often does not contain all plant parts, i.e., fruits and flowers are often missing from bulk material, and as such botanical identification can be difficult. In contrast, raw botanicals are also often provided in ground or powdered form, which makes morphological identification very difficult or virtually impossible. While microscopic and organoleptic methods do sometimes allow separating correct species from adulterants [3,11], if the material is only crushed or very coarsely ground, such an identification of powdered material or extracts is impossible. For this reason, the only possibility to later identify the source of a certain botanical securely is to count on botanical voucher specimens that can be directly linked to the material in trade. This is where plant taxonomy and trained taxonomists play an irreplaceable role in the herbal supplement industry.

4.2 VOUCHER SPECIMENS

So-called voucher specimens not only are quintessential for correct botanical identification, but can also serve as repository for the chemical compounds of a plant at any given time during its life cycle. Vouchers collected at different intervals and in different areas can thus clearly reflect compound composition as influenced by edaphic and harvest conditions. Apart from providing a clear reference for a certain batch of material, voucher specimens also allow a follow-up in case taxonomic concepts change.

The correct preparation of a voucher is the first step in correct botanical practice, and any study should make sure that reference to scientific specimens is given. Vouchers are, as a matter of fact, more important than a correct initial identification.

A good voucher specimen needs to include all plant parts necessary for correct identification (i.e., flowers, fruits, a section of the stem with leaves attached; Figure 4.1). Without fertile parts identification might be impossible. Especially if used in herbal medicine, the voucher needs to contain all plant parts (e.g., bark, roots, seeds) that are actually used for the herbal preparation. Figure 4.2 shows a complete specimen of a palm (*Bactris gasipaes* var. *chichagui* (H. Karst.) A.J. Hend.) in the field.

4.2.1 How to Get a Good Voucher

Excellent guidelines for the collection and preparation of specimens can be found in Ref. [12]. A short outline is given here. It is acceptable to make a skimpy specimen if that is all the material there is. But if sufficient material is available, it requires little more effort to make ample sheets. If only skimpy fertile material is available, a voucher can often be improved by adding extra sterile material. Since the objective of a good specimen is to provide in a convenient form an adequate representation of a plant, one should always include the full range of characters exhibited by the plant, including things such as 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 vegetative characters. If there are different types of leaves, these need to be included. Mature and immature twigs, especially in vines, need also to be included, as do sap shoots or stump sprouts and saplings often have very different characters from mature material, and can be very useful.

Field pressing is usually less efficient than collecting in plastic bags. Fragile material can be placed in a field press and sturdy things held in a plastic bag for later pressing. 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 tangled 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 of 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 height or note other characters. Many collectors who have trouble remembering

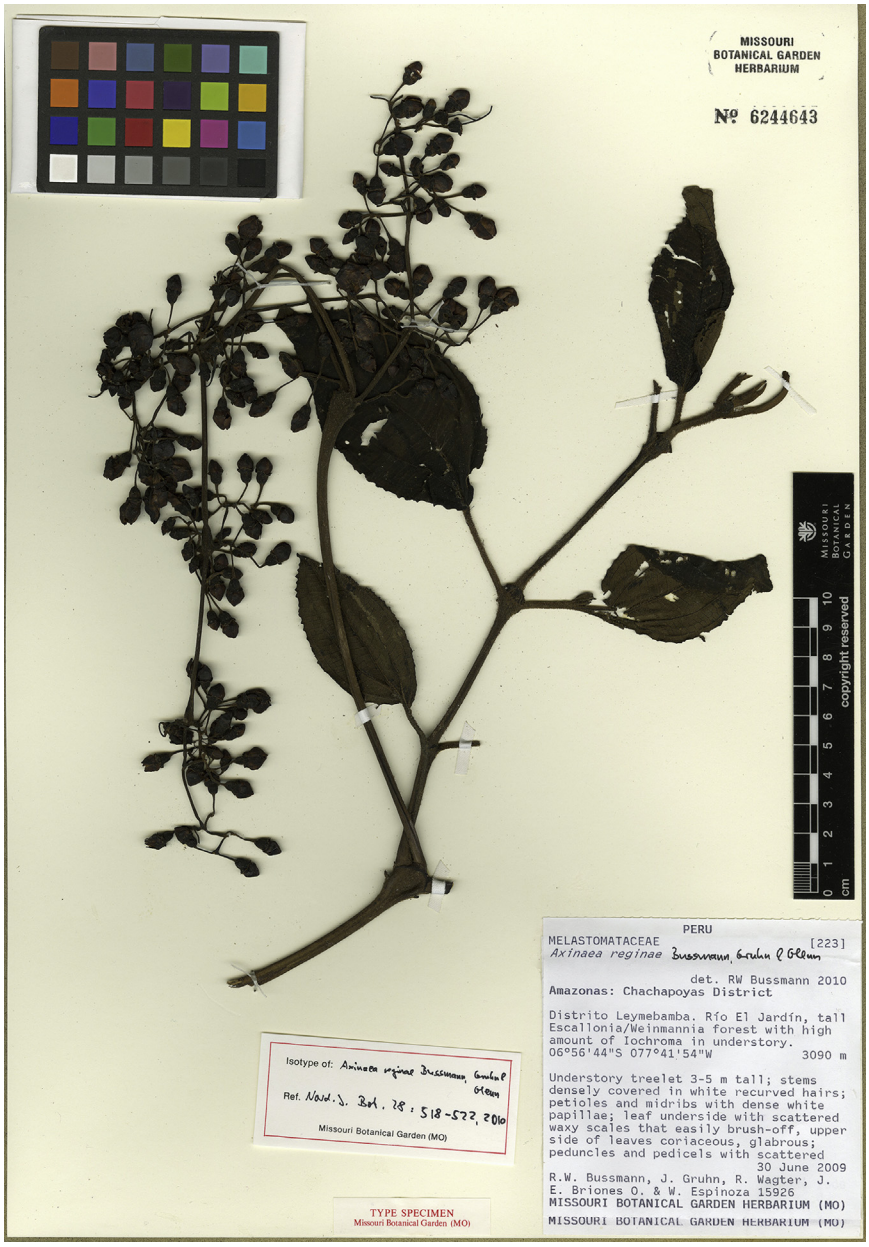


FIGURE 4.1 Complete specimen of *Axinaea reginae* Bussmann, Gruhn & Glenn, Melastomataceae, on herbarium sheet.

this information never closely examined the plant in the first place. If having trouble remembering details, one should always carry a small notebook or a marking pen to write directly on the leaves. For example, E could stand for epiphyte, T for terrestrial, S-2 for shrub 2 m, and T-4 for tree 4 m. It makes lots of sense to tag collections to prevent later mix-ups. Small white tags should be securely tied to stems or fruits, with the collector's name, collection number, and a field determination written in permanent ink or pencil. Stick-on type tags do not work well under wet and humid conditions and if field conditions are rugged.

4.2.2 Pressing Plants

Before pressing large collections it is useful to first sort the material into different species to be able to press complete sets together. This also allows to first press more fragile material. Examples for separation of different materials are given in Figure 4.3 (small high-altitude species) and Figure 4.4 (trees and shrubs). Wood presses (Figure 4.5) are the best way to process vouchers, and can be stacked quite tall with specimens. When pressing, one tries to always keep the upper surface of the specimen up. Each voucher is placed between one large newspaper or collecting paper sheet

FIGURE 4.2 A complete collection of *Bactris gasipaes* var. *chichagui* (H. Karst.) A.J. Hend., Arecaceae, with complete leaf, inflorescence, and infrutescence.

FIGURE 4.3 Sorted high-altitude vouchers in Peru.

FIGURE 4.4 Vouchers of trees and shrubs in the field in Peru.

FIGURE 4.5 Arming a large plant press for drying.

(Figure 4.6), and the number of the specimen should be noted on the outside and inside of the paper with an indelible marker or pencil (Figure 4.7). It is important to place the collector's initials by each specimen's number, e.g., RBU 15,298, directly on the newspaper used for pressing. If labels are lost, or collections mix up, it is much easier to recapture the missing data or return the specimen to its proper place. This saves time if collecting or processing plants together with someone else using the same number range, aids in replacing lost labels, and saves confusion if several collections are shipped together. The number is best put in the same place on

FIGURE 4.6 Plants between pressing sheets.

FIGURE 4.7 Plant pressing sheet with collector number RBU 15,298.

the newspaper each time, preferably along one edge. It is much easier to sort the material that way.

Each specimen for any given number needs to be as complete as possible. If flowers and fruits (or different leaf shapes, etc.) are available, each sheet or set should be representative. After drying, parts that were dried separately should be combined with the rest of the material to form complete specimens before packaging and shipping.

The specimens must be shorter than the pressing and mounting paper. More damage is done if they must be refolded later and any parts that extend beyond the newspaper will break off and be lost. Mounting paper is mostly standardized, e.g., at Missouri Botanical Garden herbarium (MO) it is 29 cm (11 1/2 inches) wide by 42 cm (16 1/2 inches) long.

When pressing material, except for vines, stems can be broken and folded into a "V" or "N" shape, but never just curved to prevent the specimen from slipping. If plants are small, several need to be collected to fill the pressing sheet. It is paramount to the quality of individual plants on each sheet, some nice, some poor (if not all in prime shape). Otherwise, one may end up with the poorest sheet for one's own institution. It simplifies later processing if the collector designates the chosen home institution sheet. Grasses and other herbs should not be "top-snatched." It is important to always collect the full specimen, roots and all. Soil must be removed completely before drying specimens by shaking or washing.

It is best to arrange plants for pressing with the same surface facing upward as will be seen once the specimen is mounted. Before drying plants need to be arranged to clearly show both surfaces of leaves and reproductive structures, paying particular attention to ferns. Some flowers should be opened for pressing, and some left closed, and others split to show the internal structure. Phyllaries (bracts) in the capitulae of Asteraceae are very important, and should be pressed so some can be clearly seen. Pubescence, stomata, and other characters are frequently more important on lower leaf surfaces than upper. If only one large leaf or fern frond sample is available, it should be folded so part of both surfaces can be seen. Flowers, fruits, or stems with leaves should never be covered. Either spread the leaves away from the other plant parts, or fold the leaf underneath them. When folding leaves, one keeps the larger part underneath so that one can still measure length, width, etc. When cutting or breaking off excess leaves, part of the petiole needs to be kept to show the original leaf position. It is important to never cut off the petiole base and the stem attachment of a compound leaf. If possible, some of the petiole bases of the other leaves and the apex of the stem need to be kept. It is important not to mistake a large compound leaf for a branch with simple leaves. Twigs should never be split, because the opposite or alternate arrangement of the leaf will no longer be evident. A specimen of two sheets or more may be necessary with very large leaves. It is usually easier to fold the stem than to try to fold all of the leaves. If a leaf tips stick out of the newspaper, it needs to be folded over or it will be broken off and lost. If folding large leaves, one can sometimes put two large leaves on a sheet rather than one. Length, width, shape, upper surface, and lower surface are still observable. With the long leaves of palms and large ferns, one takes an apical section, a midsection, and a basal portion with pinnae. The length of the leaf, length of the petiole, number of pinnae, and the arrangement of the pinnae (i.e., regular, staggered, or irregularly spaced) need to be noted in the field if the specimen does not show it. For palms, the position of the inflorescence in relation to the leaves, and whether species are solitary or colonial, needs to be described and samples of stem spines and bark included. Each specimen, at a minimum, should consist of an apex, a base, and midsection; selected parts of the inflorescence; stem; and petiole base. Photographs are

very useful, but the specimen, especially its leaves and flowers, need to be photographed from all sides.

A representative specimen of large plant may require various sheets for complete pressing. Even some temperate plants require several sheets for representative parts of leaves, stems, flowers, and fruits. In general, it is important to not overcrowd sheets. Multiple-sheet specimens contain far more information than fewer overcrowded sheets. Large fruits should be cut into 1-inch-thick slices, both longitudinally and transversely. It is preferable to have the fruits attached to the branches when the specimen is mounted. A thickness of 2.5 cm (1 inch) has been established as the maximum recommended thickness for mounted specimens at MO. It is useful to mark multiple sheets of the same specimen with the same letter. The letter can go anywhere on the sheet, but not after the number (i.e., not 15,298A) as this designates a mixed and divided collection. Large leaves need to be folded so base and apex can be seen. Part of the inflorescence, stem, flowers, etc. may be put on top of a leaf without losing any information as long as the shape, dimensions, and surface of the leaf can be seen. Even if parts are dried separately, they can be mounted together. When folding large leaves, one always starts with the largest portion of the leaf, and then the smaller parts on top; e.g., large cordate leaf: base, fold apex, then petiole and inflorescence on top. For leaves with basal lobes, one does not fold the base over; it makes it more difficult to see the shape of the leaf and to measure the lobes. Instead, one starts folding large leaves with the lower surface up. The lower surface generally has more taxonomic characters than the upper. For large symmetrical leaves such as Araceae, one side of the blade up to the midrib may be removed. In the field notes one describes the cross-section of the petiole, whether round or flattened, and whether it has ribs or sharp angles (particularly in *Anthurium* and *Philodendron* (Araceae)). Sometimes it is necessary to use multiple sheets. On large plants one needs to include at least part of the stem and one complete petiole. The stem can be sliced in half longitudinally to make it thinner. Roots and other thick or bulky plant parts can also be split to hasten drying. Bulbs or corms can be cut longitudinally or sliced crosswise, depending on what characters need to be shown. In some cases, e.g., Cyclanthaceae, the leaf base and petiole base are very important as is the depth of the leaf division, which should be indicated.

It is very useful to preserve flowers of Iridaceae, Lentibulariaceae, Burmaniaceae, Zingiberaceae, Orchidaceae, and Marantaceae in a 50% alcohol solution with a few drops of glycerine in whirlpacks or vials for later study. The glycerine prevents the material from completely drying if the fluid should be lost or evaporate. Put the collector's name and number on a piece of paper in the vials using an alcohol-proof marker, ink, or pencil. In Passifloraceae it is important to cut open at least one flower on each sheet so that the internal structure is observable. In other families with few large flowers, e.g., Cactaceae, it is useful to add cut-open flowers to each specimen, whenever possible.

4.2.3 Preserving Plants Until They Can Be Dried

Most plants will deteriorate after 2 or 3 days if they are not dried or preserved in some fashion. If they are refrigerated, they can be kept a day or two longer. Material that is supposed to serve for vouchering phytochemical profiles needs to be dried as quickly as possible, and must not be preserved chemically in order to not alter the compound composition. For regular herbarium vouchers the following preservation method can be chosen:

A 15- to 20-cm-tall bundle of plants in newspapers (Figure 4.8) can be preserved with about 1 L of 50–70% ethanol in large sealed plastic bags (Figure 4.9). If the plastic bags do not have holes, the specimens may be stored this way for several months. Holes or opening in the bag may reintroduce mold spores and allow evaporation of the alcohol. Any loss in concentration of alcohol may result in mold. Lower concentration of alcohol than 40% does not work for storage.

After the preserving solution is added to the bag of plants, the bag should be turned several times to evenly distribute the alcohol. It is best to store bags flat, and then to turn them the next day and again the following day. This ensures the alcohol or formaldehyde will thoroughly penetrate the bundle.

When pressing specimens to be preserved in alcohol, the newspapers must be numbered either with a black china marker or other marker, which is not soluble in

FIGURE 4.8 Plant package wrapped for storage.

FIGURE 4.9 Vouchers in plastic packages preserved in ethanol.

PERU
MELASTOMATACEAE [223]
Axinaea reginae Bussmann, Gruhn & Glenn

det. RW Bussmann 2010
Amazonas: Chachapoyas District

Distrito Leymebamba. Río El Jardín, tall Escallonia/Weinmannia forest with high amount of Iochroma in understory.
06°56'44"S 077°41'54"W 3090 m

Understory treelet 3-5 m tall; stems densely covered in white recurved hairs; petioles and midribs with dense white papillae; leaf underside with scattered waxy scales that easily brush-off, upper side of leaves coriaceous, glabrous; peduncles and pedicels with scattered
30 June 2009
R.W. Bussmann, J. Gruhn, R. Wagter, J. E. Briones O. & W. Espinoza 15926
MISSOURI BOTANICAL GARDEN HERBARIUM (MO)

FIGURE 4.10 Example of complete voucher label.

the preserving solution. All markers (including other colors of china markers) need to be checked before with the alcohol. Over time inks from many markers and pens will disperse into the paper, effectively erasing any data or numbers. Pencil can also be used but it may be difficult to read. Ballpoint pens are not at all permanent. Some inks that do not bleed at low preservative concentration will do so at high concentration. Labels stored with plants preserved in chemicals may eventually have to be replaced, as the ink often blurs.

Each voucher needs to be identified by a distinct number and code, and needs to contain a label with at least minimum information (Figure 4.10).

4.2.4 Locality Data

Locality data should be as specific as possible and apply to a range of collection numbers made sequentially. Someone reading the locality data should ideally be able to find their way to that general site using your description alone.

- Country: State, province or county.
- Distance and direction (kilometers or miles, N-S-E-W not "from" or "near") from the nearest city or major landmark that would appear on a map (smaller geographical localities are not seen on most maps).
- Habitat or vegetation type. Dominant, typical, or associated species if possible.
- Note if plants were preserved in alcohol, or received any other chemical treatment before drying.
- Latitude/longitude and/or township/range; altitude (meters or feet); Global Positioning System reading if available.
- Date.
- Collector(s).

For each collection:

- Plant family (capitalized), genus, and species with author.
- Life form: Tree, shrub, liana, vine, etc. Flower and/or fruit color, scent, height, and unusual features, such as shaggy bark, buttressed trunk, colored sap, any attribute that cannot be obtained from the prepared specimen.
- More specific notes on locality and habitat (near stream, on rock, in water, etc.).
- In addition, any information such as vernacular plant names, local uses, and traditional collecting and harvest process.

4.2.5 How to Dry Plants

Plant specimens need to be carefully, but quickly, dried in order to not destroy or alter the compound composition.

When filling a press, one always keeps the numbered side of the sheets up. It saves time in the long run. It is best to not turn around every other sheet to balance the press. It wastes time. Best practice is to do 20–40 sheets and then reverse the direction of the newspaper folds as necessary. It helps as you are pressing to put large fruits, stems, etc. on different parts of the sheet to balance them.

A few collectors put extra sheets around each individual sheet. It does more harm than good. It has less

structural stability than the first method. Also, the specimens have to be handled more to open and look at them. But it does pay to put extra newspaper sheets around a few extra-big specimens. It is always useful to make packets out of newspaper to hold loose fruit or flowers, which would otherwise easily fall out and be lost.

Once the plants are all dry, they need to be put into numerical order. Then all specimens of a collection will be together. This will save time and confusion. This is also a good step for catching mixed collections, rechecking the number of labels needed if necessary, and other miscellaneous problems. If there are specimens without numbers, it is important to write "specimen in bundle between number XX and number XX." This is especially important when sorting someone else's plants if you cannot solve all of the problems. Otherwise, the collector may not be able to solve the problem later, either. If there is a possibility that the plants will be fumigated later with a microwave oven, do not use staples, paper clips, or other metal objects.

Specimens with irritating hairs or resins, such as *Mucuna* (Fabaceae), *Opuntia* (Cactaceae), *Sterculia* (Malvaceae), *Urtica* (Urticaceae), or *Toxicodendron* (Anacardiaceae), need to be marked clearly with warnings on drop tags or on the outside of the bundle and the edge of the newspaper.

If shipping plants in newspaper, bundles of wrapped plants must be packed tightly in boxes rather than loosely. Even though a slight amount of damage may be incurred when packing the boxes tightly, plants in loosely packed boxes are much more easily damaged during shipping and the boxes more prone to being crushed. Reheating the paper-wrapped bundles over dryers and packing them in tightly sealed boxes or plastic bags reduces insect damage during storage or during the long trip back to the home institution. Always specimens need to be packed to withstand the possibility of moisture or pest damage during long storage and shipping times. Surface mail from some countries may take 3–12 months.

With the shipment one sends details of any special arrangements made with the host country and or other collectors (such as where duplicates are located or where they are promised to be distributed). One needs to know these arrangements before they process the plants, not after they are distributed and not available to satisfy agreements made by the collector. MO sometimes stamps special agreements on the edge of the newspaper of each collection.

With the shipment, some kind of shipping invoice needs to be included so that the receiving institution knows what is in the boxes and why they are being sent. These invoices should include the sender's name and address, receiver's name and address, list of enclosed items, number of boxes (copies of the invoice should be in each box), and any special instructions or conditions. It is also helpful to know how the plants have been chemically treated (i.e., formaldehyde, alcohol, insecticides).

When drying plants, fire is a constant danger. It is best if the plants can be dried in a separate building or fireproof area. If not, extraneous flammable materials such as paper or alcohol should be kept out of the area.

There are basically two types of drying systems. The first is a convection system, which is easier and cheaper to build, and can use a wider variety of heat sources. The second is a forced-air system, which is more complicated and expensive to build but tends to be safer, and when properly designed is usually more efficient.

In the convection system, the plant presses are placed over a heat source. The warmed, dry air rises, passes through the channels in the corrugated cardboard, and carries the moisture away. A convection dryer can be made safer by putting screen, chicken wire, or mesh between the press and the heat source. Most fires start when the press loosens as it dries and pieces fall out onto the heat source. But also remember one must have easy access to the heat source to change bulbs, add fuel, clean out fragments and dust periodically, and for any other general maintenance procedures. The press should never be put too close to the heat source. As the material in the press dries, it becomes more flammable and may ignite.

The heat source may be light bulbs, electric heating strips, kerosene stoves or lamps, propane, or any other source of heat. Propane is very readily available in most places and has been underused in the past. But note that at an elevation of over 2000 m you may need an adjustable burner to compensate for the reduced oxygen content of the air. Note also that some types of kerosene stoves fluctuate too much to be used. Specimens can be dried by putting them over the back of a propane gas stove to enable the convection currents from the oven to dry the plants. One botanist had his plants dried over the oven of the local baker. Another collector who had only half a dozen specimens every few days dried them over the exhaust tube of a kerosene refrigerator. If you have a generator or other motor running constantly, always consider the possibility of devising some sort of arrangement for taking advantage of this waste heat. The system is most efficient if the walls of the drying rack enclose both the heat source and the sides of the presses. Any part of the rack that does not have a press on it should be closed off to better distribute the flow of warm air. Heat should not escape except by going through the plant press.

Placing presses inside a closed oven is not feasible. Oven heat is often too moist and will encourage mold. The temperature cannot be satisfactorily regulated: if

not dried long enough the plants will mold, if dried at too high a temperature, too fast, or too long, the plants will darken and crumble. Utilizing the warm air from the oven by placing the press above the open oven door may work.

When press materials are the limiting factor, one may be able to dry a few more specimens by putting two thin specimens, such as thin grasses, small herbs, or small ferns, together between the blotters. This method should, however, never be used with thicker stems or fruits, because it will result in shriveled leaves and unevenly pressed specimens. Also, if you double up too many specimens, even the thin sheets may not dry, occupying the press materials for another day.

If drying space is a problem, thick fruits, stems, etc. should be sectioned. This will speed up drying. It will also result in fewer shriveled leaves and flatter specimens. A second layer of presses can be put over the first if sticks laid lengthwise separate them.

4.3 PLANT IDENTIFICATION

The main emphasis of vouchering is to correctly identify herbal medicines, i.e., link herbal material directly to a plant species. An example on how this process works is given in Figure 4.11. The correct botanical name (=binomial) represents the universally accepted determination of a certain plant species. The best way to ensure correct identification is to obtain and send duplicates of herbal materials to specialists, ideally at a larger botanical research institution that holds collections from the area where the material was collected, to enable comparisons. Such collections are, in the botanical field, called herbaria (singular: herbarium). Reputable herbaria are listed in *Index Herbariorum*, a global list of botanical collections [13]. A good example on how to successfully identify plant material is given in Ref. [14].

4.3.1 Plant Names and Nomenclature

4.3.1.1 Plant Names—A Historic Perspective

Prior to 1753, plants and animals were described by vernacular names, or long, unwieldy descriptions. The problem of this is obvious: it was never exactly clear if two researchers (or practitioners) referred to the same species with the same name, in particular if no material was available for direct comparison. In 1753, Carolus Linnaeus, a Swedish scientist developed a binomial naming system. Since Latin was the language of science at that time, all names were constructed as Latin binomials. Since then, scientists worldwide employ the

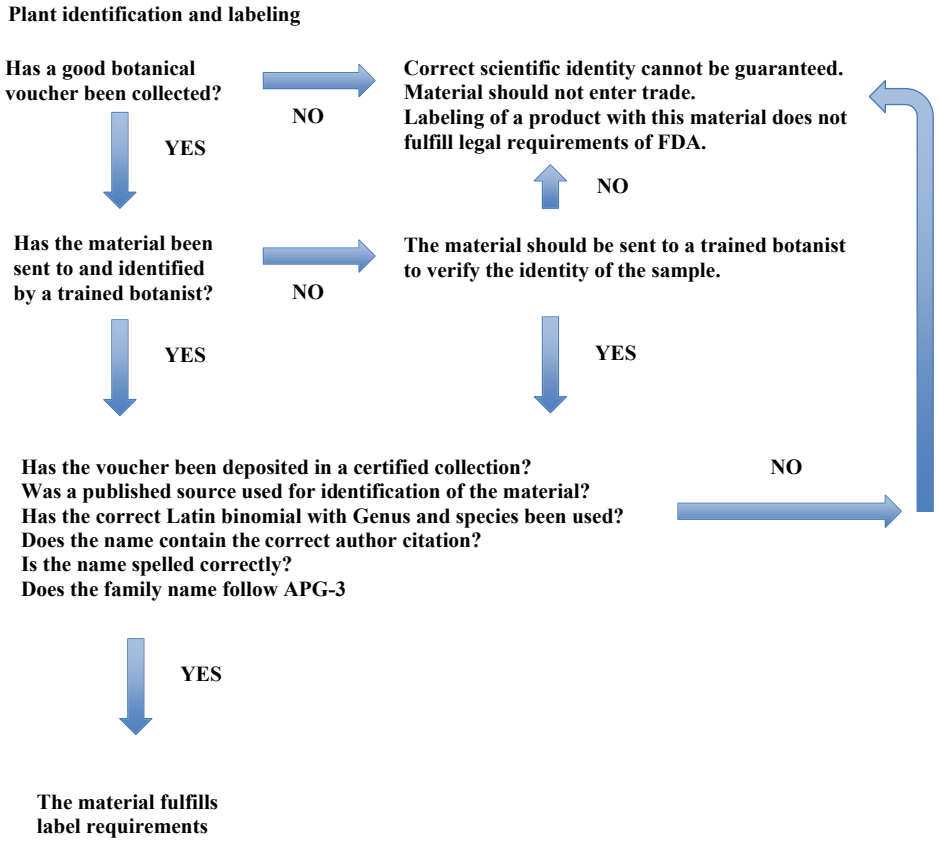


FIGURE 4.11 Correct plant identification to meet labeling requirements.

same system for naming species, based on the International Code of Nomenclature for algae, fungi, and plants, which indicates that only one correct name can refer to one taxon (e.g., species of plant). This universal use of one name allows an unambiguous classification. The Linnaean system has of course detractors, and is not perfect [e.g. 15,16]. In the binomial system, each name consists of a generic name and a species epithet, along with the citation of the author (or authors) who first described the species (e.g., *Blakea nareliana* Bussmann: genus = *Blakea*, species epithet = *nareliana*, author = Bussmann). In any publication, the whole name including the author should be referenced, but the generic name may be abbreviated once the full name has been cited. Complete plant names can be found in the literature, as well as on the Internet, e.g., under www.tropicos.org.

All Latin plant binomials are italicized. However, author names and family names are always spelled in normal font. Example: *Blakea nareliana* Bussmann (Melastomataceae).

Plant names are now governed by the International Code of Botanical Nomenclature, which is amended every 6 years by an international panel of experts. At the time of writing the Melbourne Code is valid [17]. The International Code of Nomenclature for algae, fungi, and plants was developed to provide a clear system that would allow for a unique name for each plant species. This is of great importance, because the US Food and Drug Administration (FDA) labeling guidelines for botanical products has accepted this code as reference for the validity of names in trade.

4.3.1.2 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 other names given to a species later are called synonyms. The earliest names are of course the ones assigned by Linnaeus, e.g., *Arnica montana* L., since Linnaeus developed the binomial system. Authors in parentheses, e.g., *Schisandra chinensis* (Turcz.) Baill., indicate that originally the author in parentheses (Turczaninow) described the species *Kadzur achinensis* Turcz. in 1837, but Baillon (Baill.) correctly placed the species in the genus *Schisandra* in 1868. The use of author names has often been criticized [14], but is essential in case the same name has accidentally been given to two different species.

Synonyms are names other than the correct and accepted names that have been given to a species. In the above example, *Kadzura chinensis* would be a synonym of *Schisandra chinensis*. Synonyms can originate either because the same species was described by different authors under different names or because the correct evolutionary relationship of a species was only recognized after it had already been described. The same species might be known by a wide variety of synonyms, because the species was originally collected and described by many researchers without having knowledge of each other. Quinine, for example, was originally derived from a variety of species of the genus *Cinchona*. One of the most important sources, *Cinchona calisaya* Wedd., is also known under 47 different synonyms. The significance of this becomes obvious when trying to run a search for publications about use or compounds of a certain species. "*Cinchona calisaya*" might yield only a fraction of the possible search results, and only the inclusion of all names would give a complete overview on available references. A well-known example in the United States is black cohosh [18,19]: black cohosh is correctly identified as *Actaea racemosa* L. (the species was described first by Linnaeus in 1753), but *Cimicifuga racemosa* (L.) Nutt. (described by Nuttall in 1818) is widely used. A search on Pubmed for *Actaea racemosa* yields 549 results, while a search for *Cimicifuga racemosa* yields 540 results. The combination of both names plus the vernacular "black cohosh," however, yields 661 results. Careful use of binomial names is thus important for any research on herbal medicine.

4.3.1.3 Classification Systems

Current plant taxonomy follows the so-called Angiosperm Phylogeny Group (APG)-3 system [20]. This system updates the traditional taxonomic system based on morphology with modern genetic data. APG-3 has two important advantages for herbal medicine research. First of all, by providing a unified taxonomic system, it allows for a better comparison of family overviews in herbal medicine, i.e., it can help to elucidate plant groups with similar genetics, and thus possibly more similar phytochemistry. A great example is the Mauve family (Malvaceae): Before the implementation of APG-3, Malvaceae, Tiliaceae, Sterculiaceae, and Bombacaceae were counted as independent families, although in traditional medicine many species belonging to these families had similar medicinal applications. With APG-3, all species of these families belong to Malvaceae, which provides a much better fit and a better tool for screening. Similarly, APG-3 has led to the split of lump families like Scrophulariaceae into smaller units, which in turn also allow for easier selection of screening targets. The genus *Veronica*, before in Scrophulariaceae, was found to belong to Plantaginaceae, which, from a medicinal perspective, makes much more sense. *Sambucus*, before classified in Caprifoliaceae, turned out to belong genetically to Adoxaceae. *Phyllanthus*, well known for species like *Phyllanthus stipulatus* L. and *Phyllanthus niuriri* L. (chanca piedra, for urinary problems), and *Phyllanthus*

emblica L. (amla, Indian gooseberry, known for anticancer and antiinflammatory properties) belonged formerly to the Euphorbiaceae (Spurge family, mostly with toxic latex) and now forms its own family (Phyllanthaceae), which again from a medicinal perspective makes lots of sense. *Valeriana* (formerly Valerianaceae) belongs now to the Caprifoliaceae. Although this new system does implement a considerable number of taxonomic changes, it does, however, hardly affect comparisons of the most important plant families in medicinal floras. Of 494 species cited in Bussmann et al. [21,22], only 44 changed family designation through implementation of APG-3. Another eight simply contained spelling mistakes, even after multiple checks of the complete plant database (Table 4.1). The change in family classification to APG-3 did, however, not at all alter the distribution of the first 40 (of 128) most important plant families in the medicinal flora of Northern Peru, which accounted for almost 60% of all species (Table 4.2).

4.4 LABEL REQUIREMENTS FOR BOTANICAL SUPPLEMENTS AND OTHER MATERIALS IN TRADE

In the United States, the FDA [23] regulates closely how the labels of botanical supplements and other materials in trade have to be structured. The common or usual name of ingredients of dietary supplements that are botanicals (including fungi and algae) shall be consistent with the names standardized in *Herbs of Commerce* [24].

The listing of these names on the label needs to be followed by statements of the following:

1. The part of the plant (e.g., root, leaves) from which the dietary ingredient is derived (e.g., "Garlic bulb" or "Garlic (bulb)"), except that this designation is not required for algae. The name of the part of the plant must be expressed in English (e.g., "flower" rather than "flos").
2. The Latin binomial name of the plant, in parentheses, except that this name is not required when it is available in *Herbs of Commerce* [24] for the common or usual name listed on the label, and, when required, the Latin binomial name may be listed before the part of the plant. Any name in Latin form needs to be in accordance with internationally accepted rules on nomenclature, such as those found in the *International Code of Botanical Nomenclature*, and include the designation of the author or authors who published the Latin name, when a positive identification cannot be made in its absence.
3. On labels of single-ingredient dietary supplements that do not include an ingredient list, the identification of the Latin binomial name, when needed, and the part of the plant may be prominently placed on the principal display panel or information panel, or included in the nutrition label.

An example for the correct identification and labeling flow needed to produce a voucher as basis for legal supplement identification is given in Ref. [15].

4.5 THE PROBLEM OF LACKING VOUCHERS AND INCORRECT IDENTIFICATION

Voucher specimens are of tremendous importance in herbal medicine use and research. The intraspecific (within-species) variation of phytochemicals has already been mentioned above. This variation can have significant influence on the activity and efficacy of a certain plant and preparation, and only careful vouchering of the original source allows tracing activity back to the correct plant material. Eisenman et al. [24] mention such variation for commercial preparations of *Ginkgo biloba* L.

Studies on herbal medicine frequently cite a lack of efficacy. An often-referenced example is *Echinacea purpurea* (L.) Moench. A variety of researchers found lack of efficacy in preparations of *E. purpurea* [25–29]. However, none of the studies relied on vouchered material, and thus the results are impossible to reproduce. However, such studies are often used by industry to discredit the efficacy of herbal medicine. In the case of *E. purpurea*, initial studies found compounds that exhibited bioactivity but later turned out to be compounds of *Parthenium integrifolium* L. [24].

Our own research group documented the need for vouchers to assess adulterations in herbal medicine in botanical trade [3]. Material sold as *Arnica montana* L. was often contaminated with, or replaced by, *Heterothe cainuloides* Cass.; *Matricaria chamomilla* L. was often adulterated with species of *Anthemis* (possibly causing allergic reactions), *Scutellaria laterifolia* L. (Skullcap) turned out to be adulterated with *Teucrium canadense* L. (which is hepatotoxic), and *I. verum* Hook. f. was adulterated with *I. anisatum* (which is also toxic). None of these adulterations could have been identified without the availability of vouchered material for comparison.

4.6 PROBLEMS WITH THE LACK OF TAXONOMIC ATTENTION

Latin names are often seen as anachronistic and cumbersome. Many authors are not used to Latin spelling [18]. Using a misspelled name in a bibliographic

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)

Old classification (Bussmann & Sharon 2006 [21])		Classification accoring to APG-3		
Acanthaceae	*Aphelandra cirsioides Lindau*	**Acanthaceae**	*Aphelandra cirsioides Lindau*	
Adiantaceae	*Adiantum concinnum Wild. ex H.B.K.*	**Pteridaceae**	*Adiantum concinnum Wild. ex H.B.K.*	**Change to APG-3**
Adiantaceae	*Pellaea ternifolia C. Chr.*	**Pteridaceae**	*Pellaea ternifolia C. Chr.*	**Change to APG-3**
Aizoaceae	*Tetragonia crystallina L'Herit*	**Aizoaceae**	*Tetragonia crystallina L'Herit*	
Alstroemeriaceae	*Bomarea angustifolia Benth.*	**Alstroemeriaceae**	*Bomarea angustifolia Benth.*	
Alstroemeriaceae	*Bomarea dulcis (Hook.) Beauv.*	**Alstroemeriaceae**	*Bomarea dulcis (Hook.) Beauv.*	
Amaranthaceae	*Alternanthera brasiliana (L.) Kuntze*	**Amaranthaceae**	*Alternanthera brasiliana (L.) Kuntze*	
Amaranthaceae	*Alternanthera halmifolia (Lam.) Standley & Pittier*	**Amaranthaceae**	*Alternanthera halmifolia (Lam.) Standley & Pittier*	
Amaranthaceae	*Alternanthera porrigens (Jacquin) Kuntze*	**Amaranthaceae**	*Alternanthera porrigens (Jacquin) Kuntze*	
Amaranthaceae	*Alternanthera villosa H.B.K.*	**Amaranthaceae**	*Alternanthera villosa H.B.K.*	
Amaranthaceae	*Amaranthus caudatus L.*	**Amaranthaceae**	*Amaranthus caudatus L.*	
Amaranthaceae	*Amaranthus hybridus L.*	**Amaranthaceae**	*Amaranthus hybridus L.*	
Amaranthaceae	*Iresine diffusa H.B.K. ex Willd.*	**Amaranthaceae**	*Iresine diffusa H.B.K. ex Willd.*	
Amaranthaceae	*Iresine herbstii Lindley*	**Amaranthaceae**	*Iresine herbstii Lindley*	
Amaryllidaceae	*Eustephia coccinea Cav.*	**Amaryllidaceae**	*Eustephia coccinea Cav.*	
Anacardiaceae	*Annacardium occcidentale L.*	**Anacardiaceae**	*Annacardium occcidentale L.*	
Anacardiaceae	*Loxopterygium huasango Spruce ex Engl.*	**Anacardiaceae**	*Loxopterygium huasango Spruce ex Engl.*	
Anacardiaceae	*Mangifera indica L.*	**Anacardiaceae**	*Mangifera indica L.*	
Anacardiaceae	*Mauria heterophylla H.B.K.*	**Anacardiaceae**	*Mauria heterophylla H.B.K.*	
Anacardiaceae	*Schinus molle L.*	**Anacardiaceae**	*Schinus molle L.*	
Annonaceae	*Annona muricata L.*	**Annonaceae**	*Annona muricata L.*	
Apiaceae	*Ammi visnaga (L.) Lam.*	**Apiaceae**	*Ammi visnaga (L.) Lam.*	
Apiaceae	*Apium graveolens L.*	**Apiaceae**	*Apium graveolens L.*	
Apiaceae	*Arracacia xanthorrhiza Bancroft*	**Apiaceae**	*Arracacia xanthorrhiza Bancroft*	
Apiaceae	*Coriandrum sativum L.*	**Apiaceae**	*Coriandrum sativum L.*	
Apiaceae	*Daucus montanus H. & B. ex Spreng.*	**Apiaceae**	*Daucus montanus H. & B. ex Spreng.*	
Apiaceae	*Foeniculum vulgare P. Miller*	**Apiaceae**	*Foeniculum vulgare P. Miller*	
Apiaceae	*Niphogeton dissecta (Benth.) Macbr.*	**Apiaceae**	*Niphogeton dissecta (Benth.) Macbr.*	
Apiaceae	*Petroselinum crispum (Miller) A.W. Hill*	**Apiaceae**	*Petroselinum crispum (Miller) A.W. Hill*	
Apiaceae	*Pimpinella anisum L.*	**Apiaceae**	*Pimpinella anisum L.*	
Apiaceae	*Hydrocotyle bonariensis Commerson ex Lam.*	**Araliaceae**	*Hydrocotyle bonariensis Commerson ex Lam.*	**Wrong family**
Apiaceae	*Hydrocotyle globiflora R. & P.*	**Araliaceae**	*Hydrocotyle globiflora R. & P.*	**Wrong family**
Apocynaceae	*Mandevilla antennacea (A.DC.) Schum.*	**Apocynaceae**	*Mandevilla antennacea (A.DC.) Schum.*	

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Apocynaceae	*Mandevilla cf. trianae Woodson*	**Apocynaceae**	*Mandevilla cf. trianae Woodson*	
Apocynaceae	*Nerium oleander L.*	**Apocynaceae**	*Nerium oleander L.*	
Apocynaceae	*Thevetia peruviana (Pers.) Schum.*	**Apocynaceae**	*Thevetia peruviana (Pers.) Schum.*	
Apocynaceae	*Vallesia glabra (Cav.) Link.*	**Apocynaceae**	*Vallesia glabra (Cav.) Link.*	
Aquifoliaceae	*Ilex guayusa Loes*	**Aquifoliaceae**	*Ilex guayusa Loes*	
Araliaceae	*Oreopanax eriocephalus Harms*	**Araliaceae**	*Oreopanax eriocephalus Harms*	
Araucariaceae	*Araucaria heterophylla (Salisb.) Franco*	**Araucariaceae**	*Araucaria heterophylla (Salisb.) Franco*	
Arecaceae	*Bactris spp.*	**Arecaceae**	*Bactris spp.*	
Arecaceae	*Cocos nucifera L.*	**Arecaceae**	*Cocos nucifera L.*	
Aristolochiaceae	*Aristolochia ruiziana (Klotzsch) Zahlbr.*	**Aristolochiaceae**	*Aristolochia ruiziana (Klotzsch) Zahlbr.*	
Asclepiadaceae	*Sarcostemma clausum (Jacquin) Schultes*	**Apocynaceae**	*Sarcostemma clausum (Jacquin) Schultes*	**Wrong family**
Asphodelaceae	*Aloe vera (L.) Burm f.*	**Xanthorrhoeaceae**	*Aloe vera (L.) Burm f.*	**Change to APG-3**
Asteraceae	*Acanthoxanthium spinosum (L.) Furreau*	**Asteraceae**	*Acanthoxanthium spinosum (L.) Furreau*	
Asteraceae	*Achillea millefolium L.*	**Asteraceae**	*Achillea millefolium L.*	
Asteraceae	*Achyrocline alata (H.B.K.) DC.*	**Asteraceae**	*Achyrocline alata (H.B.K.) DC.*	
Asteraceae	*Acmella cf. ciliata (H.B.K.) Cas.*	**Asteraceae**	*Acmella cf. ciliata (H.B.K.) Cas.*	
Asteraceae	*Ambrosia arborescens Miller*	**Asteraceae**	*Ambrosia arborescens Miller*	
Asteraceae	*Ambrosia peruviana Willd.*	**Asteraceae**	*Ambrosia peruviana Willd.*	
Asteraceae	*Arctium lappa L.*	**Asteraceae**	*Arctium lappa L.*	
Asteraceae	*Arnica montana L.*	**Asteraceae**	*Arnica montana L.*	
Asteraceae	*Artemisia absinthium L.*	**Asteraceae**	*Artemisia absinthium L.*	
Asteraceae	*Baccharis caespitosa (R. & P.) Pers. var. alpina (H.B.K.) Cuatr.*	**Asteraceae**	*Baccharis caespitosa (R. & P.) Pers. var. alpina (H.B.K.) Cuatr.*	
Asteraceae	*Baccharis ciliaris (Retz.) Koeler*	**Asteraceae**	*Baccharis ciliaris (Retz.) Koeler*	
Asteraceae	*Baccharis genistelloides (Lam.) Pers.*	**Asteraceae**	*Baccharis genistelloides (Lam.) Pers.*	
Asteraceae	*Baccharis glutinosa Persoon*	**Asteraceae**	*Baccharis glutinosa Persoon*	
Asteraceae	*Baccharis inidica (L.) Gaert*	**Asteraceae**	*Baccharis inidica (L.) Gaert*	
Asteraceae	*Baccharis latifolia (R. & P.) Pers.*	**Asteraceae**	*Baccharis latifolia (R. & P.) Pers.*	
Asteraceae	*Baccharis odorata H.B.K.*	**Asteraceae**	*Baccharis odorata H.B.K.*	
Asteraceae	*Baccharis salicifolia (R. & P.) Pers.*	**Asteraceae**	*Baccharis salicifolia (R. & P.) Pers.*	
Asteraceae	*Baccharis vaccinioides H.B.K.*	**Asteraceae**	*Baccharis vaccinioides H.B.K.*	
Asteraceae	*Bidens pilosa L.*	**Asteraceae**	*Bidens pilosa L.*	
Asteraceae	*Chuquiraga spinosa sp. huamanpinta C. Ezcurra*	**Asteraceae**	*Chuquiraga spinosa sp. huamanpinta C. Ezcurra*	
Asteraceae	*Chuquiragua weberbaueri Tovar*	**Asteraceae**	*Chuquiragua weberbaueri Tovar*	
Asteraceae	*Clibadium cf. sylvestre (Aubl.) Baill.*	**Asteraceae**	*Clibadium cf. sylvestre (Aubl.) Baill.*	
Asteraceae	*Cronquistianthus lavandulifolius DC.*	**Asteraceae**	*Cronquistianthus lavandulifolius DC.*	
Asteraceae	*Cynara cardunculus L.*	**Asteraceae**	*Cynara cardunculus L.*	

Continued

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Asteraceae	*Diplostephium gynoxyoides Cuatr.*	**Asteraceae**	*Diplostephium gynoxyoides Cuatr.*
Asteraceae	*Diplostephium sagasteguii Cuatrecasas*	**Asteraceae**	*Diplostephium sagasteguii Cuatrecasas*
Asteraceae	*Eupatorium gayanum Wedd.*	**Asteraceae**	*Eupatorium gayanum Wedd.*
Asteraceae	*Eupatorium triplinerve Wedd.*	**Asteraceae**	*Eupatorium triplinerve Wedd.*
Asteraceae	*Ferryanthus verbascifolius (H.B.K.) H. Robinson & Brettell*	**Asteraceae**	*Ferryanthus verbascifolius (H.B.K.) H. Robinson & Brettell*
Asteraceae	*Flaveria bidentis (L.) Kuntze*	**Asteraceae**	*Flaveria bidentis (L.) Kuntze*
Asteraceae	*Gnaphalium americanum Mill.*	**Asteraceae**	*Gnaphalium americanum Mill.*
Asteraceae	*Lactuca sativa L.*	**Asteraceae**	*Lactuca sativa L.*
Asteraceae	*Loricaria ferruginea (R. & P.) Wedd.*	**Asteraceae**	*Loricaria ferruginea (R. & P.) Wedd.*
Asteraceae	*Loricaria pauciflora Cuatr.*	**Asteraceae**	*Loricaria pauciflora Cuatr.*
Asteraceae	*Matricaria frigidum (HBK) Kunth*	**Asteraceae**	*Matricaria frigidum (HBK) Kunth*
Asteraceae	*Matricaria recutita L.*	**Asteraceae**	*Matricaria recutita L.*
Asteraceae	*Mikania leiostachya Benth.*	**Asteraceae**	*Mikania leiostachya Benth.*
Asteraceae	*Monactis flaverioides H.B.K.*	**Asteraceae**	*Monactis flaverioides H.B.K.*
Asteraceae	*Munnozia lyrata (A. Gray.) Rob. & Brett.*	**Asteraceae**	*Munnozia lyrata (A. Gray.) Rob. & Brett.*
Asteraceae	*Onoseris odorata (D. Don) Hooker & Arnott*	**Asteraceae**	*Onoseris odorata (D. Don) Hooker & Arnott*
Asteraceae	*Oritrophium peruvianum (Lam.) Cuatrec.*	**Asteraceae**	*Oritrophium peruvianum (Lam.) Cuatrec.*
Asteraceae	*Paranephelius uniflorus Poepp. & Endl.*	**Asteraceae**	*Paranephelius uniflorus Poepp. & Endl.*
Asteraceae	*Perezia multiflora (H. & B.) Lessing*	**Asteraceae**	*Perezia multiflora (H. & B.) Lessing*
Asteraceae	*Perezia pungens (H.B.K.) Cas.*	**Asteraceae**	*Perezia pungens (H.B.K.) Cas.*
Asteraceae	*Picrosia longifolia D. Don*	**Asteraceae**	*Picrosia longifolia D. Don*
Asteraceae	*Porophyllum ruderale (Jacq.) Cas.*	**Asteraceae**	*Porophyllum ruderale (Jacq.) Cas.*
Asteraceae	*Pseudogynoxis cordifolia (Cass.) Cabr.*	**Asteraceae**	*Pseudogynoxis cordifolia (Cass.) Cabr.*
Asteraceae	*Schkuhria pinnata (Lam.) Kuntze*	**Asteraceae**	*Schkuhria pinnata (Lam.) Kuntze*
Asteraceae	*Senecio canescens (H.B.K.) Cuatrecasas*	**Asteraceae**	*Senecio canescens (H.B.K.) Cuatrecasas*
Asteraceae	*Senecio chinogeton Wedd.*	**Asteraceae**	*Senecio chinogeton Wedd.*
Asteraceae	*Senecio genisianus Cuatr.*	**Asteraceae**	*Senecio genisianus Cuatr.*
Asteraceae	*Senecio hypsandinus Cuatr.*	**Asteraceae**	*Senecio hypsandinus Cuatr.*
Asteraceae	*Senecio pseudotites Grieseb.*	**Asteraceae**	*Senecio pseudotites Grieseb.*
Asteraceae	*Senecio tephrosioides Turcz.*	**Asteraceae**	*Senecio tephrosioides Turcz.*
Asteraceae	*Smallanthus sonchifolius (Poepp. & Endl) H. Rob.*	**Asteraceae**	*Smallanthus sonchifolius (Poepp. & Endl) H. Rob.*
Asteraceae	*Sonchus oleraceus L.*	**Asteraceae**	*Sonchus oleraceus L.*
Asteraceae	*Spilanthes leiocarpa DC.*	**Asteraceae**	*Spilanthes leiocarpa DC.*
Asteraceae	*Tagetes elliptica Sm.*	**Asteraceae**	*Tagetes elliptica Sm.*
Asteraceae	*Tagetes erecta L.*	**Asteraceae**	*Tagetes erecta L.*
Asteraceae	*Tagetes filifolia Lag.*	**Asteraceae**	*Tagetes filifolia Lag.*

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Asteraceae	*Tagetes patula L.*	**Asteraceae**	*Tagetes patula L.*
Asteraceae	*Taraxacum officinale Wiggers*	**Asteraceae**	*Taraxacum officinale Wiggers*
Asteraceae	*Tesaria integrifolia R. & P.*	**Asteraceae**	*Tesaria integrifolia R. & P.*
Asteraceae	*Trixis cacalioides H.B.K.*	**Asteraceae**	*Trixis cacalioides H.B.K.*
Asteraceae	*Weddelia latifolia DC.*	**Asteraceae**	*Weddelia latifolia DC.*
Asteraceae	*Werneria humilis H.B.K.*	**Asteraceae**	*Werneria humilis H.B.K.*
Asteraceae	*Werneria pygmaea H. & A.*	**Asteraceae**	*Werneria pygmaea H. & A.*
Asteraceae	*Werneria villosa A. Gray*	**Asteraceae**	*Werneria villosa A. Gray*
Balanophoraceae	*Corynaea crassa Hook. f.*	**Balanophoraceae**	*Corynaea crassa Hook. f.*
Berberidaceae	*Berberis buceronis J.F. Macbride*	**Berberidaceae**	*Berberis buceronis J.F. Macbride*
Betulaceae	*Alnus acuminata H.B.K.*	**Betulaceae**	*Alnus acuminata H.B.K.*
Bignoniaceae	*Crescentia cujete L.*	**Bignoniaceae**	*Crescentia cujete L.*
Bignoniaceae	*Cydista aequinoctialis (L.) Miers*	**Bignoniaceae**	*Cydista aequinoctialis (L.) Miers*
Bignoniaceae	*Jacaranda acutifolia H. & B.*	**Bignoniaceae**	*Jacaranda acutifolia H. & B.*
Bignoniaceae	*Tynnanthus scabra (Hoffm. ex Roem. & Schult.) Schum.*	**Bignoniaceae**	*Tynnanthus scabra (Hoffm. ex Roem. & Schult.) Schum.*
Bixaceae	*Bixa orellana L.*	**Bixaceae**	*Bixa orellana L.*
Boraginaceae	*Borrago officinalis L.*	**Boraginaceae**	*Borrago officinalis L.*
Boraginaceae	*Cordia alliodora (R. & P.) Oken*	**Boraginaceae**	*Cordia alliodora (R. & P.) Oken*
Boraginaceae	*Cordia lutea Lam.*	**Boraginaceae**	*Cordia lutea Lam.*
Boraginaceae	*Heliotropium curasavicum L.*	**Boraginaceae**	*Heliotropium curasavicum L.*
Boraginaceae	*Tiquilia paronychoides (Phil.) Rich.*	**Boraginaceae**	*Tiquilia paronychoides (Phil.) Rich.*
Brassicaceae	*Brassica oleracea L. f. sp. capitata*	**Brassicaceae**	*Brassica oleracea L. f. sp. capitata*
Brassicaceae	*Brassica rapa L.*	**Brassicaceae**	*Brassica rapa L.*
Brassicaceae	*Capsella bursa-pastoris (L.) Medic.*	**Brassicaceae**	*Capsella bursa-pastoris (L.) Medic.*
Brassicaceae	*Lepidium virginicum L.*	**Brassicaceae**	*Lepidium virginicum L.*
Brassicaceae	*Raphanus sativus L.*	**Brassicaceae**	*Raphanus sativus L.*
Brassicaceae	*Rorippa nasturtium-aquaticum (L.) Hayek*	**Brassicaceae**	*Rorippa nasturtium-aquaticum (L.) Hayek*
Bromeliaceae	*Ananas comosus (L.) Merrill*	**Bromeliaceae**	*Ananas comosus (L.) Merrill*
Bromeliaceae	*Puya hamata L.B. Sm.*	**Bromeliaceae**	*Puya hamata L.B. Sm.*
Bromeliaceae	*Puya weberbaueri Mez.*	**Bromeliaceae**	*Puya weberbaueri Mez.*
Bromeliaceae	*Tillandsia cacticola L.B. Sm.*	**Bromeliaceae**	*Tillandsia cacticola L.B. Sm.*
Bromeliaceae	*Tillandsia multiflora Bentham. var. decipiens (Andre) Sm.*	**Bromeliaceae**	*Tillandsia multiflora Bentham. var. decipiens (Andre) Sm.*
Burseraceae	*Bursera graveolens (H.B.K.) Triana & Planchon*	**Burseraceae**	*Bursera graveolens (H.B.K.) Triana & Planchon*
Burseraceae	*Commiphora myrrha (T. Nees) Engl.*	**Burseraceae**	*Commiphora myrrha (T. Nees) Engl.*
Cactaceae	*Echinopsis pachanoi (Britton & Rose) Friedrich & G. Rowley*	**Cactaceae**	*Echinopsis pachanoi (Britton & Rose) Friedrich & G. Rowley*
Cactaceae	*Opuntia ficus-indica (L.) Miller*	**Cactaceae**	*Opuntia ficus-indica (L.) Miller*

Continued

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Campanulaceae	*Centropogon articulatus Drake*	**Campanulaceae**	*Centropogon articulatus Drake*	
Campanulaceae	*Centropogon cf. cornutus L.*	**Campanulaceae**	*Centropogon cf. cornutus L.*	
Campanulaceae	*Centropogon cf. rufus. Wimm.*	**Campanulaceae**	*Centropogon cf. rufus. Wimm.*	
Campanulaceae	*Lobelia decurrens Cavaniles*	**Campanulaceae**	*Lobelia decurrens Cavaniles*	
Campanulaceae	*Siphocampylus angustiflorus Schlechtendal*	**Campanulaceae**	*Siphocampylus angustiflorus Schlechtendal*	
Campanulaceae	*Siphocampylus cutervensis A. Zahlbr.*	**Campanulaceae**	*Siphocampylus cutervensis A. Zahlbr.*	
Campanulaceae	*Siphocampylus tupaeformis Zahlbr.*	**Campanulaceae**	*Siphocampylus tupaeformis Zahlbr.*	
Capparidaceae	*Capparis crotonoides H.B.K.*	**Capparaceae**	*Capparis crotonoides H.B.K.*	**Spelling mistake**
Capparidaceae	*Capparis scabrida Kunth*	**Capparaceae**	*Capparis scabrida Kunth*	**Spelling mistake**
Caprifoliaeae	*Sambucus nigra L.*	**Adoxaceae**	*Sambucus nigra L.*	**Change to APG-3**
Caprifoliaeae	*Sambucus peruviana H.B.K.*	**Adoxaceae**	*Sambucus peruviana H.B.K.*	**Change to APG-3**
Caprifoliaeae	*Lonicera japonica Thunberg*	**Caprifoliaeae**	*Lonicera japonica Thunberg*	
Caricaceae	*Carica papaya L.*	**Caricaceae**	*Carica papaya L.*	
Caricaceae	*Jacartia digitata (Poepp. & Endl.) Solms-Lang.*	**Caricaceae**	*Jacartia digitata (Poepp. & Endl.) Solms-Lang.*	
Caryophyllaceae	*Dianthus caryophyllus L.*	**Caryophyllaceae**	*Dianthus caryophyllus L.*	
Caryophyllaceae	*Dianthus caryophyllus L.*	**Caryophyllaceae**	*Dianthus caryophyllus L.*	
Caryophyllaceae	*Stellaria media (L.) Criollo*	**Caryophyllaceae**	*Stellaria media (L.) Criollo*	
Chenopodiaceae	*Chenopodium ambrosioides L.*	**Amaranthaceae**	*Chenopodium ambrosioides L.*	**Change to APG-3**
Chenopodiaceae	*Chenopodium quinoa Willd. (wild form)*	**Amaranthaceae**	*Chenopodium quinoa Willd. (wild form)*	**Change to APG-3**
Chenopodiaceae	*Chenopodium quinoa Willd.*	**Amaranthaceae**	*Chenopodium quinoa Willd.*	**Change to APG-3**
Chloranthaceae	*Hedyosmum racemosum (R. & P.) G. Don.*	**Chloranthaceae**	*Hedyosmum racemosum (R. & P.) G. Don.*	
Chrysobalanaceae	*Coupeia sp.*	**Chrysobalanaceae**	*Couepia sp.*	**Spelling mistake Coupeia**
Clethraceae	*Clethra castaneifolia Meissner*	**Clethraceae**	*Clethra castaneifolia Meissner*	**Change to APG-3**
Clusiaceae	*Mammea americana L.*	**Calophyllaceae**	*Mammea americana L.*	**Change to APG-3**
Clusiaceae	*Clusia minor L.*	**Clusiaceae**	*Clusia minor L.*	
Clusiaceae	*Hypericum aciculare Kunth.*	**Hypericeae**	*Hypericum aciculare Kunth.*	**Change to APG-3**
Clusiaceae	*Hypericum laricifolium Jus.*	**Hypericeae**	*Hypericum laricifolium Jus.*	**Change to APG-3**
Clusiaceae	*Hypericum silenioides Jus.*	**Hypericeae**	*Hypericum silenioides Jus.*	**Change to APG-3**
Convolvulaceae	*Ipomoea batatas (L.) Lamarck*	**Convolvulaceae**	*Ipomoea batatas (L.) Lamarck*	
Convolvulaceae	*Ipomoea pauciflora M. Martens & Galeotti*	**Convolvulaceae**	*Ipomoea pauciflora M. Martens & Galeotti*	
Crassulaceae	*Echeveria peruviana Meyen*	**Crassulaceae**	*Echeveria peruviana Meyen*	
Cucurbitaceae	*Citrullus lanatus (Thunberg) Matsumura & Nakai*	**Cucurbitaceae**	*Citrullus lanatus (Thunberg) Matsumura & Nakai*	
Cucurbitaceae	*Cucumis dipsaceus Ehrenb.*	**Cucurbitaceae**	*Cucumis dipsaceus Ehrenb.*	
Cucurbitaceae	*Cucumis sativus L.*	**Cucurbitaceae**	*Cucumis sativus L.*	
Cucurbitaceae	*Cucurbita maxina Duch.*	**Cucurbitaceae**	*Cucurbita maxina Duch.*	
Cucurbitaceae	*Cucurbita moschata Duch.*	**Cucurbitaceae**	*Cucurbita moschata Duch.*	
Cucurbitaceae	*Cyclanthera pedata (L.) Schrad.*	**Cucurbitaceae**	*Cyclanthera pedata (L.) Schrad.*	

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Cucurbitaceae	*Sechium edule Swartz.*	**Cucurbitaceae**	*Sechium edule Swartz.*	
Cucurbitaceae	*Sicana odorifera (Vell.) Naud.*	**Cucurbitaceae**	*Sicana odorifera (Vell.) Naud.*	
Cucurbitaceae	*Sycos baderoa H. et A.*	**Cucurbitaceae**	*Sycos baderoa H. et A.*	
Cupressaceae	*Cupressus lusitanica Miller*	**Cupressaceae**	*Cupressus lusitanica Miller*	
Cuscutaceae	*Cuscuta foetida H.B.K.*	**Convolvulaceae**	*Cuscuta foetida H.B.K.*	**Change to APG-3**
Cyperaceae	*Cyperus articulatus L.*	**Cyperaceae**	*Cyperus articulatus L.*	
Cyperaceae	*Kyllingia pumila Michx.*	**Cyperaceae**	*Kyllingia pumila Michx.*	
Cyperaceae	*Oreobolos goeppingeri Sues*	**Cyperaceae**	*Oreobolos goeppingeri Sues*	
Cyperaceae	*Scirpus californicus (C.A. Meyer) Steudel subsp. tatora (Kunth) T. Koyama*	**Cyperaceae**	*Scirpus californicus (C.A. Meyer) Steudel subsp. tatora (Kunth) T. Koyama*	
Dioscoreaceae	*Dioscorea tambillensis Kunth*	**Dioscoreaceae**	*Dioscorea tambillensis Kunth*	
Dioscoreaceae	*Dioscorea trifida L.f.*	**Dioscoreaceae**	*Dioscorea trifida L.f.*	
Dipsacaceae	*Dipsacus jallonum L.*	**Caprifoliaceae**	*Dipsacus jallonum L.*	**Change to APG-3**
Dipsacaceae	*Scabiosa atropurpurea L.*	**Caprifoliaceae**	*Scabiosa atropurpurea L.*	**Change to APG-3**
Elaeocarpaceae	*Vallea stipularis L.f.*	**Elaeocarpaceae**	*Vallea stipularis L.f.*	
Ephedraceae	*Ephedra americana H. & B.*	**Ephedraceae**	*Ephedra americana H. & B.*	
Equisetaceae	*Equisetum bogotense (H.B.K.) Kunth*	**Equisetaceae**	*Equisetum bogotense (H.B.K.) Kunth*	
Equisetaceae	*Equisetum giganteum (Wedd.) Ulbrich*	**Equisetaceae**	*Equisetum giganteum (Wedd.) Ulbrich*	
Ericaceae	*Bejaria aestuans L.*	**Ericaceae**	*Bejaria aestuans L.*	
Ericaceae	*Gaultheria erecta Vent.*	**Ericaceae**	*Gaultheria erecta Vent.*	
Ericaceae	*Gaultheria reticulata H.B.K.*	**Ericaceae**	*Gaultheria reticulata H.B.K.*	
Eriocaulaceae	*Paepalanthus ensifolius Kunth*	**Eriocaulaceae**	*Paepalanthus ensifolius Kunth*	
Erythroxylaceae	*Erythroxylum coca Lam.*	**Erythroxylaceae**	*Erythroxylum coca Lam.*	
Euphorbiaceae	*Acalypha mandonii Muell.-Arg.*	**Euphorbiaceae**	*Acalypha mandonii Muell.-Arg.*	
Euphorbiaceae	*Chamaesyce hypericifolia (L.) Millspaugh*	**Euphorbiaceae**	*Chamaesyce hypericifolia (L.) Millspaugh*	
Euphorbiaceae	*Croton draconoides Muell.-Arg.*	**Euphorbiaceae**	*Croton draconoides Muell.-Arg.*	
Euphorbiaceae	*Croton lechleri Muell. Arg.*	**Euphorbiaceae**	*Croton lechleri Muell. Arg.*	
Euphorbiaceae	*Hura crepitans L.*	**Euphorbiaceae**	*Hura crepitans L.*	
Euphorbiaceae	*Jatropa curcas L.*	**Euphorbiaceae**	*Jatropa curcas L.*	
Euphorbiaceae	*Jatropa gosypiifolia L.*	**Euphorbiaceae**	*Jatropa gosypiifolia L.*	
Euphorbiaceae	*Jatropa multifida L.*	**Euphorbiaceae**	*Jatropa multifida L.*	
Euphorbiaceae	*Manhiot esculenta Crantz*	**Euphorbiaceae**	*Manhiot esculenta Crantz*	
Euphorbiaceae	*Ricinus communis L.*	**Euphorbiaceae**	*Ricinus communis L.*	
Euphorbiaceae	*Phyllanthus niruri L.*	**Phyllanthaceae**	*Phyllanthus niruri L.*	**Change to APG-3**
Euphorbiaceae	*Phyllanthus stipulatus (Raf.) Webster*	**Phyllanthaceae**	*Phyllanthus stipulatus (Raf.) Webster*	**Change to APG-3**
Euphorbiaceae	*Phyllanthus urinaria L.*	**Phyllanthaceae**	*Phyllanthus urinaria L.*	**Change to APG-3**
Fabaceae	*Acacia macracantha H. & B. ex Willd.*	**Fabaceae**	*Acacia macracantha H. & B. ex Willd.*	
Fabaceae	*Caesalpinia paipai R. & P.*	**Fabaceae**	*Caesalpinia paipai R. & P.*	

Continued

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Fabaceae	*Caesalpinia spinosa (Molina) Kuntze*	**Fabaceae**	*Caesalpinia spinosa (Molina) Kuntze*
Fabaceae	*Cajanus cajan (L.) Millsp.*	**Fabaceae**	*Cajanus cajan (L.) Millsp.*
Fabaceae	*Cassia fistula L.*	**Fabaceae**	*Cassia fistula L.*
Fabaceae	*Cicer arietinum L.*	**Fabaceae**	*Cicer arietinum L.*
Fabaceae	*Desmodium molliculum (H.B.K.) DC.*	**Fabaceae**	*Desmodium molliculum (H.B.K.) DC.*
Fabaceae	*Desmodium triflorum (L.) DC*	**Fabaceae**	*Desmodium triflorum (L.) DC*
Fabaceae	*Diodea virgata (Rich.) Amsh.*	**Fabaceae**	*Diodea virgata (Rich.) Amsh.*
Fabaceae	*Dolichos lablab L.*	**Fabaceae**	*Dolichos lablab L.*
Fabaceae	*Erythrina ormosia*	**Fabaceae**	*Erythrina ormosia*
Fabaceae	*Erythrina velutina Willdenow*	**Fabaceae**	*Erythrina velutina Willdenow*
Fabaceae	*Erythrina spp.*	**Fabaceae**	*Erythrina spp.*
Fabaceae	*Indigofera suffruticosa Miller*	**Fabaceae**	*Indigofera suffruticosa Miller*
Fabaceae	*Inga edulis C. Martius*	**Fabaceae**	*Inga edulis C. Martius*
Fabaceae	*Inga feuillei DC.*	**Fabaceae**	*Inga feuillei DC.*
Fabaceae	*Lathyrus odoratus L.*	**Fabaceae**	*Lathyrus odoratus L.*
Fabaceae	*Lens culinaris Medikus*	**Fabaceae**	*Lens culinaris Medikus*
Fabaceae	*Leucaena leucocephala (Lam.) De Wit*	**Fabaceae**	*Leucaena leucocephala (Lam.) De Wit*
Fabaceae	*Lupinus mutabilis Sweet*	**Fabaceae**	*Lupinus mutabilis Sweet*
Fabaceae	*Medicago sativa L.*	**Fabaceae**	*Medicago sativa L.*
Fabaceae	*Melilotus alba Medikus*	**Fabaceae**	*Melilotus alba Medikus*
Fabaceae	*Mimosa albida H. & B.*	**Fabaceae**	*Mimosa albida H. & B.*
Fabaceae	*Mimosa nothacacia Barneby*	**Fabaceae**	*Mimosa nothacacia Barneby*
Fabaceae	*Myroxylon balsamum (L.) Harms.*	**Fabaceae**	*Myroxylon balsamum (L.) Harms.*
Fabaceae	*Pisum sativum L.*	**Fabaceae**	*Pisum sativum L.*
Fabaceae	*Prosopis pallida (H. & B. ex Willd.) H.B.K.*	**Fabaceae**	*Prosopis pallida (H. & B. ex Willd.) H.B.K.*
Fabaceae	*Senna bicapsularis (L.) Roxburgh*	**Fabaceae**	*Senna bicapsularis (L.) Roxburgh*
Fabaceae	*Senna monilifera H.S. Irwin & Bowley*	**Fabaceae**	*Senna monilifera H.S. Irwin & Bowley*
Fabaceae	*Senna occidentalis (L.) Link.*	**Fabaceae**	*Senna occidentalis (L.) Link.*
Fabaceae	*Spartium junceum L.*	**Fabaceae**	*Spartium junceum L.*
Fabaceae	*Tamarindus indica L.*	**Fabaceae**	*Tamarindus indica L.*
Fabaceae	*Trifolium repens L.*	**Fabaceae**	*Trifolium repens L.*
Fabaceae	*Zornia reticulata Sm.*	**Fabaceae**	*Zornia reticulata Sm.*
Gentianaceae	*Coutoubea ramosa Aublet*	**Gentianaceae**	*Coutoubea ramosa Aublet*
Gentianaceae	*Gentianella bicolor (Wedd.) J. Pringle*	**Gentianaceae**	*Gentianella bicolor (Wedd.) J. Pringle*
Gentianaceae	*Gentianella bruneotricha (Gilg.) J.S. Pringle.*	**Gentianaceae**	*Gentianella bruneotricha (Gilg.) J.S. Pringle.*
Gentianaceae	*Gentianella crassicaulis J.S. Pringle*	**Gentianaceae**	*Gentianella crassicaulis J.S. Pringle*
Gentianaceae	*Gentianella dianthoides (H.B.K.) Fabris*	**Gentianaceae**	*Gentianella dianthoides (H.B.K.) Fabris*

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Gentianaceae	*Gentianella graminea (H.B.K.) Fabris*	**Gentianaceae**	*Gentianella graminea (H.B.K.) Fabris*	
Geraniaceae	*Erodium cicutarium (L.) L'Herit.*	**Geraniaceae**	*Erodium cicutarium (L.) L'Herit.*	
Geraniaceae	*Geranium ayavacense Willd ex H.B.K.*	**Geraniaceae**	*Geranium ayavacense Willd ex H.B.K.*	
Geraniaceae	*Geranium sesiliflorum Cavanilles*	**Geraniaceae**	*Geranium sesiliflorum Cavanilles*	
Geraniaceae	*Pelargonium odoratisimum (L.) L'Herit.*	**Geraniaceae**	*Pelargonium odoratisimum (L.) L'Herit.*	
Geraniaceae	*Pelargonium roseum Willd.*	**Geraniaceae**	*Pelargonium roseum Willd.*	
Hippocrateaceae	*Tontelea crassifolia (Mart.) Spreng.*	**Celastraceae**	*Tontelea crassifolia (Mart.) Spreng.*	
Illiciaceae	*Illicium verum Hook. f.*	**Schisandraceae**	*Illicium verum Hook. f.*	**Change to APG-3**
Isoetaceae	*Isoetes andina R. & P.*	**Isoetaceae**	*Isoetes andina R. & P.*	
Juglandaceae	*Juglans neotropica Diels*	**Juglandaceae**	*Juglans neotropica Diels*	
Krameriaceae	*Krameria lappacea (Dombey) Berdet & B. Simpson*	**Krameriaceae**	*Krameria lappacea (Dombey) Berdet & B. Simpson*	
Lamiaceae	*Hyptis sidifolia (L´Her.) Briq.*	**Lamiaceae**	*Hyptis sidifolia (L´Her.) Briq.*	
Lamiaceae	*Lavandula angustifolia Miller*	**Lamiaceae**	*Lavandula angustifolia Miller*	
Lamiaceae	*Lepechinia meyenii (Walpers) Epling*	**Lamiaceae**	*Lepechinia meyenii (Walpers) Epling*	
Lamiaceae	*Marrubium vulgare L.*	**Lamiaceae**	*Marrubium vulgare L.*	
Lamiaceae	*Melissa officinalis L.*	**Lamiaceae**	*Melissa officinalis L.*	
Lamiaceae	*Mentha x piperita L.*	**Lamiaceae**	*Mentha x piperita L.*	
Lamiaceae	*Mentha spicata L.*	**Lamiaceae**	*Mentha spicata L.*	
Lamiaceae	*Minthostachys mollis Griesebach*	**Lamiaceae**	*Minthostachys mollis Griesebach*	
Lamiaceae	*Ocimum basilicum L.*	**Lamiaceae**	*Ocimum basilicum L.*	
Lamiaceae	*Origanum majorana L.*	**Lamiaceae**	*Origanum majorana L.*	
Lamiaceae	*Origanum vulgare L.*	**Lamiaceae**	*Origanum vulgare L.*	
Lamiaceae	*Otholobium glandulosum (L.) Grimes*	**Lamiaceae**	*Otholobium glandulosum (L.) Grimes*	
Lamiaceae	*Rosmarinus officinalis L.*	**Lamiaceae**	*Rosmarinus officinalis L.*	
Lamiaceae	*Salvia ayavacensis H.B.K.*	**Lamiaceae**	*Salvia ayavacensis H.B.K.*	
Lamiaceae	*Salvia cuspidata R. & P.*	**Lamiaceae**	*Salvia cuspidata R. & P.*	
Lamiaceae	*Salvia discolor H.B.K.*	**Lamiaceae**	*Salvia discolor H.B.K.*	
Lamiaceae	*Salvia macrophylla Benth.*	**Lamiaceae**	*Salvia macrophylla Benth.*	
Lamiaceae	*Salvia officinalis L.*	**Lamiaceae**	*Salvia officinalis L.*	
Lamiaceae	*Salvia rosmarinifolia Hort. ex G. Don.*	**Lamiaceae**	*Salvia rosmarinifolia Hort. ex G. Don.*	
Lamiaceae	*Salvia sagitatta R. & P.*	**Lamiaceae**	*Salvia sagitatta R. & P.*	
Lamiaceae	*Salvia tubiflora R. & P.*	**Lamiaceae**	*Salvia tubiflora R. & P.*	
Lamiaceae	*Satureja pulchella (H.B.K.) Briquet*	**Lamiaceae**	*Satureja pulchella (H.B.K.) Briquet*	
Lamiaceae	*Scutellatia scutellarioides (Kunth) R. Harley*	**Lamiaceae**	*Scutellatia scutellarioides (Kunth) R. Harley*	
Lamiaceae	*Stachys lanata Jacq.*	**Lamiaceae**	*Stachys lanata Jacq.*	
Lamiaceae	*Thymus vulgaris L.*	**Lamiaceae**	*Thymus vulgaris L.*	
Lauraceae	*Aiouea dubia (H.B.K.) Mez.*	**Lauraceae**	*Aiouea dubia (H.B.K.) Mez.*	

Continued

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Lauraceae	*Cinnamonum verum J. Presl.*	**Lauraceae**	*Cinnamonum verum J. Presl.*	
Lauraceae	*Nectandra floribunda Nees*	**Lauraceae**	*Nectandra floribunda Nees*	
Lauraceae	*Nectandra reticulata (R. & P.) Mez.*	**Lauraceae**	*Nectandra reticulata (R. & P.) Mez.*	
Lauraceae	*Persea americana Mill.*	**Lauraceae**	*Persea americana Mill.*	
Lecytidaceae	*Gustavia augusta L.*	**Lecytidaceae**	*Gustavia augusta L.*	
Lemnaceae	*Lemna minuta H.B.K.*	**Araceae**	*Lemna minuta H.B.K.*	**Change to APG-3**
Liliaceae	*Allium odorum L.*	**Amaryllidaceae**	*Allium odorum L.*	
Liliaceae	*Allium sativum L.*	**Amaryllidaceae**	*Allium sativum L.*	
Liliaceae	*Dracaena fragrans Ker Gawl.*	**Asparagaceae**	*Dracaena fragrans Ker Gawl.*	**Change to APG-3**
Liliaceae	*Hesperoziphium niveum (Rav.) Rav.*	**Iridaceae**	*Hesperoxiphion niveum (Rav.) Rav.*	**Wrong spelling Hesperoziphium and change to APG-3**
Linaceae	*Linum sativum L.*	**Linaceae**	*Linum sativum L.*	
Linaceae	*Linum usitatissimum L.*	**Linaceae**	*Linum usitatissimum L.*	
Loganiaceae	*Buddleja utilis Kraenzl.*	**Scrophulariaceae**	*Buddleja utilis Kraenzl.*	**Change to APG-3**
Loranthaceae	*Psittacantus chanduyensis Eichler*	**Loranthaceae**	*Psittacantus chanduyensis Eichler*	
Loranthaceae	*Tristerix longibracteatus (Des.) Barlow & Wiens*	**Loranthaceae**	*Tristerix longibracteatus (Des.) Barlow & Wiens*	
Lycopodiaceae	*Huperzia crassa (H. & B. ex Willd.) Rothm.*	**Lycopodiaceae**	*Huperzia crassa (H. & B. ex Willd.) Rothm.*	
Lycopodiaceae	*Huperzia cf. columnaris B. Oellg.*	**Lycopodiaceae**	*Huperzia cf. columnaris B. Oellg.*	
Lycopodiaceae	*Huperzia hohenackeri (Herter) Holub*	**Lycopodiaceae**	*Huperzia hohenackeri (Herter) Holub*	
Lycopodiaceae	*Huperzia kuestneri (Nessel) B. Ollg.*	**Lycopodiaceae**	*Huperzia kuestneri (Nessel) B. Ollg.*	
Lycopodiaceae	*Huperzia reflexa (Lam.) Trevis.*	**Lycopodiaceae**	*Huperzia reflexa (Lam.) Trevis.*	
Lycopodiaceae	*Huperzia sellifolia B. Ollg.*	**Lycopodiaceae**	*Huperzia sellifolia B. Ollg.*	
Lycopodiaceae	*Huperzia tetragona (Hook. & Grev.) Trevis.*	**Lycopodiaceae**	*Huperzia tetragona (Hook. & Grev.) Trevis.*	
Lycopodiaceae	*Lycopodium clavatum L.*	**Lycopodiaceae**	*Lycopodium clavatum L.*	
Lycopodiaceae	*Lycopodium jussiaei Desv. ex Poir*	**Lycopodiaceae**	*Lycopodium jussiaei Desv. ex Poir*	
Lycopodiaceae	*Lycopodium thyoides H. & B. ex Willd.*	**Lycopodiaceae**	*Lycopodium thyoides H. & B. ex Willd.*	
Lythraceae	*Cuphea strigulosa H.B.K.*	**Lythraceae**	*Cuphea strigulosa H.B.K.*	
Malesherbiaceae	*Malesherbia ardens Macbr.*	**Passifloraceae**	*Malesherbia ardens Macbr.*	**Change to APG-3**
Malpighiaceae	*Banisteriopsis caapi (Spruce ex Grieseb.) Morton*	**Malpighiaceae**	*Banisteriopsis caapi (Spruce ex Grieseb.) Morton*	
Malvaceae	*Alcea rosea (L.) Cavanilles*	**Malvaceae**	*Alcea rosea (L.) Cavanilles*	
Malvaceae	*Gossypium barbadense L.*	**Malvaceae**	*Gossypium barbadense L.*	
Malvaceae	*Malva parviflora L.*	**Malvaceae**	*Malva parviflora L.*	
Malvaceae	*Malva sylvestris L.*	**Malvaceae**	*Malva sylvestris L.*	
Malvaceae	*Urena lobata L.*	**Malvaceae**	*Urena lobata L.*	
Melastomataceae	*Brachyotum tyrianthium Macbride*	**Melastomataceae**	*Brachyotum tyrianthium Macbride*	
Melastomataceae	*Miconia salicifolia (Bonpl. ex Naud.) Naud.*	**Melastomataceae**	*Miconia salicifolia (Bonpl. ex Naud.) Naud.*	

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Melastomataceae	*Tibouchina laxa (Des.) Cog.*	**Melastomataceae**	*Tibouchina laxa (Des.) Cog.*	
Menispermaceae	*Abuta grandiflora (Mart.) Sand.*	**Menispermaceae**	*Abuta grandiflora (Mart.) Sand.*	
Monimiaceae	*Peumus boldus Molina*	**Monimiaceae**	*Peumus boldus Molina*	
Monimiaceae	*Spiaruna aspera (R. & P.) A.DC.*	**Siparunaceae**	*Spiaruna aspera (R. & P.) A.DC.*	**Change to APG-3**
Monimiaceae	*Siparuna muricata (R. & P.) A. DC.*	**Siparunaceae**	*Siparuna muricata (R. & P.) A. DC.*	**Change to APG-3**
Moraceae	*Brosmium rubescens Taubert*	**Moraceae**	*Brosimum rubescens Taubert*	**Wrong spelling Brosmium**
Moraceae	*Ficus carica L.*	**Moraceae**	*Ficus carica L.*	
Moraceae	*Ficus spp.*	**Moraceae**	*Ficus spp.*	
Moraceae	*Morus alba L.*	**Moraceae**	*Morus alba L.*	
Musaceae	*Musa x paradisiaca L.*	**Musaceae**	*Musa x paradisiaca L.*	
Myricaceae	*Myrica pubescens H. & B. ex Wild.*	**Myricaceae**	*Myrica pubescens H. & B. ex Wild.*	
Myristicaceae	*Myristica fragrans L.*	**Myristicaceae**	*Myristica fragrans L.*	
Myrtaceae	*Eugenia obtusifolia Cambes.*	**Myrtaceae**	*Eugenia obtusifolia Cambes.*	
Myrtaceae	*Eucalyptus citriodora Hooker*	**Myrtaceae**	*Eucalyptus citriodora Hooker*	
Myrtaceae	*Eucalyptus globulus Labill.*	**Myrtaceae**	*Eucalyptus globulus Labill.*	
Myrtaceae	*Myrcianthes discolor (H.B.K.) Vaughn*	**Myrtaceae**	*Myrcianthes discolor (H.B.K.) Vaughn*	
Myrtaceae	*Myrcianthes fragrans (Sw) McVaugh*	**Myrtaceae**	*Myrcianthes fragrans (Sw) McVaugh*	
Myrtaceae	*Psidium guajava L.*	**Myrtaceae**	*Psidium guajava L.*	
Myrtaceae	*Scutia spicata (H. & B. ex Schultes) Weberb. var. spicata*	**Myrtaceae**	*Scutia spicata (H. & B. ex Schultes) Weberb. var. spicata*	
Myrtaceae	*Syzygium aromaticum (L.) Merr. & Perry*	**Myrtaceae**	*Syzygium aromaticum (L.) Merr. & Perry*	
Myrtaceae	*Syzygium jambos (L.) Alston*	**Myrtaceae**	*Syzygium jambos (L.) Alston*	
Nyctaginaceae	*Boerhavia coccinea Mill.*	**Nyctaginaceae**	*Boerhavia coccinea Mill.*	
Nyctaginaceae	*Mirabilis jalapa L.*	**Nyctaginaceae**	*Mirabilis jalapa L.*	
Olacaceae	*Heisteria acuminata (H. & B.) Engler*	**Olacaceae**	*Heisteria acuminata (H. & B.) Engler*	
Olacaceae	*Ximenia americana L.*	**Ximeniaceae**	*Ximenia americana L.*	**Change to APG-3**
Oleaceae	*Olea europaea L.*	**Oleaceae**	*Olea europaea L.*	
Onagraceae	*Epilobium sp.*	**Onagraceae**	*Epilobium sp.*	
Onagraceae	*Fuchsia ayavacensis H.B.K.*	**Onagraceae**	*Fuchsia ayavacensis H.B.K.*	
Onagraceae	*Oenothera rosea Aiton*	**Onagraceae**	*Oenothera rosea Aiton*	
Orchidaceae	*Aa paleacea (H.B.K.) Rchb. f.*	**Orchidaceae**	*Aa paleacea (H.B.K.) Rchb. f.*	
Orchidaceae	*Epidendrum calanthum Rchb. f.*	**Orchidaceae**	*Epidendrum calanthum Rchb. f.*	
Orchidaceae	*Lycaste gigantea Lindl.*	**Orchidaceae**	*Lycaste gigantea Lindl.*	
Orchidaceae	*Pachyphyllum pastii Krenzl. ex Weberb.*	**Orchidaceae**	*Pachyphyllum pastii Krenzl. ex Weberb.*	
Orchidaceae	*Stelis eublepharis Rchb. f.*	**Orchidaceae**	*Stelis eublepharis Rchb. f.*	
Orchidaceae	*Stelis sp.*	**Orchidaceae**	*Stelis sp.*	
Oxalidaceae	*Oxalis bulbigera Knuth.*	**Oxalidaceae**	*Oxalis bulbigera Knuth.*	

Continued

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Oxalidaceae	*Oxalis tuberosa Molina*	**Oxalidaceae**	*Oxalis tuberosa Molina*
Papaveraceae	*Argemone mexicana L.*	**Papaveraceae**	*Argemone mexicana L.*
Passifloraceae	*Passiflora caerulea L.*	**Passifloraceae**	*Passiflora caerulea L.*
Passifloraceae	*Passiflora edulis Sims.*	**Passifloraceae**	*Passiflora edulis Sims.*
Passifloraceae	*Passiflora ligularis Jus.*	**Passifloraceae**	*Passiflora ligularis Jus.*
Passifloraceae	*Passiflora quardrangularis L.*	**Passifloraceae**	*Passiflora quardrangularis L.*
Passifloraceae	*Passiflora sp.*	**Passifloraceae**	*Passiflora sp.*
Phytolaccaceae	*Gallesia integrifolia (Spreng.) Harms.*	**Phytolaccaceae**	*Gallesia integrifolia (Spreng.) Harms.*
Phytolaccaceae	*Petiveria alliacea L.*	**Phytolaccaceae**	*Petiveria alliacea L.*
Phytolaccaceae	*Phytolacca bogotensis H.B.K.*	**Phytolaccaceae**	*Phytolacca bogotensis H.B.K.*
Pinaceae	*Pinus patula Schldl. & Cham.*	**Pinaceae**	*Pinus patula Schldl. & Cham.*
Pinaceae	*Pinus radiata D. Don.*	**Pinaceae**	*Pinus radiata D. Don.*
Piperaceae	*Peperomia fraseri C. DC.*	**Piperaceae**	*Peperomia fraseri C. DC.*
Piperaceae	*Peperomia galioides H.B.K.*	**Piperaceae**	*Peperomia galioides H.B.K.*
Piperaceae	*Peperomia hartwegiana Miq.*	**Piperaceae**	*Peperomia hartwegiana Miq.*
Piperaceae	*Peperomia inaequalifolia R. & P.*	**Piperaceae**	*Peperomia inaequalifolia R. & P.*
Piperaceae	*Peperomia quadrifolia Trel.*	**Piperaceae**	*Peperomia quadrifolia Trel.*
Piperaceae	*Piper aduncum L.*	**Piperaceae**	*Piper aduncum L.*
Piperaceae	*Piper cf. aequale Vahl.*	**Piperaceae**	*Piper cf. aequale Vahl.*
Piperaceae	*Piper nigrum L.*	**Piperaceae**	*Piper nigrum L.*
Plantaginaceae	*Plantago linearis H.B.K.*	**Plantaginaceae**	*Plantago linearis H.B.K.*
Plantaginaceae	*Plantago major L.*	**Plantaginaceae**	*Plantago major L.*
Plantaginaceae	*Plantago sericea R. & P.*	**Plantaginaceae**	*Plantago sericea R. & P.*
Plantaginaceae	*Plantago sericea R. & P. var. lanuginosa Grieseb.*	**Plantaginaceae**	*Plantago sericea R. & P. var. lanuginosa Grieseb.*
Plantaginaceae	*Plantago sericea R. & P. subsp. sericans (Pilger) Rahn*	**Plantaginaceae**	*Plantago sericea R. & P. subsp. sericans (Pilger) Rahn*
Poaceae	*Arundo donax L.*	**Poaceae**	*Arundo donax L.*
Poaceae	*Cenchrus echinatus L.*	**Poaceae**	*Cenchrus echinatus L.*
Poaceae	*Cymbopogon citratus (DC.) Stapf.*	**Poaceae**	*Cymbopogon citratus (DC.) Stapf.*
Poaceae	*Cynodon dactylon (L.) Persoon*	**Poaceae**	*Cynodon dactylon (L.) Persoon*
Poaceae	*Digitaria ciliaris (Retz.) Koehler*	**Poaceae**	*Digitaria ciliaris (Retz.) Koehler*
Poaceae	*Gynerium sagittatum (Aublet.) P. Beauvois*	**Poaceae**	*Gynerium sagittatum (Aublet.) P. Beauvois*
Poaceae	*Hordeum vulgare L.*	**Poaceae**	*Hordeum vulgare L.*
Poaceae	*Oryza sativa L.*	**Poaceae**	*Oryza sativa L.*
Poaceae	*Saccharum officinarum L.*	**Poaceae**	*Saccharum officinarum L.*
Poaceae	*Triticum sativum L.*	**Poaceae**	*Triticum sativum L.*
Poaceae	*Zea mays L.*	**Poaceae**	*Zea mays L.*
Polemoniaceae	*Cantua buxifolia Jus. ex Lam.*	**Polemoniaceae**	*Cantua buxifolia Jus. ex Lam.*

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Polemoniaceae	*Cantua quercifolia Jus.*	**Polemoniaceae**	*Cantua quercifolia Jus.*	
Polygalaceae	*Monnina pterocarpa R. & P.*	**Polygalaceae**	*Monnina pterocarpa R. & P.*	
Polygalaceae	*Polygala paniculata L.*	**Polygalaceae**	*Polygala paniculata L.*	
Polygonaceae	*Muehlenbeckia tamnifolia (H.B.K.) Meisner*	**Polygonaceae**	*Muehlenbeckia tamnifolia (H.B.K.) Meisner*	
Polygonaceae	*Polygonum hydropiperoides Michaux*	**Polygonaceae**	*Polygonum hydropiperoides Michaux*	
Polygonaceae	*Rumex crispus L.*	**Polygonaceae**	*Rumex crispus L.*	
Polypodiaceae	*Grammitis moniliformis (Lag. ex Sw.) Proctor*	**Polypodiaceae**	*Grammitis moniliformis (Lag. ex Sw.) Proctor*	
Polypodiaceae	*Polypodium crassifolium L.*	**Polypodiaceae**	*Polypodium crassifolium L.*	
Polypodiaceae	*Cheilanthes myriophylla Desv.*	**Pteridaceae**	*Cheilanthes myriophylla Desv.*	**Change to APG-3**
Polypodiaceae	*Jamesonia goudotii (Hieron) C. Chr.*	**Pteridaceae**	*Jamesonia goudotii (Hieron) C. Chr.*	**Change to APG-3**
Polypodiaceae	*Jamesonia rotundifolia Fée*	**Pteridaceae**	*Jamesonia rotundifolia Fée*	**Change to APG-3**
Portulacaceae	*Portulaca oleracea L. subsp. tuberculata Danin & H.G. Baker*	**Portulacaceae**	*Portulaca oleracea L. subsp. tuberculata Danin & H.G. Baker*	
Portulacaceae	*Portulaca villosa H.B.K.*	**Portulacaceae**	*Portulaca villosa H.B.K.*	
Proteaceae	*Oreocallis grandiflora (Lam.) R.Br.*	**Proteaceae**	*Oreocallis grandiflora (Lam.) R.Br.*	
Punicaceae	*Punica granatum L.*	**Lythraceae**	*Punica granatum L.*	**Change to APG-3**
Ranunculaceae	*Laccopetalum giganteum (Wedd.) Ulbrich*	**Ranunculaceae**	*Laccopetalum giganteum (Wedd.) Ulbrich*	
Ranunculaceae	*Thalictrum decipiens Boivin*	**Ranunculaceae**	*Thalictrum decipiens Boivin*	
Rosaceae	*Alchemilla nivalis H.B.K.*	**Rosaceae**	*Alchemilla nivalis H.B.K.*	
Rosaceae	*Cydonia oblonga Miller*	**Rosaceae**	*Cydonia oblonga Miller*	
Rosaceae	*Fragaria vesca L.*	**Rosaceae**	*Fragaria vesca L.*	
Rosaceae	*Geum peruvianum Focke*	**Rosaceae**	*Geum peruvianum Focke*	
Rosaceae	*Polylepis racemosa R. & P.*	**Rosaceae**	*Polylepis racemosa R. & P.*	
Rosaceae	*Prunus serotina Ehrh.*	**Rosaceae**	*Prunus serotina Ehrh.*	
Rosaceae	*Prunus serotina Ehrhart subsp. capuli (Cav.) McVough*	**Rosaceae**	*Prunus serotina Ehrhart subsp. capuli (Cav.) McVough*	
Rosaceae	*Rosa centifolia L.*	**Rosaceae**	*Rosa centifolia L.*	
Rosaceae	*Rubus robustus C. Presl.*	**Rosaceae**	*Rubus robustus C. Presl.*	
Rosaceae	*Sanguisorba minor Scop.*	**Rosaceae**	*Sanguisorba minor Scop.*	
Rubiaceae	*Arcytophyllum nitidum (H.B.K.) Schlecht.*	**Rubiaceae**	*Arcytophyllum nitidum (H.B.K.) Schlecht.*	
Rubiaceae	*Cinchona officinalis L.*	**Rubiaceae**	*Cinchona officinalis L.*	
Rubiaceae	*Coffea arabica L.*	**Rubiaceae**	*Coffea arabica L.*	
Rubiaceae	*Uncaria tomentosa (Willdenow ex Roemer & Schultes) DC.*	**Rubiaceae**	*Uncaria tomentosa (Willdenow ex Roemer & Schultes) DC.*	
Rutaceae	*Citrus aurantium L.*	**Rutaceae**	*Citrus aurantium L.*	
Rutaceae	*Citrus grandis (L.) Osbeck*	**Rutaceae**	*Citrus grandis (L.) Osbeck*	
Rutaceae	*Citrus limetta Riso*	**Rutaceae**	*Citrus limetta Riso*	
Rutaceae	*Citrus limon (L.) Burm. f.*	**Rutaceae**	*Citrus limon (L.) Burm. f.*	
Rutaceae	*Citrus reticulata Blanco*	**Rutaceae**	*Citrus reticulata Blanco*	

Continued

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Rutaceae	*Citrus sinensis (L.) Osbeck*	**Rutaceae**	*Citrus sinensis (L.) Osbeck*	
Rutaceae	*Gardenia augusta (L.) Merr.*	**Rutaceae**	*Gardenia augusta (L.) Merr.*	
Rutaceae	*Ruta graveolens L.*	**Rutaceae**	*Ruta graveolens L.*	
Salicaceae	*Populus deltoides Bartram*	**Salicaceae**	*Populus deltoides Bartram*	
Salicaceae	*Salix chilensis Molina*	**Salicaceae**	*Salix chilensis Molina*	
Sapotaceae	*Pouteria lucuma (R. & P.) Kuntze.*	**Sapotaceae**	*Pouteria lucuma (R. & P.) Kuntze.*	
Saxifragaceae	*Escallonia pendula (R. & P.) Pers.*	**Saxifragaceae**	*Escallonia pendula (R. & P.) Pers.*	
Scrophulariaceae	*Calceolaria rugulosa Edwin*	**Calceolariaceae**	*Calceolaria rugulosa Edwin*	**Change to APG-3**
Scrophulariaceae	*Escobedia grandiflora (L.f.) Kuntze*	**Orobanchaceae**	*Escobedia grandiflora (L.f.) Kuntze*	**Change to APG-3**
Scrophulariaceae	*Galvesia fruticosa J. Gmelin*	**Plantaginaceae**	*Galvesia fruticosa J. Gmelin*	**Change to APG-3**
Scrophulariaceae	*Caprania peruviana Bentham*	**Scrophulariaceae**	*Capraria peruviana Bentham*	**Wrong spelling Caprania**
Smilacaceae	*Smilax kunthii Killip & Morton*	**Smilacaceae**	*Smilax kunthii Killip & Morton*	
Smilacaceae	*Smilax medica M.Martens & Galeotti*	**Smilacaceae**	*Smilax medica M.Martens & Galeotti*	
Solanaceae	*Brugmansia arborea (L.) Lagerheim*	**Solanaceae**	*Brugmansia arborea (L.) Lagerheim*	
Solanaceae	*Brugmansia candida Persoon*	**Solanaceae**	*Brugmansia candida Persoon*	
Solanaceae	*Brugmansia sanguinea (R. & P.) D. Don.*	**Solanaceae**	*Brugmansia sanguinea (R. & P.) D. Don.*	
Solanaceae	*Capsicum chinense L.*	**Solanaceae**	*Capsicum chinense L.*	
Solanaceae	*Capsicum rhomboideum (Dunal) Kunze*	**Solanaceae**	*Capsicum rhomboideum (Dunal) Kunze*	
Solanaceae	*Cestrum auriculatum L'Herit*	**Solanaceae**	*Cestrum auriculatum L'Herit*	
Solanaceae	*Cestrum nocturnum L.*	**Solanaceae**	*Cestrum nocturnum L.*	
Solanaceae	*Cestrum strigilatum R. & P.*	**Solanaceae**	*Cestrum strigilatum R. & P.*	
Solanaceae	*Cestrum undulatum R. & P.*	**Solanaceae**	*Cestrum undulatum R. & P.*	
Solanaceae	*Datura ferox L.*	**Solanaceae**	*Datura ferox L.*	
Solanaceae	*Jaltomata sp.*	**Solanaceae**	*Jaltomata sp.*	
Solanaceae	*Juanulloa ochracea Cuatrecasas*	**Solanaceae**	*Juanulloa ochracea Cuatrecasas*	
Solanaceae	*Lycopersicon esculentum Mill.*	**Solanaceae**	*Lycopersicon esculentum Mill.*	
Solanaceae	*Lycopersicon hirsutum Dunal*	**Solanaceae**	*Lycopersicon hirsutum Dunal*	
Solanaceae	*Lycopersicon peruvianum (L.) Mill.*	**Solanaceae**	*Lycopersicon peruvianum (L.) Mill.*	
Solanaceae	*Nicotiana tabacum L.*	**Solanaceae**	*Nicotiana tabacum L.*	
Solanaceae	*Solanum americanum Mill.*	**Solanaceae**	*Solanum americanum Mill.*	
Solanaceae	*Solanum mammosum L.*	**Solanaceae**	*Solanum mammosum L.*	
Solanaceae	*Solanum melongena L.*	**Solanaceae**	*Solanum melongena L.*	
Solanaceae	*Solanum sp.*	**Solanaceae**	*Solanum sp.*	
Solanaceae	*Solanum sp.*	**Solanaceae**	*Solanum sp.*	
Sterculiaceae	*Theobroma cacao L.*	**Malvaceae**	*Theobroma cacao L.*	**Change to APG-3**
Theaceae	*Camellia sinensis (L.) Kuntze*	**Theaceae**	*Camellia sinensis (L.) Kuntze*	
Thelypteridaceae	*Thelypteris cf. scalaris (Christ.) Alton*	**Thelypteridaceae**	*Thelypteris cf. scalaris (Christ.) Alton*	

TABLE 4.1 Plants from Bussmann & Sharon 2006 [21] and Changed Classificatilon Based on APG-3 (Gray Underlay)—cont'd

Thymeleaceae	*Daphnopsis weberbaueri Domke*	**Thymeleaceae**	*Daphnopsis weberbaueri Domke*	
Tiliaceae	*Mutingia calabura L.*	**Malvaceae**	*Mutingia calabura L.*	**Change to APG-3**
Tiliaceae	*Tilia platyphyllos Scop.*	**Malvaceae**	*Tilia platyphyllos Scop.*	**Change to APG-3**
Tropaeolaceae	*Tropaeolum minus L.*	**Tropaeolaceae**	*Tropaeolum minus L.*	
Typhaceae	*Typha angustifolia L.*	**Typhaceae**	*Typha angustifolia L.*	
Ulmaceae	*Celtis loxense C.C. Berg*	**Cannabaceae**	*Celtis loxense C.C. Berg*	
Urticaceae	*Pilea microphylla (L.) Lieberman*	**Urticaceae**	*Pilea microphylla (L.) Lieberman*	
Urticaceae	*Urtica magellanica A. Jussieu ex Poiret*	**Urticaceae**	*Urtica magellanica A. Jussieu ex Poiret*	
Urticaceae	*Urtica urens L.*	**Urticaceae**	*Urtica urens L.*	
Valerianaceae	*Belonanthus aff. hispidus (Wedd.) Graebn.*	**Caprifoliaceae**	*Belonanthus aff. hispidus (Wedd.) Graebn.*	**Change to APG-3**
Valerianaceae	*Phyllactis rigida (R. & P.) Persoon*	**Caprifoliaceae**	*Phyllactis rigida (R. & P.) Persoon*	**Change to APG-3**
Valerianaceae	*Valeriana bonplandiana Wedd.*	**Caprifoliaceae**	*Valeriana bonplandiana Wedd.*	**Change to APG-3**
Valerianaceae	*Valeriana plantaginea Kunth*	**Caprifoliaceae**	*Valeriana plantaginea Kunth*	**Change to APG-3**
Verbenaceae	*Clerodendron sp.*	**Lamiaceae**	*Clerodendron sp.*	**Change to APG-3**
Verbenaceae	*Aloysia triphylla (L. Her.) Britt.*	**Verbenaceae**	*Aloysia triphylla (L. Her.) Britt.*	
Verbenaceae	*Lantana scabiosaefolia H.B.K.*	**Verbenaceae**	*Lantana scabiosaefolia H.B.K.*	
Verbenaceae	*Lippia integrifolia (Grieseb.) Hieron*	**Verbenaceae**	*Lippia integrifolia (Grieseb.) Hieron*	
Verbenaceae	*Verbena littoralis H.B.K.*	**Verbenaceae**	*Verbena littoralis H.B.K.*	
Violaceae	*Viola tricolor L.*	**Violaceae**	*Viola tricolor L.*	
Vitaceae	*Vitis vinifera L.*	**Vitaceae**	*Vitis vinifera L.*	
Xyridaceae	*Xyris subulata R. & P.*	**Xyridaceae**	*Xyris subulata R. & P.*	
Zingiberaceae	*Zingiber officinale Roscoe*	**Zingiberaceae**	*Zingiber officinale Roscoe*	
Zygophyllaceae	*Tribulus terrestris L.*	**Zygophyllaceae**	*Tribulus terrestris L.*	

Note: The "BOLD WORDS" are the PLANT FAMILY NAMES (column 1 and 3) and the CHANGES IN CLASSIFICATION (column5).

search will, however, inevitably lead to a failure in retrieving relevant literature, similar to the above-cited problem with synonyms. A search for "*Coupeia*" in PubMed yields zero results, while the correct name "*Couepia*" yields six hits; much more striking, "*Brosmium*" yields no results, while the correct "*Brosimum*" yields 1720 reference hits.

Vernacular names are certainly not a solution. In our own research, the vernacular "Corpus Huay" referred to *Gentianella chamuchui* (Reimers) Fabris, *Gentianella bicolor* (Wedd.) Pringle, *Gentianella graminea* (H.B.K.) Fabris, *Gentianella thyrsoidea* (Hook.) Fabris., and three unidentified species of *Gentianella* (all Gentianaceae). All these species were sold and used by the local population as treatment for diabetes, and at least *G. thyrsoidea* is known to have serious toxic effects. Moreover, all these species are interchangeably called Hercampuri, Genciana, Chinchimali, Hornamo, Sumaran, AngaMacha, Amargon, and a variety of other vernacular names. Hornamo, however, also refers to species of *Senecio* and *Werneria nubigena* Kunth (both Asteraceae), as well as *Valeriana* (Valerianaceae), each of which is used traditionally for completely different applications. The term "Valeriana" might refer not only to different species of *Valeriana* (Valerianaceae), but also to *Geum peruvianum* (Rosaceae) and species of *Ranunculus* (Ranunculaceae), all of which can be used either for the same purpose (calmative) or for completely different applications [21,29–33]. Again, without careful vouchering it would never be possible to identify the correct species and adulterations in trade [34]. This has serious implications on efficacy and toxicity too. *Annona muricata* L. (Annonaceae) and *Psidium guajava* L. (Myrtaceae) are both known as "guanabana," but only the second species shows good

TABLE 4.2 Most Important Plant Families from Bussmann & Sharon 2006 [21] and New Classification under APG-3 (Gray Underlay)

Family Bussmann & Sharon 2006 [21]			Family APG-3		
Asteraceae	68	13.77	Asteraceae	68	13.77
Fabaceae	34	6.88	Fabaceae	34	6.88
Lamiaceae	25	5.06	Lamiaceae	26	5.26
Solanaceae	21	4.25	Solanaceae	21	4.25
Euphorbiaceae	13	2.63	Euphorbiaceae	10	2.02
Apiaceae	11	2.23	Apiaceae	9	1.82
Poaceae	11	2.23	Poaceae	11	2.23
Lycopodiaceae	10	2.02	Lycopodiaceae	10	2.02
Rosaceae	10	2.02	Rosaceae	10	2.02
Cucurbitaceae	9	1.82	Cucurbitaceae	9	1.82
Myrtaceae	9	1.82	Myrtaceae	9	1.82
Amaranthaceae	8	1.62	Amaranthaceae	11	2.23
Piperaceae	8	1.62	Piperaceae	8	1.62
Rutaceae	8	1.62	Rutaceae	8	1.62
Campanulaceae	7	1.42	Campanulaceae	7	1.42
Brassicaceae	6	1.21	Brassicaceae	6	1.21
Gentianaceae	6	1.21	Gentianaceae	6	1.21
Orchidaceae	6	1.21	Orchidaceae	6	1.21
Anacardiaceae	5	1.01	Anacardiaceae	5	1.01
Apocynaceae	5	1.01	Apocynaceae	6	1.21
Boraginaceae	5	1.01	Boraginaceae	5	1.01
Bromeliaceae	5	1.01	Bromeliaceae	5	1.01
Clusiaceae	5	1.01	Clusiaceae	1	0.20
Geraniaceae	5	1.01	Geraniaceae	5	1.01
Lauraceae	5	1.01	Lauraceae	5	1.01
Malvaceae	5	1.01	Malvaceae	8	1.62
Passifloraceae	5	1.01	Passifloraceae	6	1.21
Plantaginaceae	5	1.01	Plantaginaceae	6	1.21
Polypodiaceae	5	1.01	Polypodiaceae	2	0.40
Scrophulariaceae	5	1.01	Scrophulariaceae	3	0.61
Verbenaceae	5	1.01	Verbenaceae	4	0.81
Bignoniaceae	4	0.81	Bignoniaceae	4	0.81
Cyperaceae	4	0.81	Cyperaceae	4	0.81

TABLE 4.2 Most Important Plant Families from Bussmann & Sharon 2006 [21] and New Classification under APG-3 (Gray Underlay)—cont'd

Liliaceae	4	0.81	Liliaceae	0	0.00
Moraceae	4	0.81	Moraceae	4	0.81
Rubiaceae	4	0.81	Rubiaceae	4	0.81
Valerianaceae	4	0.81	Valerianaceae	0	0.00
Caprifoliaeae	3	0.61	Caprifoliaeae	7	1.42
Caryophyllaceae	3	0.61	Caryophyllaceae	3	0.61
Chenopodiaceae	3	0.61	Chenopodiaceae	0	0.00
Ericaceae	3	0.61	Ericaceae	3	0.61
Melastomataceae	3	0.61	Melastomataceae	3	0.61
Monimiaceae	3	0.61	Monimiaceae	1	0.20
Onagraceae	3	0.61	Onagraceae	3	0.61
Phytolaccaceae	3	0.61	Phytolaccaceae	3	0.61
Polygonaceae	3	0.61	Polygonaceae	3	0.61
Urticaceae	3	0.61	Urticaceae	3	0.61
Adiantaceae	2	0.40	Adiantaceae	0	0.00
Alstroemeriaceae	2	0.40	Alstroemeriaceae	2	0.40
Arecaceae	2	0.40	Arecaceae	2	0.40
Burseraceae	2	0.40	Burseraceae	2	0.40
Cactaceae	2	0.40	Cactaceae	2	0.40
Capparaceae	2	0.40	Capparaceae	2	0.40
Caricaceae	2	0.40	Caricaceae	2	0.40
Convolvulaceae	2	0.40	Convolvulaceae	3	0.61
Dioscoreaceae	2	0.40	Dioscoreaceae	2	0.40
Dipsacaceae	2	0.40	Dipsacaceae	0	0.00
Equisetaceae	2	0.40	Equisetaceae	2	0.40
Linaceae	2	0.40	Linaceae	2	0.40
Loranthaceae	2	0.40	Loranthaceae	2	0.40
Nyctaginaceae	2	0.40	Nyctaginaceae	2	0.40
Olacaceae	2	0.40	Olacaceae	1	0.20
Oxalidaceae	2	0.40	Oxalidaceae	2	0.40
Pinaceae	2	0.40	Pinaceae	2	0.40
Polemoniaceae	2	0.40	Polemoniaceae	2	0.40
Polygalaceae	2	0.40	Polygalaceae	2	0.40
Portulacaceae	2	0.40	Portulacaceae	2	0.40
Ranunculaceae	2	0.40	Ranunculaceae	2	0.40

Continued

TABLE 4.2 Most Important Plant Families from Bussmann & Sharon 2006 [21] and New Classification under APG-3 (Gray Underlay)—cont'd

Salicaceae	2	0.40	Salicaceae	2	0.40
Smilacaceae	2	0.40	Smilacaceae	2	0.40
Tiliaceae	2	0.40	Tiliaceae	0	0.00
Acanthaceae	1	0.20	Acanthaceae	1	0.20
Aizoaceae	1	0.20	Aizoaceae	1	0.20
Amaryllidaceae	1	0.20	Amaryllidaceae	3	0.61
Annonaceae	1	0.20	Annonaceae	1	0.20
Aquifoliaceae	1	0.20	Aquifoliaceae	1	0.20
Araliaceae	1	0.20	Araliaceae	3	0.61
Araucariaceae	1	0.20	Araucariaceae	1	0.20
Aristolochiaceae	1	0.20	Aristolochiaceae	1	0.20
Asclepiadaceae	1	0.20	Asclepiadaceae	0	0.00
Asphodelaceae	1	0.20	Asphodelaceae	0	0.00
Balanophoraceae	1	0.20	Balanophoraceae	1	0.20
Berberidaceae	1	0.20	Berberidaceae	1	0.20
Betulaceae	1	0.20	Betulaceae	1	0.20
Bixaceae	1	0.20	Bixaceae	1	0.20
Chloranthaceae	1	0.20	Chloranthaceae	1	0.20
Chrysobalanaceae	1	0.20	Chrysobalanaceae	1	0.20
Clethraceae	1	0.20	Clethraceae	1	0.20
Crassulaceae	1	0.20	Crassulaceae	1	0.20
Cupressaceae	1	0.20	Cupressaceae	1	0.20
Cuscutaceae	1	0.20	Cuscutaceae	0	0.00
Elaeocarpaceae	1	0.20	Elaeocarpaceae	1	0.20
Ephedraceae	1	0.20	Ephedraceae	1	0.20
Eriocaulaceae	1	0.20	Eriocaulaceae	1	0.20
Erythroxylaceae	1	0.20	Erythroxylaceae	1	0.20
Hippocrateaceae	1	0.20	Hippocrateaceae	0	0.00
Illiciaceae	1	0.20	Illiciaceae	0	0.00

TABLE 4.2 Most Important Plant Families from Bussmann & Sharon 2006 [21] and New Classification under APG-3 (Gray Underlay)—cont'd

Isoetaceae	1	0.20	Isoetaceae	1	0.20
Juglandaceae	1	0.20	Juglandaceae	1	0.20
Krameriaceae	1	0.20	Krameriaceae	1	0.20
Lecythidaceae	1	0.20	Lecythidaceae	1	0.20
Lemnaceae	1	0.20	Lemnaceae	0	0.00
Loganiaceae	1	0.20	Loganiaceae	0	0.00
Lythraceae	1	0.20	Lythraceae	2	0.40
Malesherbiaceae	1	0.20	Malesherbiaceae	0	0.00
Malpighiaceae	1	0.20	Malpighiaceae	1	0.20
Menispermaceae	1	0.20	Menispermaceae	1	0.20
Musaceae	1	0.20	Musaceae	1	0.20
Myricaceae	1	0.20	Myricaceae	1	0.20
Myristicaceae	1	0.20	Myristicaceae	1	0.20
Oleaceae	1	0.20	Oleaceae	1	0.20
Papaveraceae	1	0.20	Papaveraceae	1	0.20
Proteaceae	1	0.20	Proteaceae	1	0.20
Punicaceae	1	0.20	Punicaceae	0	0.00
Sapotaceae	1	0.20	Sapotaceae	1	0.20
Saxifragaceae	1	0.20	Saxifragaceae	1	0.20
Sterculiaceae	1	0.20	Sterculiaceae	0	0.00
Theaceae	1	0.20	Theaceae	1	0.20
Thelypteridaceae	1	0.20	Thelypteridaceae	1	0.20
Thymeleaceae	1	0.20	Thymeleaceae	1	0.20
Tropaeolaceae	1	0.20	Tropaeolaceae	1	0.20
Typhaceae	1	0.20	Typhaceae	1	0.20
Ulmaceae	1	0.20	Ulmaceae	0	0.00
Violaceae	1	0.20	Violaceae	1	0.20
Vitaceae	1	0.20	Vitaceae	1	0.20

Continued

TABLE 4.2 Most Important Plant Families from Bussmann & Sharon 2006 [21] and New Classification under APG-3 (Gray Underlay)—cont'd

Xyridaceae	1	0.20	Xyridaceae	1	0.20
Zingiberaceae	1	0.20	Zingiberaceae	1	0.20
Zygophyllaceae	1	0.20	Zygophyllaceae	1	0.20
Adoxaceae	0	0.00	Adoxaceae	2	0.40
Araceae	0	0.00	Araceae	1	0.20
Asparagaceae	0	0.00	Asparagaceae	1	0.20
Calceolariaceae	0	0.00	Calceolariaceae	1	0.20
Calophyllaceae	0	0.00	Calophyllaceae	1	0.20
Cannabaceae	0	0.00	Cannabaceae	1	0.20
Celastraceae	0	0.00	Celastraceae	1	0.20
Hypericaeae	0	0.00	Hypericaeae	3	0.61
Iridaceae	0	0.00	Iridaceae	1	0.20
Orobanchaceae	0	0.00	Orobanchaceae	1	0.20
Phyllanthaceae	0	0.00	Phyllanthaceae	3	0.61
Pteridaceae	0	0.00	Pteridaceae	5	1.01
Schisandraceae	0	0.00	Schisandraceae	1	0.20
Siparunaceae	0	0.00	Siparunaceae	2	0.40
Xanthorrhoeaceae	0	0.00	Xanthorrhoeaceae	1	0.20
Ximeniaceae	0	0.00	Ximeniaceae	1	0.20

Note: Column 2 and 5: Number of species; Column 3 and 6: Percentage of species.

antibacterial activity, although both are used for wound infections [35]. *Gynerium sagittatum* (Aubl.) Pers and *Arundo donax* (L.) are both called "carrizo." The ethanolic extract of *A. donax* is highly toxic, while its aqueous extract is benign. *G. sagittatum* shows exactly the opposite toxicity. A wide variety of species of *Huperzia* (Lycopidiaceae) are known as "condor," but all exhibit completely different toxicity, from nontoxic to highly toxic, again sometimes depending on preparation. *Notholaena sulphurea* (Cav.) L. Sm. and *Argyrochosma nivea* (Poir.) Windham (both Pteridaceae) are called Doradilla (as well as CutiCuti) and used against diabetes in aqueous extract. *Notholaena* is relatively nontoxic, but *Argrochosma* is highly toxic [36]. Recent studies in the markets of La Paz, Bolivia, focused on plants used for the treatment of urinary infections. One of the most frequently mentioned herbs was "cola de caballo," horsetail, which normally signifies species of *Equisetum*. However, every single vendor in La Paz sold instead a species of *Ephedra*. Not only does *Ephedra* not have any properties related to treating urinary infections or inflammations, but also its main compounds can lead to serious side effects. Without the collection of botanical vouchers, this serious health risk would not have been discovered.

4.7 CONCLUSIONS

The correct identification of plant material and the preparation of voucher specimens that serve for future research and documentation are relatively simple, and require only a few easy steps [19]. Voucher specimens are simply a permanent reference on which all research

and commercialization of herbal medicine needs to be placed. After the collection of fertile specimens, these need to be carefully processed and deposited in recognized herbarium. Any publication related to herbal medicines should cite such voucher specimens. Many professional journals already require authors to provide information about how the plant materials used in their research was documented, and where the vouchers are stored. Failure to comply with such provisions inevitably leads to the rejection of the paper in such cases. In addition, trade regulations, as, e.g., increasingly enforced by the US FDA and similar agencies, require producers and sellers of herbal medicinal products to provide correct names on their labels and to provide information about voucher specimens. The current guidelines state: "A suitable voucher specimen (reference specimen) for each of the botanical raw materials should be established, along with a reference standard for the drug substance and drug product," and in current practice these guidelines are more and more turned into a requirement. Both researchers and producers in the field of herbal medicine need to ask themselves if they are completely sure that the material they are working with really represents the species they think it is.

Most taxonomic errors are simply caused by orthographic mistakes, by citing synonyms instead of the correct scientific name, wrong or missing author citations, and mistakes in family classification. Most of these problems can simply be avoided by using open access online databases. The identification of vouchering material should, however, always be done or at least confirmed by specialists. As such, taxonomy is a crucial tool for the validation and safety of herbal medicine.

References

[1] Eisenman SW, Struwe L. The global distribution of wild tarragon (*Artemisia dracunculus* L.; Asteraceae) cytotypes with twenty-seven new records from North America. 2011.

[2] Eisenman SW, Poulev A, Struwe L, Raskin I, Ribnicky DM. Qualitative variation of antidiabetic compounds in different tarragon (*Artemisia dracunculus* L.) cytotypes. Fitoterapia 2011;82:1062–74.

[3] Walker KM, Applequist W. Adulteration of selected unprocessed botanicals in the U.S. retail herbal trade. Econ Bot 2012;66(4):321–7.

[4] Soller RW, Bayne HJ, Shaheen C. The regulated dietary supplement industry: myths of an unregulated industry dispelled. HerbalGram 2012;93:42–57.

[5] Low M-Y, Zeng Y, Li L, Ge X-W, Lee R, Bloodworth B-C, et al. Safety and quality assessment of 175 illegal sexual enhancement products seized in red-light districts in Singapore. Drug Saf 2009;32:1141–6.

[6] Cooper K, Noller B, Connell D, Yu J, Sadler R, Olszowy H, et al. Public health risks from heavy metals and metalloids present in traditional Chinese medicines. J Toxicol Environ Health 2007;70:1694–9.

[7] Slifman NR, Obermeyer WR, Aloi BK, Musser SM, Correll Jr WA, Cichowicz SM, et al. Contamination of botanical dietary supplements by *Digitalis lanata*. N Engl J Med 1998;339:806–11.

[8] GarzoFernández C, GómezPintado P, BarrasaBlanco A, MartínezArrieta R, RamírezFernández R, RamónRosa F, et al. Casos de enfermedad de sintomatología neurológica asociados al consumo de anís estrellado empleado como carminativo. Anales Españoles de Pediatria 2002;57:290–4.

[9] Johanns ES, vanderKolk LE, vanGemert HM, Sijben AE, Peters PW, de Vries I. Een epidemie van epileptische aanvallen na drinken van kruidenthee. Ned Tijdschr Geneeskd 2002;146:813–6.

[10] Bryson PD, Watanabe AS, Rumack BH, Murphy RC. Burdock root tea poisoning. Case report involving a commercial preparation. J Am Med Assoc 1978;239:2157.

[11] Applequist W. The identification of medicinal plants: a handbook of the morphology of botanicals in commerce. Austin, TX: American Botanical Council; 2006.

[12] Bridson D, Forman L. The herbarium handbook. Kew, UK: Kew Publishing; 2010.

[13] Index Herbariorum, http://sciweb.nybg.org/science2/IndexHerbariorum.asp; 2014.

[14] Nesbitt M, McBurney RPH, Broin M, Beentje HJ. Linking biodiversity, food and nutrition: the importance of plant identification and nomenclature—a review. J Food Compos Anal 2010;23:486–98.

[15] Godfray Jr HCJ. Linnaeus in the information age. Nature 2007;446:259–60.

[16] Rieppel O. The PhyloCode: a critical discussion of its theoretical foundation. Cladistics 2006;22:186–97.

[17] McNeill J, Barrie FR, Buck WR, Demoulin V, Greuter W, Hawksworth DL, et al. International code of nomenclature for algae, fungi, and plants (Melbourne Code). Regnum Vegetabile154. Koeltz Scientific Books; 2012., http://www.iapt-taxon.org/nomen/main.php.

[18] Bennett BC, Balick MJ. Does the name really matter? The importance of nomenclature and plant taxonomy in biomedical research. J Ethnopharmacol 2013;152(3):387–92.

[19] Bennett BC, Balick MJ. Phytomedicine 101: plant taxonomy for preclinical and clinical medicinal plant researchers. J Soc Integr Oncol 2008;6:150–7.

[20] The Angiosperm Phylogeny Group. An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG III. Bot J Linn Soc 2009;161(2):105–21.

[21] Bussmann RW, Sharon D. Traditional plant use in Northern Peru: tracking two thousand years of health culture. J Ethnobiol.Ethnomed 2006;2:47.

[22] Bussmann RW, Sharon D. Plants of the four winds – the magic and medicinal flora of Peru. Plantas de los cuatro vientos – La flora mágica y medicinal del Perú. Trujillo, Peru: Graficart; 2007.

[23] Food and Drug Administration (FDA). Guidance for Industry – botanical drug products. 2004. http://www.fda.gov/ohrms/dockets/98fr/04-13031.htm. and, http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=101.4.

[24] Eisenman SW, Tucker AO, Struwe L. Voucher specimens are essential for documenting source material used in medicinal plant investigations. J Medicinally Active Plants 2012;1(1):30–43.

[25] Naser B, Lund B, Henneicke-von Zepelin HH, Köhler G, Lehmacher W, Scaglione F. A randomized, double-blind, placebo-controlled, clinical dose-response trial of an extract of Baptisia, Echinacea and Thuja for the treatment of patients with common cold. Phytomedicine 2005;12(10):715–22.

[26] Köhler G, Bodinet C, Freudenstein J. Pharmacodynamic effects and clinical effectiveness of a combination of herbal substances comprised of cone flower, wild indigo and white cedar. Wien Med Wochenschr 2002;152(15–16):393–7.

[27] Schulten B, Bulitta M, Ballering-Brühl B, Köster U, Schäfer M. Efficacy of *Echinacea purpurea* in patients with a common cold. A placebo-controlled, randomised, double-blind clinical trial. Arzneimittel forschung 2001;51(7):563–8.

[28] Henneicke-von Zepelin H, Hentschel C, Schnitker J, Kohnen R, Köhler G, Wüstenberg P. Efficacy and safety of a fixed combination phytomedicine in the treatment of the common cold (acute viral respiratory tract infection): results of a randomised, double blind, placebo controlled, multicentre study. Curr Med Res Opin 1999;15(3):214–27.
[29] Wüstenberg P, Henneicke-von Zeppelin HH, Köhler G, Stammwitz U. Efficacy and mode of action of an immunomodulator herbal preparation containing Echinacea, wild indigo, and white cedar. Adv Ther 1999;16(1):51–70.
[30] Bussmann RW. The globalization of traditional medicine in northern Peru – from shamanism to molecules. Evidence Based Complementary and Altern Med 2013:46. http://dx.doi.org/10.1155/2013/291903. Article ID 291903.
[31] Bussmann RW, Paniagua Zambrana N, Rivas Chamorro M, Molina Moreira N, Cuadros Negri ML, Olivera J. Peril in the market – classification and dosage of species used as antidiabetics in Lima, Peru. J Ethnobiol Ethnomed 2013;9:37.
[32] Monigatti M, Bussmann RW, Weckerle CS. Medicinal plant use in two Andean communities located at different altitudes in the Bolivar Province, Peru. J Ethnopharmacol 2013;145(2):450–64.
[33] Bussmann RW, Glenn A, Meyer K, Rothrock A, Townesmith A. Herbal mixtures in traditional medicine in Northern Peru. J.Ethnobiol Ethnomed 2010;6(10).
[34] McGuffin M, Tucker A, Leung AY, Kartesz T. Herbs of commerce. American Natural Products Association; 2010.
[35] Bussmann RW, Malca G, Glenn A, Sharon D, Chait G, Díaz D, et al. Minimum inhibitory concentration of medicinal plants used in Northern Peru as antibacterial remedies. J Ethnopharmacol 2010;132:101–8.
[36] Bussmann RW, Malca G, Glenn A, Sharon D, Nilsen B, Parris B, et al. Toxicity of medicinal plants used in Northern Peru. J Ethnopharmacol 2011;137:121–40.

CHAPTER

5

Validation of Medicinal Herbs for Skin Aging

Pulok K. Mukherjee, Shiv Bahadur, Sushil K. Chaudhary, Ranjit K. Harwansh, Neelesh K. Nema

School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

OUTLINE

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00005-2

5.1 INTRODUCTION

Aging is a universal biological process that leads to some progressive and deleterious changes. The meaning of antiaging has been changed from simply prolonging lifespan to increasing health span, which emphasizes more on the quality of life. It is now widely accepted that aging is a multifarious event that results from the collective effects of genetic variation, environmental risk factors, nutritional factors, and lifestyle [1]. Skin aging may be divided into two basic processes, intrinsic aging and photoaging. Photoaging can be described as premature skin aging in chronically photodamaged skin. Intrinsic aging is characterized by smooth, dry, pale, and finely wrinkled skin. Photoaging is characterized by coarse, deep, severe wrinkling, and pigmentary changes, on exposed areas such as the face, neck, and forearm [2]. Skin aging is associated with a progressive increase in extensibility and a reduction in elasticity. With increasing age, the skin also becomes more fragile and susceptible to trauma, and this leads to more lacerations and bruising [3].

The skin is the largest organ of the body with a surface area of approximately 1.5–2.0 m^2. It protects the internal organs of the body by acting as an effective barrier against the detrimental effects of environmental and xenobiotic agents (e.g., extrinsic harmful chemicals and genotoxics). In modern life, the skin is often expressed to environmental accidents, including excess ultraviolet (UV) exposure and augmented air pollution, which usually leads to oxidative stress. In these stages, high reactive oxygen species (ROS) can damage the skin and induce skin disease [4]. It can cause an imbalance between ROS and endogenous antioxidant systems, such as reduced glutathione (GSH), and results in a severe decrease of its antioxidant content and in a striking formation of active oxygen intermediates [5]. ROS formed as a result of UV irradiation may oxidize and damage cellular lipids, proteins, and deoxyribonucleic acid (DNA). This leads to changes and often to obliteration of skin structures and can result in the hindrance of its regular function [6]. A small amount of UV radiation is required for the production of vitamin D; however, extensive exposure to the sun increases the risk of developing sunburn, accelerates skin aging, and causes wrinkles and several malignant skin cancers [7]. In addition, excessive UV exposure results in a lot of chronic skin diseases such as (1) cutaneous malignant melanoma, a life-threatening malignant skin cancer; (2) squamous cell carcinoma of the skin, a malignant cancer, which generally progresses less rapidly than melanoma does and is less likely to cause death; (3) basal cell carcinoma of the skin, a slow-growing skin cancer appearing predominantly in older people; and (4) photoaging, which causes loss of skin tightness and the development of solar keratoses [8,9].

Several botanicals have potent antioxidant properties and are also associated with reduced incidence of ROS and photoaging. Medicinal plants attracted considerable attention because they play a crucial role in skin photoprotective effects. This has led to great interest in nutraceuticals rich in antioxidants for the prevention of photocarcinogenesis and photoaging [10]. Antioxidants contain phenolic acids, flavonoids, and high molecular weight polyphenols. Natural bioactive molecules exhibit several therapeutic activities, which include anticancer, antiwrinkle, antiaging, skin whitenening, antiinflammatory, wound healing, and immunomodulating activities. Topical administration of antioxidants offers a better way to develop the endogenous cutaneous protection system and thus may be an effective strategy for decreasing UV-mediated oxidative damage in the skin tissues [5].

5.2 CONSEQUENCES OF HERBAL COSMETIC

There has been a very long history in human civilization for the use of herbal medicine for skin care. From ancient times, Egyptians were the first to use whipped ostrich eggs, olive oil, and resin mixed with milk for the treatment of various skin problems or for beautifying [11]. The topical application of herbal products serves as cosmetics for the care of the body, and bioactive molecules influence the biological functions of the skin, and provide the nutrients necessary for a healthy skin [12]. Actually, they are enriched with vitamins,

antioxidants, various oils, essential oils, hydrocolloids, proteins, terpenoids, and other constituents. *Ayurveda* (wisdom of life), the Indian system of medicine, has its origin in prehistoric antiquity. One of its compilations *Charak Samhita* (~900 BC) has listed out 10 antiaging drugs. Among those drugs, seven are plant-derived products that are most commonly used in *Rasaayan* (rejuvenation) therapy [13]. Those herbal products have been subjected to scientific validation and have possessed antiaging effects with significant free radical scavenging and other antioxidant activities [14].

In 1820, Sir Everad Home (from England) was the first to propose that melanin acts as a sunscreen and is protective to the skin, a concept that is now being challenged. In 1878, Otto Veiel (from Austria) discovered that tannins may work as sun protectors. However, their persistent staining of skin precluded their commercial development for that application. In 1962, Franz Greiter (from Switzerland) introduced the concept of sun protection factor (SPF) by developing a method to measure the effectiveness of a sunscreen to suppress sunburn. In 1969, Albert Kligman (from the USA) published the first evidence that sun exposure causes structural damage to the skin that can be differentiated from the intrinsic aging process. In 1986, Albert Kligman developed the concept of photoaging. In the 1990s, several studies unveiled the molecular basis of photoaging response. In 2006, the first evidence highlighted by Rachel Haywood (from the UK) that visible light (400–700 nm) also contributes to skin damage via induction of radical production. In 2007, a report from the World Health Organization linked indoor tanning to melanoma. In 2010, research from Andrea Vierkötter and Jean Krutmann redefined the causes of extrinsic aging to include some environmental factors other than light [15]. Recently, *Emblica officinalis* has been reported as an antiaging agent by promoting procollagen production and inhibiting (MMP-1) in human and mouse skin fibroblasts [16]. *E. officinalis* produces its activities through diverse mechanisms, as shown by green tea tannins through the involvement of mitogen-activated protein kinases (MAPKs), modulation of matrix metalloproteinase (MMP) expression, and production through activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) activation. This suggests that it is a strong antiphotoaging agent [17–19]. Therefore, numerous significant research and development opportunities exist to discover novel and effective skin care products.

5.3 SKIN AGING

Skin aging is a complex biological process that affects various layers of the skin, with major damage seen in the connective tissue of the dermis due to the accumulation of the disorganized structure of elastin and loss of interstitial collagens. Solar irradiation induces MMPs in the epidermis and dermis, and the results supported the theory that MMPs are the primary mediators for the damage of connective tissue in skin exposed to UV irradiation and in the premature aging of skin [20]. Deficiencies and alterations of collagen fiber are the major structural components of skin and have been suggested to be a cause for the skin wrinkling observed in photoaged and naturally aged skin [21]. On excessive matrix degradation, MMPs secreted by various cells, including keratinocytes, fibroblasts, and inflammatory cells, contribute substantially to the connective tissue damage that occurs during photoaging [22]. During the natural skin aging process, the collagen content in the dermis decreases approximately 1% per year throughout adult life [23]. Sunscreens play an important role in avoiding skin damage by solar radiation. Some antioxidants reduce the rate of formation of secondary UV-induced damage, particularly those induced by singlet oxygen [24–26].

5.3.1 Concept of Photoaging

UV rays may cause sunburn of the cells, premature skin aging, which may also lead to a risk of several skin cancers [27]. The most commonly used commercially available UV filter in the industry is octal methoxycinnamate [28], octyl-dimethylaminobenzoate, *p*-amino benzoic acid, *p*-amyl dimethyl *p*-amino benzoic acid (padimate-A), 2-ethoxyethyl-*p*-methoxycinnamate that absorb UV radiations. UV rays have several serious effects on exposed skin and cause accelerated aging, including wrinkling, dryness, telangiectasia, scaling, and mottled pigment including lentigines as well as guttate hypermelanosis and hypomelanosis. UV radiation damage up to the cellular level can exhibit changes in DNA, including oxidation of nucleic acids [29]. These oxidative processes can also cause structural damages by changing the function of proteins and lipids by which lipid peroxide is synthesized. The formation of these accumulations can lead to severe changes to the skin such as mitochondrial damages, DNA damages, wrinkles, and aging [10]. With a loss of polarity, keratinocytes and melanocytes become inconsistent, unequal with abnormalities in pocket size. In actinic skin, Langerhans cells are reduced due to UV radiation, which is completely absorbed in the epidermis [30]. Epidermal keratinocytes are protected from different oxidative stress by antioxidants that are generally low molecular weight organic molecules or vitamins. Topical administration of antioxidants in a novel formulation may be a flourishing tactic for minimizing UV radiation-mediated oxidative damage to the skin [31].

Consequently, containment of inflammatory cutaneous damage and timely initiation of cellular antioxidant capacity may give a mechanistic basis for the prevention of photoaging and photocarcinogenesis [32]. Phytoconstituents are suitable effective alternative in cosmetic formulations as they can protect the skin from harmful UV rays [33].

5.3.2 UV Index and Sun Protection Factor

The global solar UV index (UVI) is a simple measure of the level of solar UV radiation received at a particular time on the earth's surface and an indicator of the potential for skin damage. The UVI also serves as a vehicle to raise public awareness and to alert people about the need to adopt protective measures when exposed to UV radiation. It is reported along with the weather forecast during the summer months in many countries. Encouragement of individuals to protect themselves by seeking shade and wearing suitable clothes is the important key for protection against UV radiations [18,32].

The UVI is designed to indicate the potential for adverse health effects and to encourage people to protect themselves. The higher the UVI value, the greater the potential for damage to the skin and eyes, and the less time it takes to harm the skin [34]. The SPF is defined as the ratio of the "minimal erythema dose" of protected skin and unprotected skin. The SPF value usually represents the first approach to evaluate the ability of suncare to prevent skin damage by UV irradiations. A number of methods for the assessment of the level of protection have been developed and standardized [35].

5.3.3 Etiology of Skin Aging

The skin is made up of different tissues and has different functions, and mammalian skin is composed of three primary layers, namely, the epidermis, dermis, and hypodermis [36]. When the skin is environmentally exposed, some histological changes occur in the epidermis and dermis.

5.3.3.1 Epidermis

This is a stratified squamous, keratinized epithelium, and it forms a waterproof, protective wrap over the body and serve as the interface between the organism and its environment [37]. The following strata (beginning with the outermost layer): corneum, lucidum (only in the palms of the hands and bottom of the feet), granulosum, spinosum, and basale are the different layers of human skin. The deepest layer is called stratum basale, and it gives rise to new cells through mitosis, which are still undifferentiated in this layer [38]. The daughter cells move up, change their shape and composition as they can be differentiated and get filled with keratinase. They die due to isolation from the blood source. Cytoplasm is released, and the protein keratin is inserted. They eventually reach the corneum and slough off (desquamation), and this process is called keratinization [22]. This keratinized layer of the skin is responsible for retaining water in the body and keeping other harmful chemicals and pathogens out. This makes the skin a natural barrier to infection. The dermal–epidermal junction (DEJ) undergoes changes as well. The DEJ surface area becomes smaller with age. Further, it causes decreased transfer of nutrients between the skin layers and the dermal papillae, and epidermal ridges become less prominent [38].

5.3.3.2 Dermis

The dermis is the layer of skin beneath the epidermis and consists of collagen fibrils and elastic tissues that, respectively, provide support and flexibility to the dermis. It contains hair follicles; sweat, sebaceous, and apocrine glands; lymphatic vessels; and blood vessels. Sebum secreted by sebaceous glands associated with the follicles is composed of free fatty acids, triglycerides, and waxes that play a vital role in lubricating the skin surface and maintaining the surface pH. The sweat glands originating in the dermis secrete sweat (a dilute salt solution of pH 5) in response to physical and emotional stress [36]. Collagen fibers run parallely to the surface of the skin and are responsible for the resilience of the dermis, whereas elastin fibers form a network. Aging brings with it a decline in procollagen type-I and -III mRNA and protein expression. Besides the impairment in collagen synthesis, an increase in the production of some MMPs also occurs with aging, especially of MMP-1, MMP-2, MMP-3, and MMP-9. Oxidative damage to cells accumulates with years, and the free radicals and ROS from aerobic metabolism initiate the aging process.

5.4 FACTORS ASSOCIATED WITH SKIN AGING

5.4.1 Intrinsic Aging

Intrinsic aging is also called chronological aging, and it occurs as a natural consequence of physiological changes with time. Intrinsic skin aging occurs due to telomere shortening; telomeres are DNA sequences located at the ends of the chromosomes of eukaryotes, which protect the genome and lifespan of cells. Skin epidermis contains telomerase that plays a significant role in lowering oxidative damage in cells [3]. Telomeres, a small DNA sequence present at the ends of chromosomes, are considered as essential elements in the intrinsic aging process. The processes associated with intrinsic skin aging are deliberating to the result

from a combination of several events including (1) decreased proliferative capacity of skin derived cells, (2) decreased matrix synthesis in the dermis, and (3) increased expression of enzymes that degrade the collagenous matrix [39]. Several significant factors that are associated with intrinsic aging are described briefly.

5.4.1.1 Ethical Background

The main effect of ethnicity on aging relates to the difference in pigmentation. High levels of melanin pigmentation protect from the cumulative effects of photoaging. Black skin is more compact, has a greater amount of lipids, and is also considered as a factor that influences the increased resistance to aging. Asian subjects were observed to develop wrinkles later and to a lesser degree of intensity as compared to Caucasians [40].

5.4.1.2 Anatomic Variations

Some areas of the skin are thinner than are others, and in those thinner skin regions, aging becomes more evident. This is especially noted on the eyelids, the thinnest area of the skin in the human body. There is also variability in both the composition and the distribution of lipids in the skin. The high lipid area is less affected in comparison to the low lipid area [40].

5.4.2 Extrinsic Aging

Extrinsic aging is due to environmental factors and occurs to different degrees of intensity, due to solar exposure, smoking, and to other general lifestyle factors, such as diet, sleep, and overall health. It has been suggested that as much as 80% of facial aging is attributable only to sun exposure [41]. Histological changes occur in the epidermis of photodamaged skin, including both an increase and decrease of epidermal thickness and loss of epidermal polarity. Changes in the proportion and functionality of the dermal extracellular components are associated with UV-induced extrinsic damage. There are three basic principal molecular components involved in the extrinsic aging such as collagen fibers, elastic fibers, and glycosaminoglycans. Sunburned cells undergo apoptosis, which is aided by caspase-3, which is used as a biomarker to determine the level of UV-induced cutaneous damage. Various potential factors associated with extrinsic aging are discussed in brief [40].

5.4.2.1 UV Radiations

Among all environmental factors, accelerating aging process, UV radiation contributes up to 80% in skin aging. UV-B (290–320 nm) and UV-A (320–400 nm) are both responsible for skin aging, but UV-B has 1000 times stronger biological effects than UV-A does. UV-B is mostly absorbed in the epidermis and predominantly affects epidermal cells; however, about 10–30% of UV-B can penetrate the epidermis and reach the upper dermis and affect fibroblasts [15]. UV radiation damages the skin through the formation of thymine and pyrimidine dimers, commencement of an inflammatory response, and the increased production of collagenase, the enzyme that breaks down collagen. The clinical characteristics of sun exposure related to aging include increased wrinkle formation and loss of skin elasticity. More specifically, loss of dermal collagen is the hallmark of sun-related skin aging [42]. Increased synthesis and expression of MMP-1 by dermal fibroblasts after UV radiation through the increased ROS formation are believed to play a critical role in photoaging by enhancing the degradation of type-1 and type-3 collagen [43]. UV radiation generates severe oxidase stress in skin cells via interaction with intracellular chromophores and photosensitizers, and results in transient and permanent genetic damage and in the activation of cytoplasmic signal transduction pathways that are related to growth, differentiation, senescence, and connective tissue degradation [29]. UV rays induce production of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF), which has been considered attributable to these changes. In addition, UV irradiation is known to stimulate both keratinocytes and fibroblasts to induce basic fibroblast growth factor that is responsible for the proliferation of melanocytes and keratinocytes [44].

5.4.2.2 Infrared Radiation

Infrared Radiation (IR) radiation causes an imbalance between MMP-1 and TIMP Metallopeptidase Inhibitor-1 (TIMP-1) expressions in favor of MMP-1 and at the same time decreases collagen-1 and collagen-2 expressions and thereby leads to a rarefaction of collagen fibers, which eventually leads to wrinkle formation [1]. IR radiation is primarily absorbed by copper atoms in complex IV of the mitochondrial respiratory chain. The first detectable signaling event is the subsequent intramitochondrial generation of ROS. This intramitochondrial signal is then transmitted to the cytoplasm where it causes an increase in calcium levels, followed by an activation of MAPKs and the subsequent intranuclear transcriptional activation of IR responsive genes. The mitochondrially targeted antioxidants are highly effective in blocking this signaling cascade in preventing IR radiation-induced MMP-1 upregulation in human skin [45]. IR comprises of IRA (700–1400 nm), IRB (1400–3000 nm), and IRC (3000 nm–1 mm). IRB and IRC do not penetrate the skin deeply, but IRA produces harmful effects. Among the total radiations, IRA represents about 30%, of which 65% reaches the dermis and 10% reaches the hypodermis [13]. IRA radiation generates ROS within the skin and the relative contribution of IRA to free radical generation in sunlight has been

estimated to be around one-fourth of that of UV radiation. IRA also induces unbalanced gene expression of MMP and decreases collagen gene expression, favors angiogenesis, involved in photoaging, may promote carcinogenesis, and also affects mitochondrial integrity. IRA interaction with Cytochrome C Oxidase could lead to disruption of the mitochondrial electron transport chain, and result in inadequate energy production and increased generation of ROS. IRB and IRC are mainly responsible for the generation of heat in the skin. Keratinocytes, fibroblasts, and melanocytes express various thermosensitive receptors at their membrane, including the transient receptor potential vanilloid-1 (TRPV-1), which was recently proposed to be activated by IR radiation. TRPV1 is a cell membrane channel that opens upon stimulation, and allows a flux of calcium ions to cross the membrane and rush into the cell. TRPV1 activation in fibroblasts induces MMP-1 expression at the mRNA and protein levels, which results in an increase in collagen degradation and premature skin aging [1].

5.4.2.3 *Ambient Conditions*

Higher temperatures lead to increased water evaporation, while low temperatures provide a hardening and reduced water loss through the same mechanism, even with abundant air moisture. The appropriate formation of structural proteins and lipids in the skin depends on the environmental temperature [40].

5.4.2.4 *Tobacco Smoking*

Smoking is strongly associated with a number of systemic diseases, and is also related to many dermatological conditions, including premature skin aging, acne, poor wound healing, squamous cell carcinoma, melanoma, oral cancer, psoriasis, and hair loss. Several studies report that smoking increases wrinkles, tissue laxity, and pigmentary changes in human skin. A research report indicates that a 10-year smoking difference in twins corresponds roughly to one twin appearing 2.5 years older [15]. Smoking induces several harmful modifications such as elastosis, telangiectasias, and decrease of blood flow in the capillary vessels, and leads to the deprivation of nutrients in cutaneous tissues. There is a reduction of collagen fibers and elastin in the dermis, free radical increase in the lung, increase in keratinocytic dysplasia, and skin roughness [40]. Several studies indicate that tobacco smoke extract impairs the production of collagen and increases the production of tropoelastin and MMPs, which degrade matrix proteins and also cause an abnormal production of elastosis material. Smoking increases the MMP level, which leads to the degradation of collagen, elastic fibers, and proteoglycans; this suggests an imbalance between biosynthesis and degradation in dermal connective tissue metabolism [46]. The biosynthesis of new collagen is decreased significantly by tobacco smoke extracts in cultured skin fibroblasts [47].

5.4.2.5 *Habitats*

Several epidemiological studies indicate the influence of lifestyle factors on skin aging and skin health. Eating unhealthy food is associated with all kinds of premature skin aging, ranging from acne to signs of skin aging. On the contrary, when a healthy diet containing high antioxidants is consumed, chronological aging effects may get delayed. Thus, twins who avoid excessive alcohol intake have a younger apparent age. Proper rest and number of sleeping hours needed are also very important; a person having regular sleeping habits tends to be associated with healthier and younger appearance of the skin. Regular exercise is also beneficial for the skin; it improves skin tone and stimulates blood circulation and helps to evacuate metabolic wastes and brings oxygen to skin tissues [15]. One of the worst common habits is sun bed tanning, which can further damage skin. The intensity of solar radiation emitted by a powerful tanning bed is 10–15 times higher than what we normally get under the normal summer sun [48].

5.5 PHOTOPROTECTIVE MECHANISM OF BIOACTIVE MOLECULES

Several plant bioactive molecules have been reported to interact with cellular signaling pathways that are directly or indirectly involved in skin aging. Most of these pathways are, in fact, signaling pathways involved in cell survival and programed cell death.

The several molecular mechanisms of bioactive compounds from natural resources are depicted in Figure 5.1 and discussed in brief as follows.

5.5.1 ROS Regulation

ROS as a result of UV irradiation may oxidize and damage cellular lipids, proteins, and DNA. This leads to changes and often to destruction of skin structures and results in hindrance of regular function [6]. An imbalance between ROS and endogenous antioxidant systems, such as reduced GSH, results in a severe decrease of its antioxidant content and in a striking formation of active oxygen intermediates as a result [5]. Senescence of eukaryotic cells may be linked to mutations of their mitochondrial genome (mDNA). mDNA synthesis takes place near the inner mitochondrial membrane in close proximity where highly ROS are generated from normal respiration. The resulting mitochondrial destruction and concomitant decline in

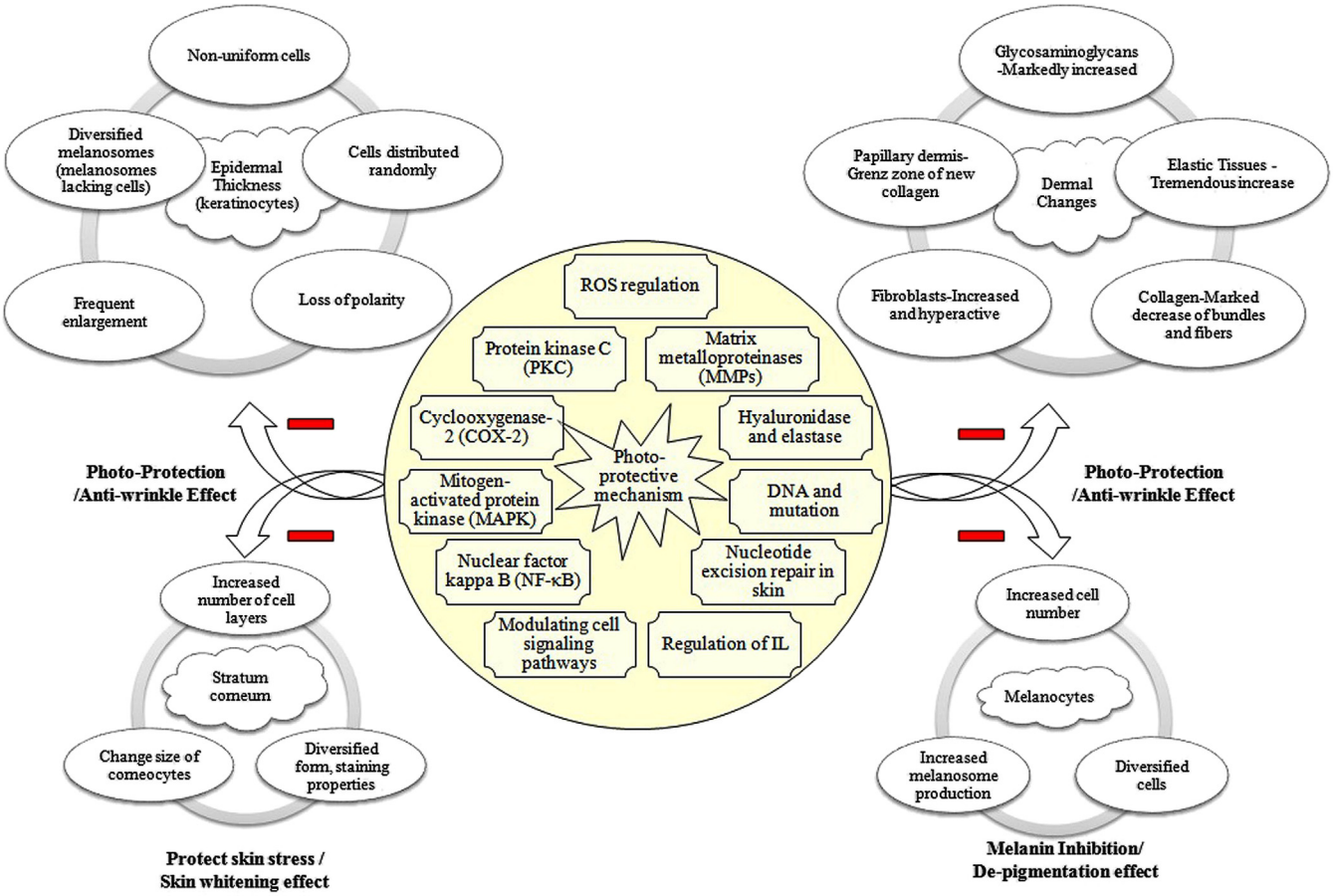


FIGURE 5.1 Photoprotective mechanisms of botanicals.

cell bioenergetic capabilities may cause senescent loss of physiological function and degeneration of cell, resulting in aging [49]. The excited photosensitizer subsequently reacts with oxygen, resulting in the generation of ROS including the superoxide anion (O_2^-) and singlet oxygen ($^1O_2$). O_2^- and O_2 are also produced by neutrophils that are accelerated in the case of photoaged skin and contribute to the overall prooxidant state. Superoxide dismutase (SOD) converts O_2^- to hydrogen peroxide (H_2O_2). H_2O_2 is able to cross cell membranes easily and in conjunction with transitional Fe (II) drives the generation of the highly toxic hydroxyl radical (OH). Both singlet oxygen and OH• can initiate lipid peroxidation of cellular membranes with the generation of carbonyls and to date are poorly understood consequences [50].

5.5.2 Matrix Metalloproteinases

MMPs are calcium (Ca)-dependent zinc (Zn)-containing endopeptidases that belong to the "Metzincin" superfamily. MMP production, transcription, pro-MMP activation, regulation, and inhibition are dynamic equilibrium processes as presented in Figure 5.2. It consists of 33 members sharing a regular catalytic core or domain with a Zn metal in their active site [51]. MMPs play an important role in several physiological processes, such as the degradation of extracellular matrix (ECM) including collagens, elastins, gelatin, matrix glycoproteins, proteoglycan, wound healing, cell migration, angiogenesis, and tumor progression. Collagenase, MMP-1 (fibroblast collagenase), is the key collagenase, which is involved in the physiological and pathological turnover of the ECM of skin [52]. Irradiation of human skin causes increased generation of hydrogen peroxide, which activates the MAPK signal transduction pathway. This leads to decreased transforming growth factor-β(TGF-β), and increased AP-1 expression further induces MMP genes that stimulate the production of collagenase, gelatinase, and stromelysin in the exposed skin [53].

Collagenase attacks and degrades collagen, long-term elevations in the levels of collagenase, and other MMPs likely yield the disorganized and clumped collagen identified in photoaged skin [54]. UV radiation is absorbed by skin molecules and generates ROS causing "oxidative damage," which further changes hyaluronidase, elastase, and MMPs levels (Figure 5.2).

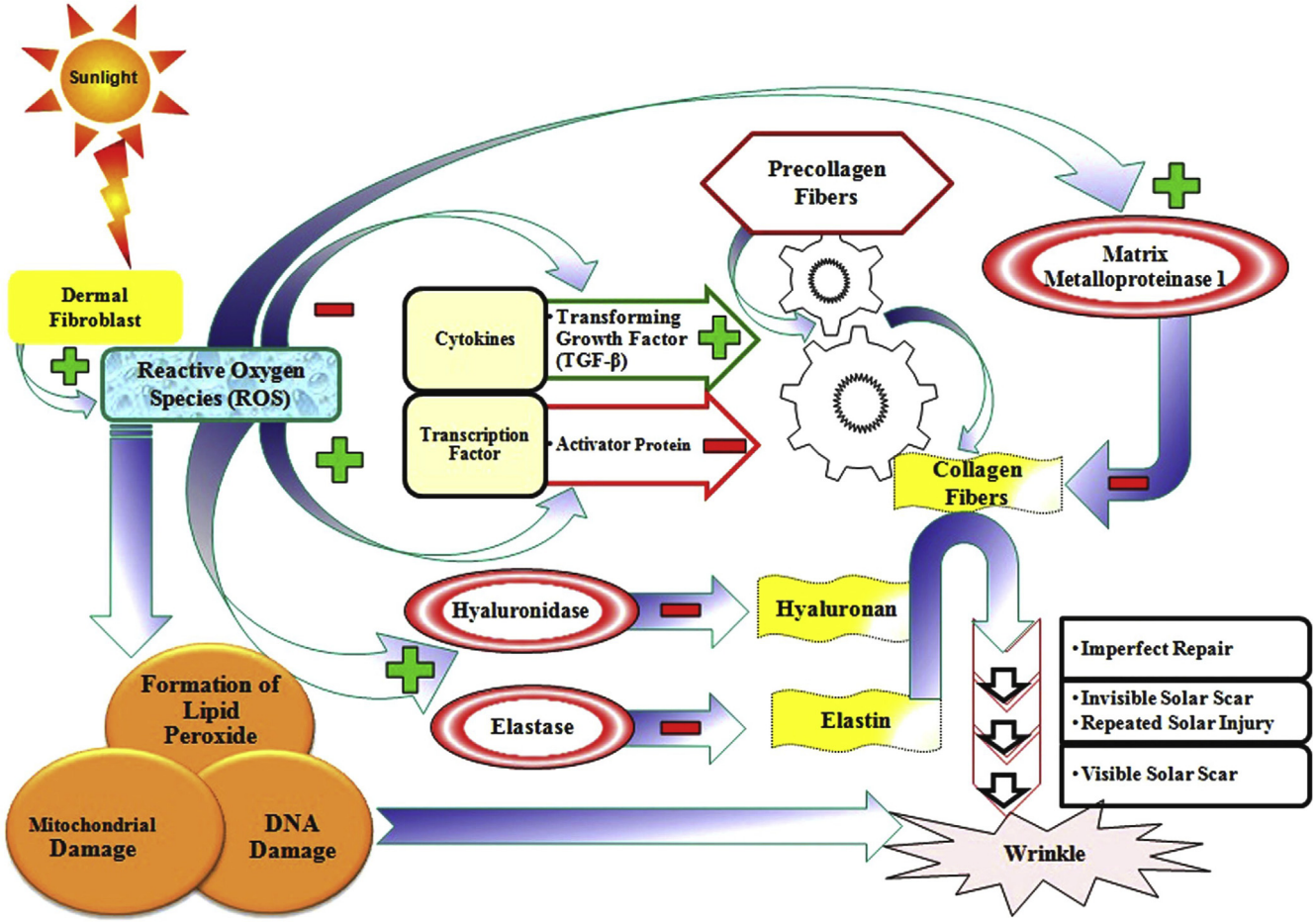


FIGURE 5.2 Pathway of premature skin aging [+, induction; −, inhibition]. *Reproduced with permission from Mukherjee et al., 2011, Phytomedicine 19, 64–73.*

5.5.3 Hyaluronidase and Elastase

Hyaluronidase is an enzyme belonging to the family of endolytic glycoside hydrolases that depolymerize the β1-4 linkages between N-acetylglucosamine and glucuronic acid of hyaluronan, and also takes part in the structural constituent of the pericellular matrix synthesized by skin fibroblasts [55]. The Hyaluronan content of the skin decreases due to age-related factors. Lysosomal enzyme known as hyaluronidase regulates the turnover of hyaluronan [7]. Therefore, hyaluronidase synthesis/degradation might be strongly altered in favor of hyaluronan degradation (Figure 5.2). Secretions of hyaluronidase increase with aging and cause degradation of hyaluronan and thereby decrease hydration and ion transport in the extracellular space [56]. Increased hyaluronic acid catabolism induced by UV-B stimulation of hyaluronidase activity might be one of the main factors involved in skin photoaging [57].

Elastase belongs to the family of chymotrypsin, which is another enzyme that is capable of hydrolyzing materials such as elastin and fibrillin, which are the fiber materials found within the ECM. The secretion and activation of elastase from dermal fibroblasts in response to UV irradiation and/or to cytokines released by keratinocytes are responsible for the degeneration of the three-dimensional structure of elastic fibers during the formation of wrinkles. Elastase has a significant impact in the metabolism of elastic fibers in skin tissues during photoaging. It was also found that the transcriptional activity of NF-κB is induced by UV irradiation and greatly contributes to the photoaging process [58].

5.5.4 DNA Damage and Mutation

DNA is a major UV-B-absorbing cellular chromophore in the skin. Cyclobutane pyrimidine dimers (CPDs) and pyrimidine photodimers are the most common and frequent intermediate products that are formed in the skin after UV-B exposure, and result in DNA strand ruptures [59]. Studies propose that most of the UV-B-induced CPDs are seen in the epidermis, but a considerable amount is also found in the dermis

[60]. Photoinduced carcinogenesis is a complex multistage process involving three distinct stages exemplified by initiation, promotion, and progression. UV-B irradiated in extensive amounts has been found to be associated with the formation of sunburn cells, initiated via a p53-mediated pathway, which causes the removal of damaged cells, and thus diminishes the risk of skin cancer. The lesser amount of UV-B permits cell survival and repair of genetic damage, but the cell survival response is a state of local and systemic immunosuppression, which in itself may be harmful and contributes to the process of development of skin cancers [61].

5.5.5 Nucleotide Excision Repair in Skin

Photoaging of human skin cells may cause potential mutation in sites are completely repaired by nucleotide excision repair (NER) before replication or synthesize DNA using postreplication repair-specific DNA polymerase, which is free from error. NER is a highly conserved approach for renovating a variety of bulky DNA damages, such as CPDs. Base change, such as 8-hydroxy 2′ -deoxyguanosine (8-OHdG), is repaired by base excision repair system using glycosylase in combination with replication protein-A, proliferating cell nuclear antigen, and AP endonuclease. The significance of NER for prevention of UV-stimulated skin cancer and benign tumors is well recognized by an extremely high occurrence of skin cancer and seborrheic keratoses on sun-exposed areas in xeroderma pigmentosum-affected patients who have faulty NER. This is a complex process involving >30 gene products including some important steps: (1) recognition of a DNA lesion; (2) single strand incision at both sides flanking the lesion; (3) excision of a single stranded DNA nucleotide (possibly 24−32 bases); (4) DNA repair synthesis to replace the excised DNA lesion; and (5) ligation of remaining single stranded nick [59].

5.5.6 Regulation of IL

Exposure to sunlight especially UV-B radiation results in the prevention of contact hypersensitivity (CHS) stimulated by contact allergens that is a prototypic T-cell-mediated immune response. In the skin, interleukin-12 (IL-12) may be produced locally either by keratinocytes or by macrophages. UV-B-exposed skin inhibits antigen appearance, and thereby downregulates CHS responses. IL-12 suppresses UV-induced apoptosis by stimulating DNA repair both in vitro and in vivo [59]. It is also shown to overcome UV-B-induced systemic immunosuppression of CHS and Delayed Type Hypersensitivity (DTH) reactions. The mechanisms of IL-12 to recover or inhibit UV-B-induced systemic immunosuppression are not obvious still, while IL-12 enhances the accessory cell function of Langerhans cells (LCs) in a mixed epidermal cell/lymphocyte reaction resulting in augmentation of interferon gamma (IFN-g) production. In addition, using an in vitro system, the effects of IL-12 on antigen presenting cell (APC) function in UV-B-induced systemic immunosuppression and demonstrated that IL-12-pretreated APC could not restore the reduced IFN-g production [60].

5.5.7 Modulating Cell Signaling Pathways

Several plant bioactive molecules have been reported to interact with cellular signaling pathways that are directly or indirectly involved in photoaging. Most of these pathways are signaling pathways that are involved in cell survival and programed cell death. In this respect, polyphenols have been reported to act at phosphoinositide 3-kinase (PI-3K), protein kinase B, tyrosine kinases, protein kinase C (PKC), and MAPK signaling cascades. They affect cellular function by altering the phosphorylation mode and expression level of targeted molecules within the mentioned pathways [62].

5.5.8 Nuclear Factor Kappa B

NF-κB, a transcription factor, is activated by UV radiation, which induces neutrophil attraction bringing neutrophil collagenase (MMP-8) into the UV incident site to further aggravate matrix degradation. Both AP-1 and NF-κB are activated by ROS that may provide the common denominator for driving these complex biologic interactions. Oxidative stress increases elastin mRNA levels in dermal fibroblasts and results in elastotic changes observed in photoaged skin cell [60].

5.5.9 Mitogen-activated Protein Kinase

Xu and Fisher [62] demonstrated that UV stimulation of diverse cell surface receptors results in simultaneous activation of multiple receptor-coupled signal transduction pathways, including the three MAPKs, signaling modules (extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38), PI-3 kinase, and NF-kB pathway. MAPKs play a critical role in transmitting environmental cues via cell surface receptors to transcriptional machinery in the nucleus [63−65]. The three different MAPK signal transduction pathways have been recognized including ERK1/2, JNK, and p38 MAPK [66]. The mechanisms behind UV-B-induced COX-2 expression in cultured cells [67] as well as in mouse skin in vivo [68] involve intracellular signaling mediated by different upstream kinases, such as MAPKs and PI3K. Further, the inhibition of MAP kinase kinase

(MEK), which is upstream of ERK1/2, inhibited UV-B-induced COX-2 expression in human epidermal keratinocytes (HaCaT) [69]. Human or mouse skin, in contact with UV light stimulates MMPs, which have been concerned with aging. UV radiation activates AP-l that stimulates transcription of MMP genes encoding MMP-l (collagenase), MMP-9 (gelatinase), and MMP-3 (stromelysin-l) in skin cells. These modifications apparently happen through induction of AP-1 that is activated by a series of MAPKs [70,71]. Altogether, MMPs are capable of degrading the collagen framework and other components of skin connective tissue.

5.5.10 Cyclooxygenase-2

Kundu et al. [32] discussed that inadequate expression and/or activity of COX-2, a rate-limiting enzyme involved in the biosynthesis of prostaglandins, has been concerned in UV-B-induced skin carcinogenesis [72]. The UV-B-induced ROS production may be responsible for irregular induction of COX-2. Molecular mechanisms underlying UV-B-induced Cyclooxygenase (COX-2) expression in cultured cells [73] as well as in mouse skin in vivo [68] involve intracellular signaling mediated by different upstream kinases, including MAP kinases, and PI3K. The blockade of MAP kinase (MEK), which is upstream of extracellular signal-regulated protein kinase1/2 (ERK1/2), prevents UV-B-induced COX-2 expression in HaCaT [69]. Similarly, pretreatment of HaCaT cells with inhibitors of p38 MAP kinase [73], JNK, and PI3K [69] resulted in a significant decline in the UV-B-induced expression of COX-2 [74]. Therefore, suppression of inflammatory subcutaneous (SC) damage and timely induction of cellular antioxidant capacity may provide systemic approaches for the prevention of photoaging and photocarcinogenesis.

5.5.11 Protein Kinase C

Ichihashi et al. [59] described that PKC is a serine/threonine−protein kinase family consisting of >10 isoforms, which is involved in a diverse signal transduction pathway. PKC isoforms have regulatory and catalytic domains in the amino and carboxyl-terminal halves, respectively. Further PKC can be classified into three categories, namely, cPKC, nPKC, and aPKC, based upon structural differences in their regulatory domains, which require specific cofactors in each category. The PKC isoform causes growth inhibition and differentiation in cultured cells when it is overexpressed. In addition, a proteolytic fragment of dPKC containing its catalytic domain is produced in cells in response to UV. The activation of aPKC isoforms is inhibited by UV exposure, and overexpression of an aPKC isoforms can guard against UV-induced apoptosis. aPKC isoforms have also been concerned with the activation of AP-1 by UV light. cPKC and nPKC isoforms are molecular targets for tumor-promoting phorbol esters in skin chemical carcinogenesis.

5.6 NATURAL BIOACTIVE MOLECULES AGAINST SKIN AGING

The use of bioactive molecules from natural sources is very beneficial in combating the harmful effects of solar light. Antioxidants and photoprotective agents include phenolic acids, flavonoids, and high molecular weight polyphenols. Flavonoids are natural antioxidants derived from the plant kingdom and are widely present in human diet in the form of numerous edible fruits and vegetables [75]. This has generated great interest in using dietary botanical supplements rich in antioxidants for the prevention of photocarcinogenesis and photoaging [10]. Although avoiding excessive exposure to the sun, wearing protective clothing, and using sunscreen lotions are popular recommendations, there is a need for the development of effective phytochemicals that are capable of ameliorating the adverse effects of UV radiations. Various plant-derived phytochemicals possess photoprotective properties by several mechanisms as represented in Figure 5.1. Understanding how such phytochemicals exert their effects is not only important but essential to the development of better and more effective photochemopreventive products for general human use [60]. The biological effects, postulated mechanism(s), and phytoconstituents of these botanical antioxidants are listed in Table 5.1. Presently, medicinal plants have a vast experiential-evidence base for prevention and treatment of skin aging.

5.7 FEW MEDICINAL PLANTS USEFUL IN SKIN AGING

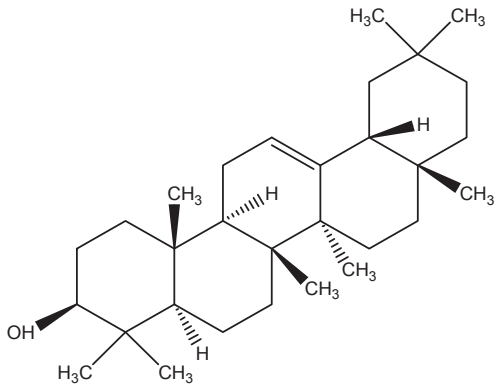
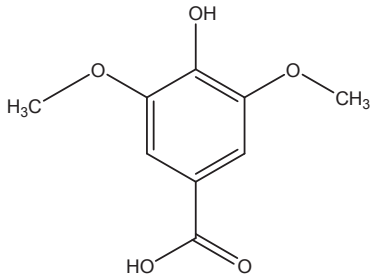
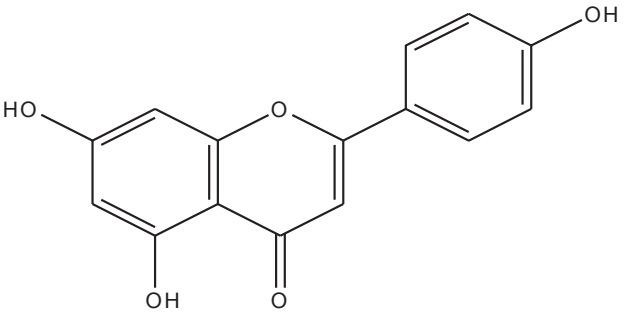
5.7.1 *Tagetes erecta* (Fam: Asteraceae)

Flowers contain provitamin A "-carotene," β-smyrin and syringic acids responsible for photoprotection. Methanol extract of flowers has been found to possess in vitro inhibition of hyaluronidase and elastase and MMP-1, which suggested the potential of this plant as an antiwrinkle agent [20].

5.7.2 *Citrus sinensis* (Fam: Rutaceae)

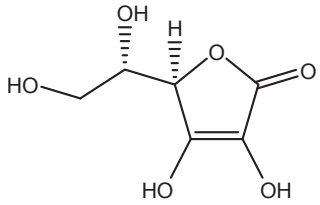
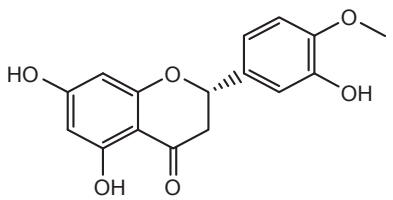
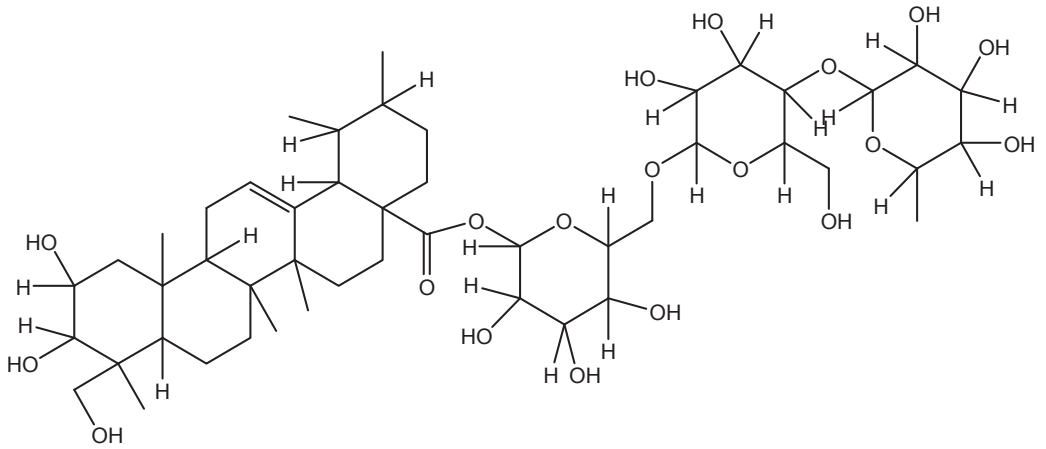
Cimino et al. [107] have reported that phenolic compounds, such as anthocyanins, hesperetin, flavanones, hydroxycinnamic acids, and ascorbic acid, are

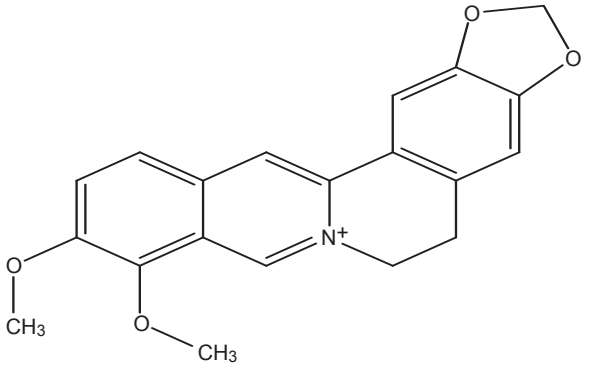
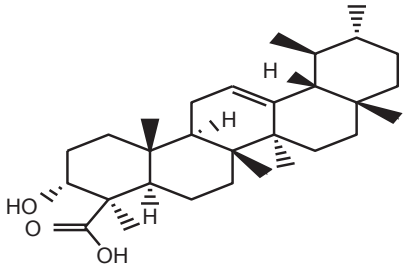
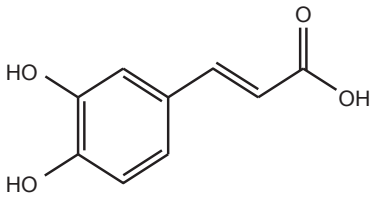
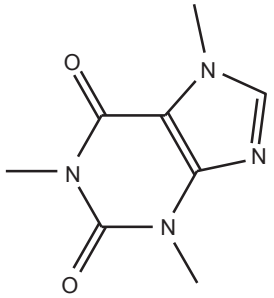
TABLE 5.1 Some Natural Bioactive Leads Against Photodamage

Bioactive compounds	Chemical structure	Plant name (family)	Mechanism	Reference
β-Amyrin, syringic acid	 β-Amyrin  Syringic acid	*Tagetes erecta* (Asteraceae) Part used: Leaves	Inhibition of elastase, hyaluronidase, and MMP-1, Inhibition of reactive oxygen species (ROS)	[7,12]
Apigenin		*Matricaria recutita* (Asteraceae) Part used: Flowering head	Inhibition of UV-mediated induction of ornithine decarboxylase (ODC) activity, reduction in cancer incidence, and increase in tumor-free survival Inhibition of MMP-1 activity and downregulation of MMP-1 expression and activator protein-1 (AP-1) activation	[60,76]

Continued

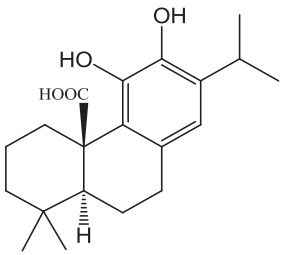
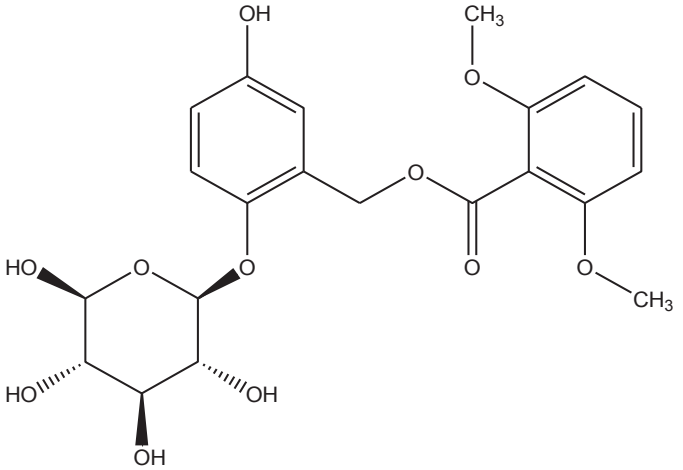
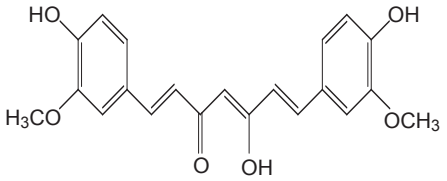
TABLE 5.1 Some Natural Bioactive Leads Against Photodamage—cont'd

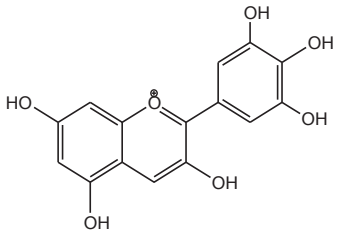
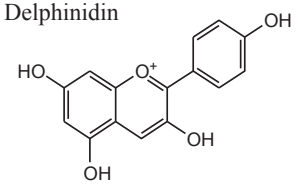
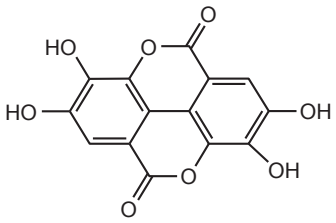
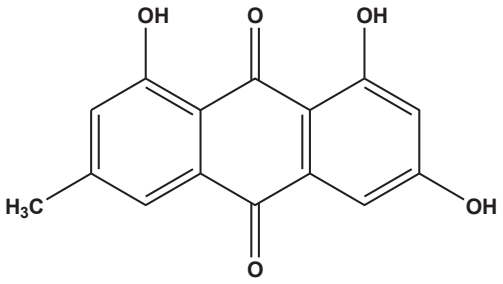
Bioactive compounds	Chemical structure	Plant name (family)	Mechanism	Reference
Ascorbic acid		*Citrus sinensis* (Rutaceae) Part used: Fruit	Nuclear factor kappa B (NF-κB) and AP-1 translocation and procaspase-3 cleavage Inhibition of the hyaluronidase and elastase enzyme and free radical scavenging activity	[7,77,78]
Hesperetin		*C. sinensis* (Rutaceae) Part used: Fruit	Potential antityrosinase and antioxidant activity Protection against UV-A-induced necrotic cell in human skin fibroblasts (FEK4)	[7,79,80]
Asiaticoside		*Centella asiatica* (Umbelliferae) Part used: Whole plant	Improvement of the clinical score for deep and superficial wrinkles, suppleness, firmness, roughness, and skin hydration and induction of type-I collagen synthesis	[7,81]

Berberine		*Berberis aristata* (Berberidaceae) Part used: Berries	Inhibition of the expression of MMP-9 and suppression of TPA-induced Interleukin (IL-6) expression Type-I procollagen expression increased	[7,82]
Boswellic acids		*Boswellia serrata* (Burseraceae) Part used: Resin	Interference with the production of leukotrienes by inhibition of 5-lipoxygenase and inhibition of the MMP transcription in fibroblasts and endothelial cells	[83]
Caffeic acid		*Hibiscus rosa sinensis* (Malvaceae) Part used: Leaves and flower	Topical protective agents against UV radiation-induced skin damage Potent ROS scavenging activity Inhibitory effect on MMP-9	[84,85]
Caffeine		*Camellia sinensis* (Theaceae) Part used: Young leaf	UV-B-induced mutations in the p53 gene in early mutant p53-positive patches inhibited UV-B-induced carcinogenesis.	[86]

Continued

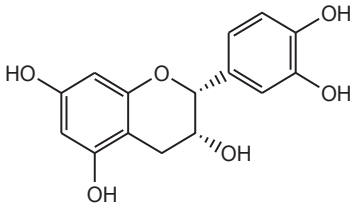
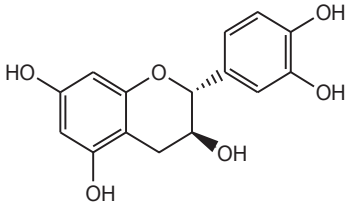
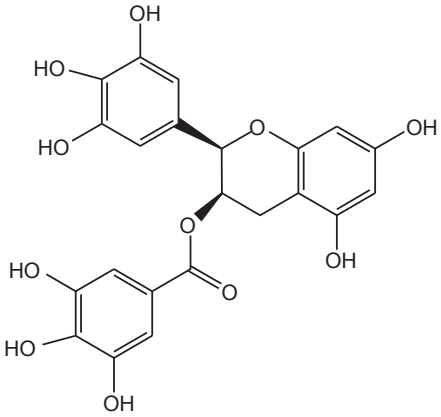
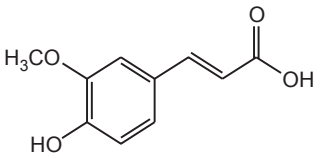
TABLE 5.1 Some Natural Bioactive Leads Against Photodamage—cont'd

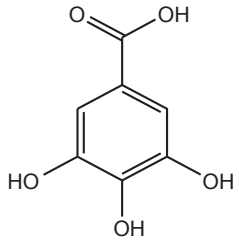
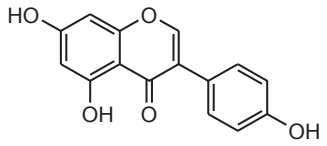
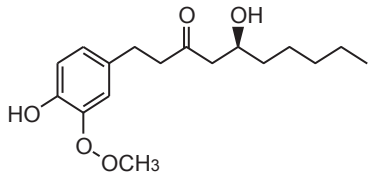
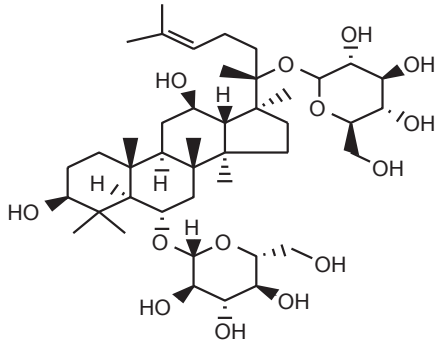
Bioactive compounds	Chemical structure	Plant name (family)	Mechanism	Reference
Carnosic acid		*Rosmarinus officinalis* (Lamiaceae) Part used: Leaves, whole plant	Scavenging of free radicals and modulation of xenobiotic metabolizing enzymes Protection against UV-A-induced photoaging by inhibition of MMP-1 mRNA expression in human fibroblasts	[87,88]
Curcoligoside		*Curculigo orchioides* (Hypoxidaceae) Part used: Rhizome	Inhibition of MMP-1 expression	[7,89]
Curcumin		*Curcuma longa* (Zingiberaceae) Part used: Rhizome	Inhibition of MMP-1 and MMP-2 expressions Induction of apoptosis, activation of caspase-3, -8, and -9, release of cytochrome *c* Inhibition of Cyclooxygenase (COX)-2 mRNA and protein expressions, c-Jun N-terminal Kinase, and p38 in UV-B-irradiated Human keratinocyte (HaCaT) cells	[7,60]

Delphinidin, Pelargonidin	 Delphinidin  Pelargonidin	*Punica granatum* (Punicaceae) Part used: Fruit	Protection of HaCaT cells against UV-B-mediated decrease in cell viability, induction of apoptosis, increase in lipid peroxidation Inhibition of UV-B-mediated apoptosis and markers of DNA damage such as Cyclobutane pyrimidine dimer and 8-hydroxy 2′-deoxyguanosine (8-OHdG) in SKH-1 hairless mouse skin [10,60]
Ellagic acid		*Terminalia chebula* (Combretaceae) Part used: Fruit	Attenuation of the UV-B-induced toxicity of HaCaT keratinocytes and human dermal fibroblasts Inhibition of MMP production in UV-B-exposed fibroblasts Inhibition of the UV-A-induced apoptosis of HaCaT cells, as measured by a reduction of DNA fragmentation, mitochondria dysfunction, ER stress, caspase-3 activation, and Bcl-2/Bax deregulation Upregulation of the HO-1 and Nrf-2 antioxidant genes Inhibition of tyrosinase enzyme [90–92]
Emodin		*Aloe vera* (Liliaceae) Part used: Leaves	Inhibition of stimulated granulocyte MMPs, inhibition of tyrosine hydroxylase and 3,4dihydroxy-phenylalanine oxidase Elevation of levels of RNA and protein expression of superoxide dismutase [7,93,94]

Continued

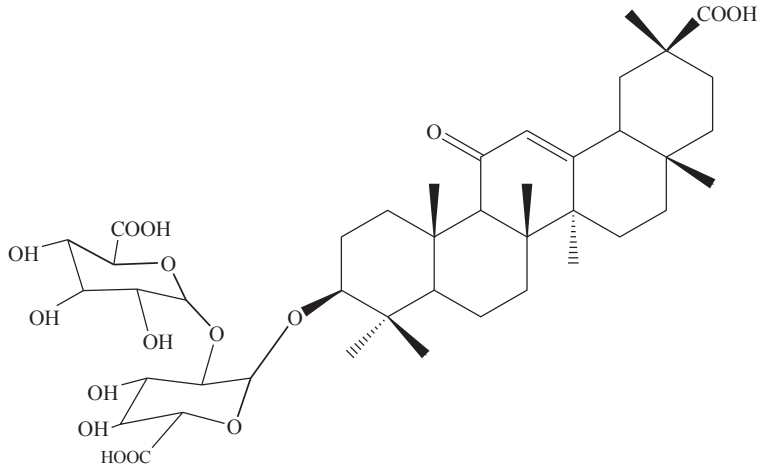
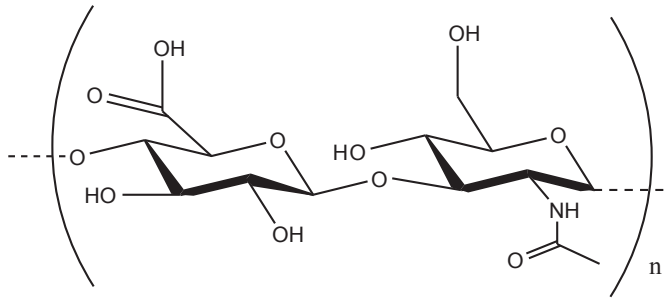
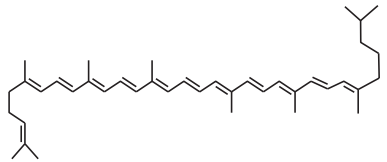
TABLE 5.1 Some Natural Bioactive Leads Against Photodamage—cont'd

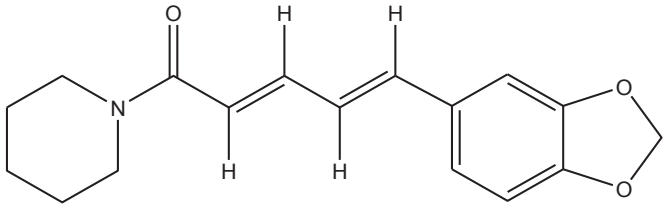
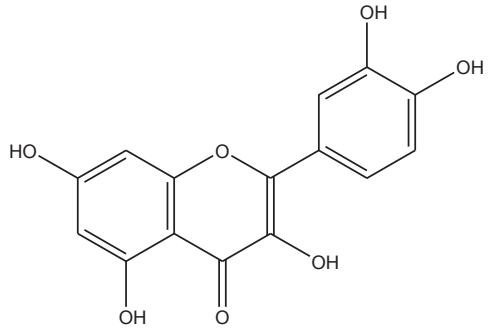
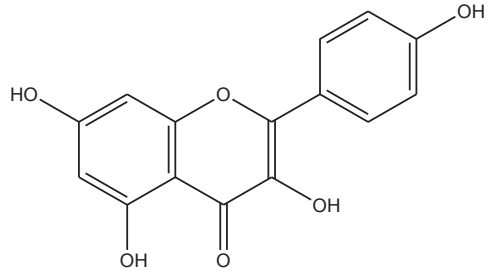
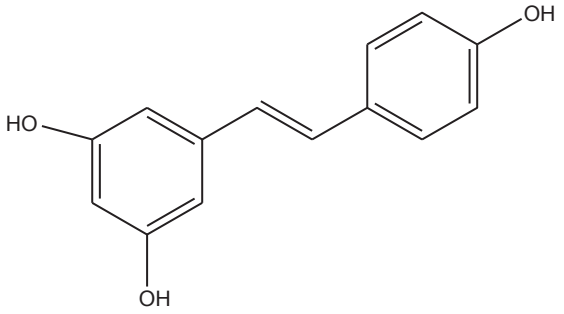
Bioactive compounds	Chemical structure	Plant name (family)	Mechanism	Reference
Epicatechin, Catechin, epigallocatechin gallate	 Epicatechin  Catechin  Epigallocatechin gallate	*Camellia sinensis* (Theaceae) Part used: Leaves	Suppression of UV irradiation-induced cutaneous erythema, thickening of the epidermis, overexpression of CK5/6, CK16, MMP-2, MMP-9 modulations in NF-κB and Mitogen-activated protein kinase (MAPK) pathways Decrease in H_2O_2 and NOS-expressing cells and inhibition of H_2O_2 and nitric oxide production in both the dermis and epidermis Restoration of UV-B-induced decrease in glutathione (GSH) level Inhibition of skin tumors, tumor regression, inhibition of DNA synthesis, enhancement of apoptosis	[60,95]
Ferulic acid		*Theobroma cacao* (Sterculiaceae) Part used: Seed, bean	Downregulation of hydroxyproline and pepsin-resistant hydroxyproline content An antioxidant to prevent damage from UV radiation and skin carcinogenesis	[7,96]

Gallic acid		*T. chebula* (Combretaceae) *Emblica officinalis* (Phyllanthaceae) Part used: Fruit	Tyrosinase and MMP-2 inhibition of elastase, hyaluronidase, MMP-2 enzyme inhibition Inhibition of type-I collagen collagenase, increase in TIMP-1 level	[90,97,98] [7,10,60]
Genistein, Daidzein		*Glycine max* (Fabaceae) Part used: Seed	Inhibition of melanosome phagocytosis, prevention of the activation of caspase-3 pathway Inhibition of UV-induced DNA damage. In mice, inhibition of UV-B-induced H_2O_2 and Malondialdehyde (MDA) in skin and 8-OHdG in the epidermis	
Gingerol		*Zingiber officinale* (Zingiberaceae) Part used: Rhizome	Reduction in UV-B-induced intracellular ROS levels, activation of caspase-3, -8, -9, and Fas expression, inhibition of the induction of COX-2 mRNA and protein and NF-κB translocation Inhibition of fibroblast-derived elastase	[7,60]
Ginsenoside		*Panax ginseng* (Araliaceae) Part used: Root	Type-I procollagen gene and protein expression, prevention of MMP-9 gene induction, and elongation of the fibrillin-1 fiber length Increase of expression of procollagen type-I and decrease MMP-1	[7,99]

Continued

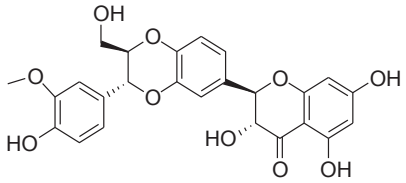
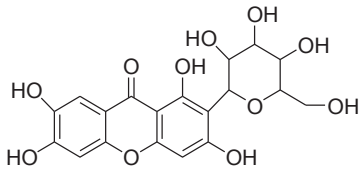
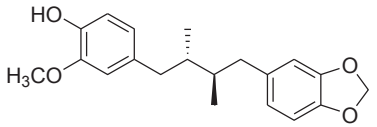
TABLE 5.1 Some Natural Bioactive Leads Against Photodamage—cont'd

Bioactive compounds	Chemical structure	Plant name (family)	Mechanism	Reference
Glycyrrhizin		*Glycyrrhiza glabra* (Fabaceae) Part used: Root and rhizome	Inhibition of the activation of NF-κB and the activities of phosphoinositide-3-kinase and reduction of the production of LPS-induced TNF-α, IL-6, and IL-1β Reduction of the ROS	[100]
Hyaluronic acid		Glycyrrhiza glabra (Fabaceae) Part used: Root and rhizome	Increase of the content of hyaluronic acid, increase of the cellular expression of telomerase reverse transcriptase	[7]
Lycopene		*Solanum lycopersicum* (Solanaceae) Part used: Fruit	Inhibition of UV-B-induced ODC and myeloperoxidase and prevention of the cleavage of caspase-3 and significant reversal of UV-B-induced proliferating cell nuclear antigen inhibition Elevation of the actinic erythema threshold and a general reduction in UV-induced erythemas, reduction in UV-induced p53 expression and sunburn cells, and a parallel reduction in lipoperoxide levels and increase in pigmentation	[60]

Piperine		*Piper betel* (Piperaceae) Part used: Leaves	Protection of photosensitization-mediated Lipid peroxidation [7,101]
Quercetin, Kaempferol	 Quercetin  Kaempferol	*Calendula officinalis* (Asteraceae) Part used: Flower and leaves	Control of the secretion of MMP-2 and MMP-9. Improvement in the collagen synthesis in the subepidermal connective tissue Potent inhibition of UV-B-induced oxidative skin damage Inhibition of the expression of MMP-1, at mRNA and protein levels Scavenging of oxygen radicals, protection against lipid peroxidation by reducing the amount of MDA, and complexation of transition metal ions to form inert chelate complexes [75,102,103]
Resveratrol		*Vitis vinifera* (Vitaceae) Part used: Fruit	Inhibition of UV-B-induced skin edema and decrease in UV-B-mediated generation of H_2O_2, and activation of NF-κB, network, and MAPK pathway Inhibition of the expression of MMP-2 and MMP-9 and inhibition of the proliferation in multiple myeloma cell lines [60,104,105]

Continued

TABLE 5.1 Some Natural Bioactive Leads Against Photodamage—cont'd

Bioactive compounds	Chemical structure	Plant name (family)	Mechanism	Reference
Silymarin (silybin, silibini)		*Silybum marianum* (Asteraceae) Part used: Fruit	Reduction in GSH depletion, ROS production, lipid peroxidation, formation of UV-A-induced DNA single strand breaks, caspase-3 activity Reduction in the UV-B-induced enhancement of the levels of IL-10, in the skin and draining lymph nodes and enhancement in the levels of IL-12	[60,106]
Mangiferin		*Mangifera indica* (Anacardiaceae) Part used: Leaves, bark, fruits	Prevention of neuronal death, oxidative stress, and mitochondrial depolarization Quenching of reactive oxygen intermediates Erk1/2 pathway, protein kinase signaling pathway, NF-β signaling pathway	[94,107]
Macelignan		*Myristica fragrans* (Myristicaceae) Part used: Rhizomes	Protection of skin keratinocytes from UV-B-induced damage and inhibition of MMP-9 and COX-2 expression by attenuation of the activation of MAPKs and PI3K	[108]

responsible for the antiphotoaging activity of three different varieties of *Citrus sinensis* in modulating cellular responses such as NF-κB and AP-1 translocation and procaspase-3 cleavage to UV-B in human keratinocytes. Thus, *C. sinensis* has been proposed as a useful natural standardized extract in skin photoprotection with promising applications in the field of dermatology.

5.7.3 *Centella asiatica* (Fam: Apiaceae)

Centella asiatica contains several active triterpenoids, saponins, including madecassoside, asiaticoside, centelloside, and asiatic acid, which have been reported to increase cellular hyperplasia, collagen production, granulation tissue levels of DNA, protein, total collagen, hexosamine, rapid maturation, and crosslinking of collagen [109]. Haftek et al. [110] have performed a randomized double-blind clinical trial and found significant improvement of the clinical score for wrinkles, suppleness, firmness, roughness, and skin hydration. Asiaticoside is another active saponin, which induced type-I collagen synthesis in human dermal fibroblast cells. Triterpenes including asiatic acid, madecassic acid, and asiaticoside extracted from *C. asiatica* were screened on human fore skin fibroblast monolayer cultures; it was observed that collagen synthesis was increased in a dose-dependent manner, whereas the specific activity of neosynthesized collagen was decreased [111].

5.7.4 *Berberis aristata* (Fam: Berberidaceae)

Topical application of *Berberis aristata* has been reported to prevent acne vulgaris in patients suffering from skin disorders [112]. Berberine isolated from *B. aristata* has been reported to inhibit basal and TPA-induced expression and activity of MMP-9 and also suppressed TPA-induced IL-6 expression, ERK activation and AP-1 DNA binding activity in UV-induced skin inflammation, aging process, and degradation of extracellular matrix proteins [55]. Kim and Chung [82] reported that MMP-1 and type-I procollagen expression in human dermal fibroblasts regulated by berberine.

5.7.5 *Curcuma longa* (Fam: Zingiberaceae)

The effect of a *Curcuma longa* extract has been found to bring about potential changes in skin thickness, increased elasticity, decreased pigmentation, and wrinkling caused by long-term, low-dose UV-B irradiation. It prevents the formation of wrinkles and melanin and increases the diameter and length of skin blood vessels and decreases the expression of MMP-2 [7,60].

5.7.6 *Terminalia chebula* (Fam: Combretaceae)

In vitro skin cell protective activity of *Terminalia chebula* has been evaluated through antioxidative and tyrosinase inhibition activity as well as the antiproliferative and MMP-2 inhibition activity on early aged human skin fibroblasts. The plant showed 1.37 times more potent MMP-2 inhibition than did ascorbic acid on fibroblasts when determined by zymography [92].

5.7.7 *Zingiber officinale* (Fam: Zingiberaceae)

Topical application of *Zingiber officinale* extract to hairless mouse skin significantly inhibited the wrinkle formation induced by chronic UV-B irradiation at a suberythemal dose accompanied by a significant prevention of the decrease in skin elasticity [7,60].

5.7.8 *Aloe vera* (Fam: Xanthorrhoeaceae)

Emodine and aloin A and B have been shown to inhibit *Clostridium histolyticum* collagenase reversibly and noncompetitively. Both aloe gel and aloin are also effective inhibitors of stimulated granulocyte MMPs [113]. Aloesin from *Aloe vera* has been reported to modulate melanogenesis via the competitive inhibition of tyrosinase. Tyrosine hydroxylase and 3, 4-dihydroxyphenylalanine oxidase activities of tyrosinase from normal human melanocyte cell lysates were inhibited by aloesin in a dose-dependent manner [93].

5.7.9 *Theobroma cacao* (Fam: Malvaceae)

Cacao bean and cola nut are popular edible plants that contain polyphenols and xanthine derivatives, which protective effects against UV-induced erythema when topically applied to the dorsal skin of hairless mice [114].

5.7.10 *Glycine max* (Fam: Fabaceae)

Anthocyanin isolated from *Glycine max* seed is responsible for the downregulation of in vitro and in vivo UV-B-induced ROS levels and apoptotic cell death through the prevention of caspase-3 pathway activation and reduction of proapoptotic Bax protein levels [10].

5.7.11 *Panax ginseng* (Fam: Araliaceae)

Bioactive constituents, ginsenoside, have antiskin aging activities. Red ginseng extract improved type-I procollagen gene and protein expression, prevented MMP-9 gene induction, and elongated the fibrillin-1 fiber length, and thereby reduced facial wrinkles [73]. Red Ginseng also inhibits the increase of epidermal thickness and skin TGF-1 content induced by UV-B irradiation, which

may be due to partial inhibition of the increase of skin TGF-1 [89,99].

5.7.12 *Vitis vinifera* (Fam: Vitaceae)

Vitis vinifera fruit has a stronger in vitro antioxidant capacity than vitamin C or vitamin E has on cultured normal human keratinocytes and also in vivo photoaging activity in combination with a biotechnological extract (Ronacare Hydroine) [60]. The dermatologic evaluation showed that application of a serum containing the combination improved the main clinical signs of photoaged skin. Resveratrol a bioactive constituent inhibits the expression of MMP-2 and MMP-9 and inhibits the proliferation in multiple myeloma cell lines [104].

5.7.13 *Piper betel* (Fam: Piperaceae)

Allylpyrocatechol, piperine, and chavibetol isolated from *Piper betel* leaves have been established to effectively protect photosensitization-mediated lipid peroxidation of rat liver mitochondria. Allylpyrocatechol also prevented the unfavorable effects of the type-II photosensitization-induced toxicity to mouse fibroblast L929 cells [7].

5.7.14 *Calendula officinalis* (Fam: Asteraceae)

Oral treatment of hairless mice maintained GSH levels close to those of nonirradiated control mice and affected the activity/secretion of MMP-2 and MMP-9 stimulated by exposure to UV-B irradiation [95].

5.7.15 *Astragalus membranaceus* (Fam: Fabaceae)

Astragalus membranaceus has been found to increase the content of hyaluronic acid in cultures of keratinocytes and fibroblasts by elevating the hyaluronan synthase-3 and hyaluronan synthase-2 mRNA expressions [115].

5.7.16 *Curculigo orchioides* (Fam: Hypoxidaceae)

Curculigoside isolated from rhizomes of *Curculigo orchioides* has been reported to possess strong inhibitory activity against MMP-1 in cultured human skin fibroblasts and suggests its skin improvement property [89].

5.7.17 *Emblica officinalis* (Fam: Phyllanthaceae)

E. officinalis shows significant type-I collagen promotion and anticollagenase effects on primary mouse fibroblast cells, as determined by immunocytochemistry and Western blot analysis [16]. Fruit extract has been reported to stimulate the proliferation of fibroblasts and induced production of procollagen in a concentration- and time-dependent manner. On the contrary, MMP-1 production from fibroblasts was dramatically decreased, whereas TIMP-1 was significantly increased [116].

5.7.18 *Camellia sinensis* (Fam: Theaceae)

Sunscreen formulated with 2–5% *Camellia sinensis* extract has been reported to protect UV irradiation-induced photoaging, photoimmunosuppression, cutaneous erythema, thickening of the epidermis, overexpression of CK5/6, CK16, MMP-2, MMP-9, etc. [117,118]. Nichols and Katiyar [52] reported that green tea polyphenols, catechin, epigallocatechin, and epigallocatechin-3-gallate, function favorably as sunscreen supplements to protect the skin from the adverse effects of UV radiation-induced inflammation, oxidative stress, and DNA damage including the risk of developing skin cancers. Caffeine an active constituent of the *C. sinensis* inhibits the formation of early patches of epidermal cells [7].

5.7.19 *Theobroma cacao* (Fam: Malvaceae)

Cacao bean and cola nut contain polyphenols and xanthine derivatives, which have protective effects against UV-induced erythema when topically applied to the dorsal skin of hairless mice. Ferulic acid chemical constituent is a strong antioxidant that prevents damage from UV radiation and skin carcinogenesis [96]. The total hydroxyproline and pepsin-resistant hydroxyproline content downregulated markedly increased after UV irradiation [114].

5.7.20 *Silybum marianum* (Fam: Asteraceae)

Silymarin a flavonolignan from *Silybum marianum* has antioxidant and antiinflammatory properties. Oxidative stress is one of the major mechanisms for skin aging and dermatologic conditions; phytochemicals with proven antioxidant activity, such as silymarin, could be useful for treating many dermatologic conditions as well as skin aging [119]. It has been shown that concentration-dependent reduction of UV-A caused oxidative stress and ROS production as well as lipid peroxidation in irradiated cells [60].

5.7.21 *Matricaria recutita* (Fam: Asteraceae)

Apigenin active constituent of *Matricaria recutita* plants is most commonly used in topical health and

beauty products for its soothing and antiinflammatory effects on skin. It also inhibits the UV-mediated induction of ornithine decarboxylase (ODC) activity as well as reduction in cancer incidence with an increase in tumor-free survival [120]. Apigenin has been shown to inhibit UV-induced COX-2 expression and downregulate MMP-1 expression via the inhibition of AP-1 activation [121].

5.7.22 *Solanum lycopersicum* (Fam: Solanaceae)

Solanum lycopersycum fruit mainly contains lycopene, β-carotene, α-Lipoic acid, vitamin C, and vitamin. These compounds are able to prevent and retain free radical formation, which can cause aging and chronic diseases. Lycopene shows dose-dependent inhibition of UV-B-induced ODC and myeloperoxidase activities and significantly reduced bifold skin thickness [60].

5.7.23 *Glycyrrhiza glabra* (Fam: Fabaceae)

Glycyrrhiza glabra is a well-known herb in Indian and Chinese traditional medicines and is commonly known as liquorice. The major phytoconstituents of liquorice having antioxidant activity are glycyrrhizin (glycyrrhizic acid) and flavonoids. The role of *G. glabra* extract on skin is mainly attributed to its antioxidant activity particularly to its potent antioxidants triterpene saponins and flavonoids [122].

5.7.24 *Mangifera indica* (Fam: Anacardiaceae)

Mangifera indica extract contains a defined mixture of components including polyphenols, triterpenes, phytosterols, fatty acids, and microelements. Various pharmacological activities have been reported such as antioxidant, analgesic, and antiinflammatory properties. The extract has a powerful in vitro scavenger activity of hydroxyl radicals and hypochlorous acid and acts as an iron chelator [108].

5.7.25 *Rosmarinus officinalis* (Fam: Lamiaceae)

Rosmarinus officinalis has been used extensively in traditional medicine. Anthropologists and archaeologists have found evidence that *R. officinalis* herbs have been used as cosmetics in ancient Egypt, Mesopotamia, China, and India. It is also used in traditional medicine for digestive disorders, headaches and migraine, dysmenorrhea, amenorrhea and oligomenorrhea, states of exhaustion, dizziness, and poor memory. Its astringent and antiseptic nature stimulates metabolic processes in the skin's dermal layer and is very beneficial in cases of acne, dermatitis, and eczema [123].

5.7.26 *Punica granatum* (Fam: Punicacea)

Pomegranate (*Punica granatum*) has strong antioxidant activity and good potency for cancer prevention. Extract of *P. granatum* inhibits UV-B-mediated apoptosis and markers of DNA damage, such as CPD and 8-OHdG, in SKH-1 hairless mice skin [10].

5.7.27 *Boswellia serrata* (Fam: Burseraceae)

Boswellic acid is pentacyclic triterpenes with strong antiinflammatory activity; their most important source is the extract of the gum resin of *Boswellia serrata*. The molecular mechanism of boswellic acid is the inhibition of MMP transcription in fibroblasts and endothelial cells [44]. Another well-known biological activity of boswellic acid is inhibition of kappa B kinases as the molecular target, with consequent inhibition of NF-kappa B activation and TNF-α release from activated monocytes [83].

5.7.28 *Hibiscus rosa sinensis* (Fam: Malvaceae)

Hibiscus rosa sinensis have various pharmacological activities such as antioxidants, antiinflammatory, antidiabetic, and hyperglycemic activities. Sajia et al. (2000) [85] reported that extracts of *H. rosa sinensis* have potent ROS scavenging activity and inhibitory effect on MMP-9.

5.7.29 *Myristica fragrans* (Fam: Myristicaceae)

Several pharmacological studies of *Myristica fragrans* show the antiinflammatory properties of myristicin from mace, and also have antifungal, antibacterial, larvicidal, and antioxidant potential. The main constituents of *M. fragrans* fruit have been found to be alkyl benzene derivatives (myristicin, elemicin, safrole, etc.), terpenes, alpha-pinene, myristic acid, trimyristicin, volatile oil, fixed oil, and starch. Protection of skin keratinocytes from UV-B-induced damage attained by inhibiting MMP-9 and COX-2 expression by attenuating the activation of MAPKs and PI3K [108].

5.8 MANAGEMENT OF SKIN AGING

Skin aging is a complex biological process and is influenced by a combination of several endogenous and exogenous factors. Healthy and beautiful skin is considered as one of the principal factors representing overall well-being and perception of good health in

humans. The main goal for prevention and treatment of skin aging is to increase the biosynthetic capacity of fibroblasts, which induces the reconstruction of an optimal physiologic environment; enhancement of cell activity; synthesis of collagen, elastin, and hyaluronic acid [124].

During 2004–2014, several antiaging strategies have been developed including preventive measurements, cosmetological strategies, topical and systemic therapeutic agents, invasive procedures, etc. The schematic diagram is presented in Figure. 5.3. The skin antiaging strategies to reverse the dermal and epidermal signs of photoaging and chronological aging can be grouped under the following approaches.

5.8.1 Cosmetological Care

Skin barrier itself is an important defender against dehydration; infiltration of microorganisms, allergens, irritants, ROS, and radiation. Therefore, daily skin care is required and that may increase skin regeneration, elasticity, and smoothness [125]. It is compulsory to stop the degradation of the skin primary structural constituents, such as collagen and elastin, to prevent the formation of wrinkles. Another essential approach for preventing skin aging is the reduction of inflammation by topical or systemic antioxidants that may be used in combination with sunscreens and retinoids to enhance their protective effects [41].

5.8.2 Systemic Agents

The main objective to prevent skin aging includes sun avoidance, sun protection using sunscreens to block or reduce skin exposure to UV radiation, retinoids to inhibit collagenase synthesis and to promote collagen production, and antioxidants to reduce and neutralize free radicals [126]. Some important studies indicate that it is possible to delay skin aging to improve skin conditions through administration of some selected nutritional supplements. Nutritional antioxidants act through different mechanisms in skin aging but they are mainly free radical scavengers. Nutritional supplements are the most important source of antioxidants and the well-known systemic antioxidants belong to vitamin C, vitamin E, and carotenoids [41]. It is well known that there is a progressive decrease in the hormone level with the age. The main

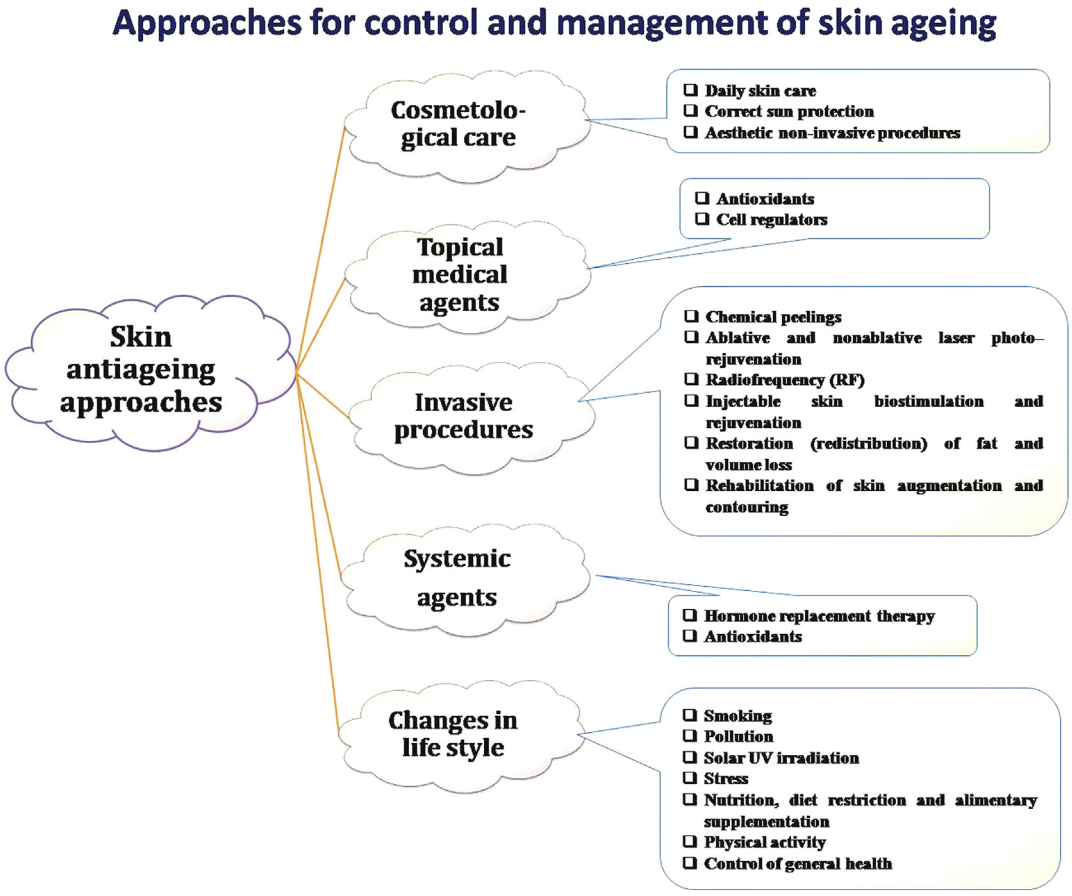


FIGURE 5.3 Strategies toward control and management of skin aging.

hormonal deficits in humans are menopause, andropause, and partial androgen deficiency for aging. Improvement of the skin health, libido, and osseous density has been observed in men and woman on systemic administration of dehydroepiandrosterone and growth hormone [127].

5.8.3 Topical Medicinal Agents

There are two main groups of agents, such as the antioxidants and cell regulators, that can be used as antiaging cream components. Different antioxidants, for example, vitamins, polyphenols, and flavonoids, reduce collagen degradation by reducing the concentration of free radicals in tissues. Several cell regulators, such as retinols, peptides, and growth factors, have direct effects on the metabolism and production of collagen fiber. Vitamins C, B3, and E are the most important antioxidants that have the ability to penetrate the skin through their small molecular weight [128]. L-Ascorbic acid (vitamin C) has a skin antiaging effect by inducing the production of Collagen-1 and Collagen-3, as well as enzymes important for the production of collagen and inhibition of the property of MMP-1. Clinical studies indicate that vitamins C and E in combination have a higher antioxidative property than that with vitamin C or E alone [110]. Retinol (Vitamin A) and its derivatives such as retinaldehyde and tretinoin have antioxidant effects and also encourage the biosynthesis of collagen and reduce the expression of MMP-1 (collagenase 1) [41].

5.8.4 Invasive Procedures

There are several persistent procedures, and most of these are intended to "resurface" the epidermis layer of the skin to remove the damaged epidermis and replace the tissue with remodeled skin layers. The chemical peeling method causes a chemical ablation of defined skin layers to induce an even and tight skin as a result of the regeneration and repair mechanisms after the inflammation of the epidermis and dermis. There are several research reports to indicate that improvements in skin elasticity and wrinkles after chemical peeling may be attributed to increase of collagen-1 with collagen-3 elastic fibers [129]. The desired antiaging effect can also be achieved by microinjections into the superficial dermis; these contain only one active ingredient or a combination of different compounds that are perfectly biocompatible and totally absorbable. These include vitamins, nutrients, hormones, growth factor, hyaluronic acid, amino acids, minerals, autologous cultured fibroblasts, homeopathic products, etc. [124].

5.9 CONCLUSION

A better understanding of the molecular and cellular mechanisms underlying aging, as well as providing a growing list of factors that can be targeted for specific interventions should aimed to prevent or delay the aging. Oxidative stress and inflammation are among the principal mechanisms likely to be involved in the aging process. It is clear that there are readily discernible differences between intrinsically aged skin and that aged by habitual exposure to sunlight, particularly at the macromolecular level. Changes seen with intrinsic aging such as decreased cellular lifespan, reduced response to growth factors, and disruption of matrix synthesis and elevation of proteolytic activity are all evident in photodamaged skin. There are so many bioactive molecules, with powerful antioxidant properties, which exert antiinflammatory, anticancer, and antiphotoaging activities in skin cells. Several research reports suggested that phytopharmaceuticals might be useful for the prevention and treatment of a variety of human skin disorders. Natural bioactive molecules can used to protect against UV radiation and can be ideal photochemoprotective agents for photoaging.

Acknowledgments

The authors are thankful to the Department of Science and Technology, Drug and Pharmaceutical Research Program, Government of India, New Delhi [DST-DPRP, File No. VI-D&P/287/08-09/TDT], for financial support for this project to the School of Natural Product Studies, Jadavpur University, Kolkata, India.

References

[1] Benedetto AVD. The environment and skin aging. Clin dermatol 1998;16:129–39.
[2] Seo JY, Chung JH. Thermal aging: a new concept of skin aging. J Dermatol Sci 2006;(Suppl. 2):13–22.
[3] Calleja-Agius J, Brincat M, Borg M. Skin connective tissue and ageing. Best Pract Res Clin Gastroenterol 2013;27:727–40.
[4] Chen-yu G, Chun-fen Y, Qi-lu L, Qi T, Yan-wei X, Wei-na L, et al. Development of a quercetin-loaded nanostructured lipid carrier formulation for topical delivery. Int J Pharm 2012;430:292–8.
[5] Saija A, Tomaino A, Cascio RL, Rapisarda P, Dederen JC. In-vitro antioxidant activity and in vivo photoprotective effect of a red orange extract. Int J Cosmet Sci 1998;20:331–42.
[6] Campanini MZ, Pinho-Ribeiro FA, Ivan ALM, et al. Efficacy of topical formulations containing *Pimenta pseudocaryophyllus* extract against UVB-induced oxidative stress and inflammation in hairless mice. J Photochem Photobiol 2013;127:153–60.
[7] Mukherjee PK, Maity N, Nema NK, Sarkar BK. Bioactive compounds from natural resources against skin aging. Phytomedicine 2011;19:64–73.
[8] WHO. Ultraviolet radiation and human health. 2009.
[9] Lucas RM, McMichael AJ, Armstrong BK, Smith WT. Estimating the global disease burden due to ultraviolet radiation exposure. Int J Epidemiol 2008;37:654–67.

[10] Afaq F, Mukhtar H. Botanical antioxidants in the prevention of photocarcinogenesis and photoaging. Exp Dermatol 2006;15: 678–84.
[11] Pandey S, Meshya N, Viral D. Herbs play an important role in the field of cosmetics. Int J Pharm Tech Res 2010;2:632–9.
[12] Dureja H, Kaushik D, Gupta M, Kumar V, Lather V. Cosmeceuticals: an emerging concept. Indian J Pharmacol 2005;37:155–9.
[13] Sukh Dev. Ayurveda materia medica: a treasure trove of biologically active molecules. In: Proceedings of international conference on new developments in drug discovery from natural products and traditional medicine. S.A.S. Nagar, India: NIPER; 2008.
[14] Kapoor VK, Dureja J, Chadha R. Herbals in the control of ageing. Drug Discovery Today 2009;14:992–8.
[15] Dupont E, Gomez J, Bilodeau D. Beyond UV radiation: a skin under challenge. Int J Cosmet Sci 2013;35:224–32.
[16] Chanvorachote P, Pongrakhananon V, Luanpitpong S, Chanvorachote B, Wannachaiyasit S, Nimmannit U. Type I procollagen promoting and anti-collagenase activities of *Phyllanthus emblica* extract in mouse fibroblasts. J Cosmet Sci 2009;60: 395–404.
[17] Bae JY, Choi JS, Choi YJ, Shin SY, Kang SW, Han SJ, et al. (–) Epigallocatechin gallate hampers collagen destruction and collagenase activation in ultraviolet-B-irradiated human dermal fibroblasts: involvement of mitogen-activated protein kinase. Food Chem Toxicol 2008;46:1298–307.
[18] Adil MD, Kaiser P, Satti NK, Zargar AM, Vishwakarma RA, Tasduq SA. Effect of *Emblica officinalis* (fruit) against UVB-induced photo-aging in human skin fibroblasts. J Ethnopharmacol 2010;132:109–14.
[19] Lall N, Kishore N. Are plants used for skin care in South Africa fully explored? J Ethnopharmacol 2014;153:61–84.
[20] Maity N, Nema NK, Abedy MK, Sarkar BK, Mukherjee PK. Exploring *Tagetes erecta* Linn flower for the elastase, hyaluronidase and MMP-1 inhibitory activity. J Ethnopharmacol 2011; 137:1300–5.
[21] Heenen M, Giacomoni PU, Goldstein P. Erythema, a link between UV-induced DNA damage, cell death and clinical effects? In: Giacomoni PU, editor. Sun protection in man. Amsterdam: Elsevier; 2001. p. 277–85.
[22] Wulf HC, Sandby-Møller J, Kobayasi T, Gniadecki R. Skin aging and natural photoprotection. Micron 2004;35:185–91.
[23] Huang R, Wu J, Fan Y, Adamson E. UV activates growth factor receptors via reactive oxygen intermediates. J Cell Biol 1996; 133:211–20.
[24] Giacomoni PU, Declerq L, Hellemans L, Maes D. Aging of human skin: review of a mechanistic model and first experimental data. IUBMB-Life 2000;49:259–63.
[25] Giacomoni PU. Advancement in skin aging: the future cosmeceuticals. Clin Dermatol 2008;26:364–6.
[26] Smijs TG, Pavel S. Titanium dioxide and zinc oxide nanoparticles in sunscreens: focus on their safety and effectiveness. Nanotechnol Sci Appl 2011;4:95–112.
[27] Couteau C, Faure A, Fortin J, Paparis E, Coiffard LJM. Study of the photostability of 18 sunscreens in creams by measuring the SPF in vitro. J Pharmaceut Biomed Anal 2007;44:270–3.
[28] Yang YWC, Chen YT, Li CC, Yu HC, Chuang YC, Su JH, et al. Preparation of UV-filter encapsulated mesoporous silica with high sunscreen ability. Mater Lett 2011;65:1060–2.
[29] Chainiaux F, Magalhaes J, Eliaers F, Remacle J, Toussaint O. UVB-induced premature senescence of human diploid skin fibroblasts. Int J Biochem Cell B 2002;34:1331–9.
[30] Mishra AK, Mishra A, Chattopadhyay P. Herbal cosmeceuticals for photoprotection from ultraviolet B radiation: a review. Trop J Pharm Res 2011;10:351–60.
[31] Abdulmajed K, Heard CM. Topical delivery of retinyl ascorbate co-drug. Synthesis, penetration into and permeation across human skin. Int J Pharm 2004;280:113–24.
[32] Kundu JK, Choi KS, Fujii H, Sun B, Surh YJ. Oligonol, a lychee fruit-derived low molecular weight polyphenol formulation, inhibits UVB-induced cyclooxygenase-2 expression, and induces NAD (P) H: quinine oxidoreductase-1 expression in hairless mouse skin. J Funct Food 2009;1:98–108.
[33] Saraf S, Kaur CD. Phytoconstituents as photoprotective novel cosmetic formulations. Pharmacog Rev 2010;4:1–11.
[34] Mishra AK, Mishra A, Ghosh AK, Chattopadhyay P. Evaluation of skin irritation of herbal o/w sunscreen cream on rabbit model. J Pharm Cosmetol 2011;1:44–9.
[35] Sayre RM, Agin PP, Levee GJ, Marlowe E. Comparison of in vivo and in vitro testing of sunscreening formulas. Photochem Photobiol 1979;29:559–66.
[36] Thakur R, Batheja P, Kaushik D, Michniak B. Structural and biochemical changes in aging skin and their impact on skin permeability barrier. In: Dayan N, editor. Skin aging handbook-an integrated approach to biochemistry and product development. Norwich, New York: William Andrew Inc; 2008.
[37] Cynthia AW. Aging skin and wound healing. Dermatology Nursing 2006;18:265–6.
[38] Hensley K, Floyd RA. Reactive oxygen species and protein oxidation in aging: a look back, a look ahead. Arch Biochem Biophys 2002;397:377–83.
[39] Jenkins G. Molecular mechanisms of skin ageing, mechanisms of ageing and development. Mech Ageing Dev 2002;123:801–10.
[40] Ramos-e-Silva M, Celem LR, Ramos-e-Silva S, Fucci-da-Costa AP. Anti-aging cosmetics: facts and controversies. Clin Dermatol 2013;31:750–8.
[41] Ganceviciene R, Liakou A, Theodoridis A, Makrantonai E, Zouboulis CC. Skin anti-aging strategies. Dermato-Endocrinol 2012;4:308–19.
[42] Zeng J, Bi B, Chen L, Yang P, Guo Y, Zhou Y, et al. Repeated exposure of mouse dermal fibroblasts at a sub-cytotoxic dose of UVB leads to premature senescence: a robust model of cellular photoaging. J Dermatol Sci 2013;73:49–56.
[43] Lan E, Wu C, Yu H. Solar-simulated radiation and heat treatment induced metalloproteinase-1 expression in cultured dermal fibroblasts via distinct pathways: Implications on reduction of sun-associated aging. J Dermatol Sci. 2013;26:29–40.
[44] Tanaka K, Hasegawa J, Asamitsu K, Okamoto T. Prevention of the ultraviolet B-mediated skin photoaging by a nuclear factor–B inhibitor, parthenolide. J Pharmacol Exp Ther 2005; 315:624–30.
[45] Krutmann J. Skin ageing. In: Humbert Krutmann P, editor. Nutrition for healthy skin strategies for clinical and cosmetic practice; 2011. p. 15–25.
[46] Morita A. Tobacco smoke causes premature skin aging. J Dermatol Sci 2007;48:169–75.
[47] Yin L, Morita A, Tsuji T. Alterations of extracellular matrix induced by tobacco smoke extract. Arch Dermatol Res 2000; 292:188–94.
[48] Dore JF, Chignol MC. Tanning salons and skin cancer. Photochem Photobiol Sci 2012;11:30–7.
[49] Chung JH, Hanft VN, Kang S. Aging and photoaging. J Am Acad Dermatol 2003;49:690–7.
[50] Kochanek KS, Brenneisen P, Wenk J, Herrmann G, Ma W, Kuhr L, et al. Photoaging of the skin from phenotype to mechanisms. Exp Gerontol 2000;35:307–16.
[51] Losso JN, Munene CN, Bansode RR, Bawadi HA. Inhibition of matrix metalloproteinase-1 activity by the soybean Bowman–Birk inhibitor. Biotechnol Lett. 2004;26:901–5.

[52] Nichols JA, Katiyar SK. Skin photoprotection by natural polyphenols: antiinflammatory, antioxidant and DNA repair mechanisms. Arch Dermatol Rese 2010;302:71–83.
[53] Balcombe NR, Sinclair A. Ageing: definitions, mechanisms and the magnitude of the problem. Best Pract Res Clin Gastroenterol 2001;15:835–49.
[54] Bendova H, Akrman J, Krejci A, Kubac L, Jírova D, Kejlova K, et al. In vitro approaches to evaluation of sun protection factor. Toxicol in Vitro 2007;21:1268–75.
[55] Kim JH, Byun JC, Bandi AKR, Hyun CG, Lee NH. Compounds with elastase inhibition and free radical scavenging activities from *Callistemon lanceolatus*. J Med Plants Res 2009;3: 914–20.
[56] Kim YS, Lee YK, Min KR. Inhibitory effects of herbal medicines on hyaluronidase activity. J Korean Pharm 1995;26:265–72.
[57] Labat RJ, Fourtanier A, Boyer LB, Robert L. Age dependent increase of elastase type protease activity in mouse skin: effect of UV-irradiation. J. Photochem. Photobiol B 2000;57:113–8.
[58] Nishimori Y, Kenjoh Y, Matsumoto K, Kawai M. UVB-induced ultrastructural changes of collagen bundles in hairless mouse skin. J Dermatol Sci 1997;15:125.
[59] Ichihashi M, Ueda A, Budiyanto T, Bito M, Oka M, Fukunaga K, et al. UV-induced skin damage. Toxicology 2003;189:21–39.
[60] Adhami VM, Syed DN, Khan N, Afaq F. Phytochemicals for prevention of solar ultraviolet radiation-induced damages. J Photochem Photobiol 2008;84:489–500.
[61] Yin L, Morita A, Tsuji T. Skin premature aging induced by tobacco smoking: the objective evidence of skin replica analysis. J Dermatol Sci 2001;27:26–31.
[62] Xu Y, Fisher GJ. Ultraviolet (UV) light irradiation induced signal transduction in skin photoaging. J Dermatol Sci 2005;(Supp 1): S1–8.
[63] Cobb M, Goldsmith E. How MAP kinases are regulated. J Biol Chem 1995;270:43–6.
[64] Karin M, Hunter T. Transcriptional control by protein phosphorylation: signal transmission form the cell surface nucleus. Curr Biol 1995;5:747–57.
[65] Marshall C. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179–85.
[66] Su B, Karin M. Mitogen-activated protein kinase cascades and regulation of gene expression. Curr Opin Immunol 1996;8: 402–11.
[67] Cho JW, Park K, Kweon GR, Jang BC, Baek WK, Suh MH, et al. Curcumin inhibits the expression of COX-2 in UVB-irradiated human keratinocytes (HaCaT) by inhibiting activation of AP-1: p38 MAP kinase and JNK as potential upstream targets. Exp Mol Med 2005;37:186–92.
[68] Bachelor MA, Cooper SJ, Sikorski ET, Bowden GT. Inhibition of p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase decreases UVB-induced activator protein-1 and cyclooxygenase-2 in a SKH-1 hairless mouse model. Mol Cancer Res. 2005;3:90–9.
[69] Ashida M, Bito T, Budiyanto A, Ichihashi M, Ueda M. Involvement of EGF receptor activation in the induction of cyclooxygenase-2 in HaCaT keratinocytes after UVB. Expl Dermatol 2003;12:445–52.
[70] Koon HW, Zhao D, Zhan Y, Rhee SH, Moyer MP, Pothoulakis C. Substance P stimulates cyclooxygenase-2 and prostaglandin E2 expression through JAK–STAT activation in human colonic epithelial cells. J Immun 2006;176:5050–9.
[71] Ahsan H, Aziz MH, Ahmad N. UVB exposure activates Stat3 signaling via phosphorylation at tyrosine705 in skin of SKH1 hairless mouse: a target for the management of skin cancer? Biochem Biophys Res Commun 2005;333:241–6.
[72] An KP, Athar M, Tang X, Katiyar SK, Russo J, Beech J, et al. Cyclooxygenase-2 expression in murine and human nonmelanoma skin cancers: implications for therapeutic approaches. J Photochem Photobiol 2002;76:73–80.
[73] Cho YH, Kim JH, Sim GS, Lee BC, Pyo HB, Park HD. Inhibitory effects of antioxidant constituents from *Melothria heterophylla* on matrix metalloproteinase-1 expression in UVA-irradiated human dermal fibroblasts. J Cosmet Sci 2006;57:279–89.
[74] Tang Q, Gonzales M, Inoue H, Bowden GT. Roles of Akt and glycogen synthase kinase 3beta in the ultraviolet B induction of cyclooxygenase-2 transcription in human keratinocytes. Cancer Res 2001;61:4329–32.
[75] Bose S, Du Y, Takhistov P, Michniak-Kohn B. Formulation optimization and topical delivery of quercetin from solid lipid based nanosystems. Int J Pharm 2013;441:56–66.
[76] Kaur CD, Saraf S. In vitro sun protection factor determination of herbal oils used in cosmetics. Pharmacogn Res. 2010;2:22–5.
[77] Zenner L, Callait MP, Granier C, Chauve C. In vitro effect of essential oils from *Cinnamomum aromaticum, Citrus limon* and *Allium sativum* on two intestinal flagellates of poultry, *Tetratrichomonas gallinarum* and *Histomonas meleagridis*. Parasite 2003;10:153–7.
[78] Nema NK, Maity N, Sarkar B, Mukherjee PK. *Cucumis sativus* fruit-potential antioxidant, anti-hyaluronidase, and anti-elastase agent. Arch Dermatol Res 2011;303:247–52.
[79] Tsai YH, Lee KF, Huang YB, Huang CT, Wu PC. In vitro permeation and in vivo whitening effect of topical hesperetin microemulsion delivery system. Int J Pharm 2010;388:257–62.
[80] Proteggente AR, Basu-Modak S, Kuhnle G, Gordon MJ, Youdim K, Tyrrell R, et al. Hesperetin glucuronide, a photoprotective agent arising from flavonoid metabolism in human skin fibroblasts. J Photochem Photobiol 2003;78:256–61.
[81] Ullah MO, Sultana S, Haque A, Tasmin S. Antimicrobial, cytotoxic and antioxidant activity of *Centella asiatica*. Eur J Sci Res 2009;30:260–4.
[82] Kim S, Chung JH. Berberine prevents UV-induced MMP-1 and reduction of type I procollagen expression in human dermal fibroblasts. Phytomedicine 2008;15:749–53.
[83] Husch J, Bohnet J, Fricker G, Skarke C, Artaria C, Appendino G, et al. Enhanced absorption of boswellic acids by a lecithin delivery form (Phytosome®) of Boswellia extract. Fitoterapia 2013;84: 89–98.
[84] Mandade R, Sreenivas SA, Sakarkar DM, Choudhury A. Radical scavenging and antioxidant activity of *Hibiscus rosasinensis* extract. Afr J Pharm Pharmacol 2011;5:2027–34.
[85] Saija A, Tomaino A, Trombetta D, Pasquale AD, Uccella N, Barbuzzi T, et al. In vitro and in vivo evaluation of caffeic and ferulic acids as topical photoprotective agents. Int J Pharm 2000;199:39–47.
[86] Conney AH, Kramata P, Lou YR, Lu YP. Effect of caffeine on UVB-induced carcinogenesis, apoptosis and the elimination of UVB-induced patches of p53 mutant epidermal cells in SKH-1 mice. J Photochem Photobiol 2008;84:330–8.
[87] Svobodová A, Psotová J, Walterová D. Natural phenolics in the prevention of UV-induced skin damage. A review. Biomed Papers 2003;147:137–45.
[88] Offord EA, Gautier JC, Avanti O, Scaletta C, Runge F, Amer KK, et al. Photoprotective potential of lycopene, β-carotene, vitamin-E, vitamin-C and carnosic acid in UVA-irradiated human skin fibroblasts. Free Rad Biol Med 2002;32:1293–303.
[89] Lee SY, Kim MR, Choi HS, Moon HI, Chung JH, Lee DG, et al. The effect of curculigoside on the expression of matrix metalloproteinase-1 in cultured human skin fibroblasts. Arch Pharm Res 2009;32:1433–9.
[90] Naik GH, Priyadarsini KI, Mohan H. Radioprotecting ability and phytochemical analysis of an Indian medicinal plant: *Terminalia chebula*. BARC Newsl 2002;1:22–6.

[91] Bae JY, Choi JS, Kang SW, Lee YJ, Park J, Kang YH. Dietary compound ellagic acid alleviates skin wrinkle and inflammation induced by UV-B irradiation. Exp Dermatol 2010;19: 182–90.
[92] Hseu YC, Chou CW, Kumar KJS, Fu KT, Wang HM, Hsu LS, et al. Ellagic acid protects human keratinocyte (HaCaT) cells against UVA-induced oxidative stress and apoptosis through the upregulation of the HO-1 and Nrf-2 antioxidant genes. Food Chem Toxicol. 2012;50:1245–55.
[93] Guo Y, Ji R, Lü X, Wan YF, Jiang X. The protective effects of sodium selenite and aloin against ultraviolet-A radiation. Sichuan Da Xue Xue Bao Yi Xue Ban 2011;42:61–4.
[94] Ebrahimi A, Schluesener H. Natural polyphenols against neurodegenerative disorders: potentials and pitfalls. Ageing Res Rev 2012;11:329–45.
[95] Luximon-Ramma A, Bahorun T, Crozier A, Zbarsky V, Datla K, Dexter D, et al. Characterization of the antioxidant functions of flavonoids and proanthocyanidins in Mauritian black teas. Food Res Int 2005;38:357–67.
[96] Bourne LC, Rice-Evans C. Bioavailability of ferulic acid. Biochem Biophys Res Commun 1998;253:222–7.
[97] Yilmaz Y, Toledo RT. Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic acid. J Agric Food Chem 2004;52:255–60.
[98] Kim J, Hwang JS, Cho YK, Han Y, Jeon YJ, Yang KH. Protective effects of (−)-epigallocatechin-3-gallate on UVA-and UVB-induced skin damage. Skin Pharmacol Appl Skin Physiol 2001;14:11–9.
[99] Bhattacharya S. Phytosomes: emerging strategy in delivery of herbal drugs and nutraceuticals. PharmaTimes 2009;41:9–12.
[100] Marianecci C, Rinaldi F, Mastriota M, Pieretti S, Trapasso E, Paolino D, et al. Anti-inflammatory activity of novel ammonium glycyrrhizinate/niosomes delivery system: human and murine models. J Control Rel 2012;164:17–25.
[101] Koul IB, Kapil A, Barthakur MNN, Arnold NP. Evaluation of the liver protective potential of piperine, an active principle of black and long peppers. Planta Med 1993;59:413–7.
[102] Fonseca YM, Catini CD, Vicentini FTMC, Cardoso JC, Junior RLCA, Fonseca MJV. Efficacy of marigold extract-loaded formulations against UV-induced oxidative stress. Indian J Pharm Sci 2011;100:2182–93.
[103] Bonina F, Puglia C, Ventura D, Aquino R, Tortora S, Sacchi A, et al. In vitro antioxidant and in vivo photoprotective effects of a lyophilized extract of *Capparis spinosa* L. buds. J Cosmet Sci 2002;53:321–35.
[104] Waffo-Teguo P, Fauconneau B, Deffieux G, Huguet F, Vercauteren J, Merillon JM. Isolation, identification and antioxidant activity of three stilbene glucosides newly extracted from *Vitis vinifera* cell cultures. J Nat Prod 1998;61:655–7.
[105] Mukherjee PK, Maity N, Nema NK, Sarkar BK. Natural matrix-metallo-proteinase inhibitors—leads from herbal resources. In: Rahman Atta-ur-, editor. Studies in natural products chemistry, 36. Amsterdam: Elsevier Science; 2013. p. 91–113.
[106] Gu M, Singh RP, Dhanalakshmi S, Agarwal C, Agarwal R. Silibinin inhibits inflammatory and angiogenic attributes in photocarcinogenesis in SKH-1 hairless mice. Cancer Res 2007; 67:3483–91.
[107] Rai S, Basak S, Mukherjee K, Saha BP, Mukherjee PK. Oriental medicine *Mangifera indica*. Ori Pharm Expl Med 2007;7:1–10.
[108] Yanti A, Hwang JK. Effects of macelignan isolated from *Myristica fragrans* Houtt. on UVB-induced matrix metalloproteinase-9 and cyclooxygenase-2 in HaCaT cells. J Dermatolog Sci 2010;57: 114–22.
[109] Shetty BS, Udupa SL, Udupa AL, Somayaji SN. Effect of *Centella asiatica* L (Umbelliferae) on normal and dexamethasone-suppressed wound healing in Wistar Albino rats. Int J Low Extrem Wounds 2006;5:137–43.
[110] Haftek M, Mac-Mary S, Bitoux MA, Creidi P, Seité S, Rougier A. Clinical, biometric and structural evaluation of the long-term effects of a topical treatment with ascorbic acid and madecassoside in photoaged human skin. Exp Dermatol 2008;17:946–52.
[111] Kumar S, Parameshwaraiah S, Shivakumar HG. Evaluation of topical formulations of aqueous extract of *Centella asiatica* on open wounds in rats. Indian J Exp Biol 1998;36:569–72.
[112] Mamgain RK. Acne vulgaris and its treatment by indigenous drugs SK-34 (Purim) and SK-235 (Clarina). The Antiseptic 2000;97:76–8.
[113] Barrantes E, Guinea M. Inhibition of collagenase and metalloproteinases by aloins and aloe gel. Life Sci 2003;72:843–50.
[114] Mitani H, Ryu A, Suzuki T, Yamashita M, Arakane K, Koide C. Topical application of plant extracts containing xanthine derivatives can prevent UV induced wrinkle formation in hairless mice. Photodermatol Photoimmunol Photomed 2007;23: 86–94.
[115] Hsu MF, Chiang BH. Stimulating effects of *Bacillus subtilis* natto-fermented *Radix astragali* on hyaluronic acid production in human skin cells. J Ethnopharmacol 2009;125:474–81.
[116] Takashi F, Masanori W, Takao I, Morio S. Amla (*Emblica officinalis* Gaertn.) extract promotes procollagen production and inhibits matrix metalloproteinase-1 in human skin fibroblasts. J Ethnopharmacol 2008;119:53–7.
[117] Li YH, Wu Y, Wei HC, Xu YY, Jia LL, Chen J, et al. Protective effects of green tea extracts on photoaging and photoimmunosuppression. Skin Res Technol 2009;15:338–45.
[118] Chiu AE, Chan JL, Kern DG, Kohler S, Rehmus WE, Kimball AB. Double-blinded, placebo-controlled trial of green tea extracts in the clinical and histologic appearance of photoaging skin. Dermatol Surg 2005;31:855–60.
[119] Singh RP, Agarwal R. Cosmeceuticals and silibinin. Clinics Dermatol 2009;27:479–84.
[120] Birt DF, Mitchell D, Gold B, Pour P, Pinch HC. Inhibition of ultraviolet light induced skin carcinogenesis in SKH-1 mice by apigenin, a plant flavonoid. Anticancer Res 1997;17:85–91.
[121] Li B, Robinson DH, Birt DF. Evaluation of properties of apigenin and [G-3H] apigenin and analytic method development. J Pharm Sci 1997;86:721–5.
[122] Russell LR, Shirley FM, Charles OY. Lipids in citrus fruit juice lipid content during orange ripening in eastern Sicily. J Agric Food Chem 1987;35:1023–7.
[123] Stefanovits-Bányai E, Tulok M, Hegedûsm A, Renner C, Szöllôsi VI. Antioxidant effect of various rosemary (*Rosmarinus officinalis* L.) clones. Acta Biologica Szegediensis 2003;47:111–3.
[124] Iorizzo M, De Padova MP, Tosti A. Biorejuvenation: theory and practice. Clin Dermatol 2008;26:177–81.
[125] Tabata NO, Zhen YX, Kligman AM, Tagami H. Biophysical assessment of persistent effects of moisturizers after their daily applications: evaluation of corneotherapy. Dermatol 2000;200: 308–13.
[126] Trautinger F. Mechanisms of photodamage of the skin and its functional consequences for skin ageing. Clinic Exp Dermatol 2001;26:573–7.
[127] Heutling D, Lehnert H. Hormone therapy and antiaging: is there an indication? Internist 2008;49:570–80.
[128] Bissett DL, Miyamoto K, Sun P, Li J, Berge CA. Topical niacinamide reduces yellowing, wrinkling, red blotchiness and hyperpigmented spots in aging facial skin. Int J Cosmet Sci 2004;26: 231–8.
[129] Han SH, Kim HJ, Kim SY, Kim YC, Choi GS, Shin JH. Effects of chemical peeling in photoaged hairless mice. Int J Dermatol 2011;50:1075–82.
[130] Cimino F, Cristani M, Saija A, Bonina FP, Virgili F. Protective effects of a red orange extract on UVB-induced damage in human keratinocytes. Biofactors 2007;30:129–38.

[131] Xia Q, Ma Q, Shi JA, Duan TTX, Dong KW, Tsim K. Chemical analysis of *Radix astragali* (Huangqi) in China: a comparison with its adulterants and seasonal variations. J Agric Food Chem 2002;50:4861−6.

LIST OF ABBREVIATIONS

8-OHdG 8-hydroxy 2′-deoxyguanosine
AP-1 Activator protein-1
COX Cyclooxygenase
CPD Cyclobutane pyrimidine dimer
EGFR Epidermal growth factor receptor
ERK Extracellular signal-regulated kinase
GSH Total glutathione;
HaCaT Human keratinocyte
HO-1 Heme oxygenase 1
IL Interleukin
JNK c-Jun N-terminal Kinase
LPO Lipid peroxidation
MAPK Mitogen-activated protein kinase;
MDA Malondialdehyde
MMP Matrix metalloproteinase
NF-κB Nuclear factor kappa B
Nrf2 NF-E2-related factor-2
ODC Ornithine decarboxylase
PARP Poly (ADP-ribose) polymerase
PCNA Proliferating cell nuclear antigen
PI-3K Phosphatidylinositol-3kinase
PUVA Psoralen plus ultraviolet A radiation
ROS Reactive oxygen species
TPK Tyrosine protein kinase

This page intentionally left blank

CHAPTER

6

Proangiogenic Potential of Medicinal Plants in Wound Healing

Deepak Kasote, Aijaz Ahmad, Alvaro Viljoen

Department of Pharmaceutical Sciences, Tshwane University of Technology, Pretoria, South Africa

OUTLINE

6.1 INTRODUCTION

A wound is defined as a disruption of cellular, anatomical, and functional continuity of living tissue, and wounds are broadly classified as open or closed. When the skin is torn, cut, or punctured, it is known as an open wound, and when blunt force or trauma causes a contusion, it is termed a closed wound. Wounds are generally caused by physical, chemical, thermal, microbial, or immunological assault to the tissue. Burns are the most common type of wounds caused by fire, heat, radiation, chemicals, electricity, and sunlight [1]. Although wounds affect all sectors of the society, elderly patients are the most severely affected, which tremendously impacts their quality of life. Unhealed wounds continually produce inflammatory mediators that cause discomfort to these patients due to pain and swelling at the wound site. To exacerbate the situation, there is a rapid global increase in impaired wound healing associated with injuries such as burns, frostbite, wounds resulting from military and civil combat, diabetes, and cardiovascular disease [2].

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00006-4

Normally, the body initiates a cascade of physiological and anatomical actions to heal a wound, collectively termed the wound healing process. According to the Wound Healing Society (USA), wound healing is defined as a complex dynamic process that results in the restoration of anatomical continuity and function [3]. Depending on the intensity of wound healing, wounds are classified as acute or chronic wounds. Acute wounds heal over a prolonged period of time (e.g., burns, traumatic injuries, and surgical wounds), whereas the chronic wound healing process is grossly impaired, e.g., ulcers [4,5]. Compared to acute wounds, chronic wounds require an extended healing period, and often healing does not occur or the wound reoccurs. It is estimated that at any given time, 6 million people suffer from chronic wounds causing extensive physiological and mental trauma [6]. Irrespective of the type of wound, healing comprises four different phases detailed in the next section. Apart from superficial wounds, all wounds require angiogenesis for healing, which is a process in which new blood vessels sprout from preexisting blood vessels. Angiogenesis is an integral process in fetal development, the female reproductive cycle, chronic inflammation, as well as tissue and wound repair [7–9]. It is important to define the role of angiogenesis in wound healing, as reduced angiogenesis is always associated with impaired wound healing, which has an undesirable clinical impact on the patient. The process of angiogenesis is highly regulated by various proangiogenic and antiangiogenic factors. Several factors are known to impact the wound healing process by hampering the progression of angiogenesis, which leads to improper or impaired wound healing characterized by tissue damage.

6.2 PHASES OF WOUND HEALING

Wound healing is a convoluted process of repairing the skin and other organ tissues after injury. It is an intricate process involving four complex and highly controlled phases such as hemostasis, inflammation, proliferation/granulation, and maturation/tissue remodeling (Figure 6.1). For efficient wound healing, all four phases and their biophysiological functions must progress in the proper sequence at a specific time with optimal intensity [6,10]. These phases are characterized by multifarious and synchronized biological events such as chemotaxis, phagocytosis, neocollagenesis, collagen degradation and remodeling, angiogenesis, epithelialization, as well as production of new glycosaminoglycans and proteoglycans that assist in proper

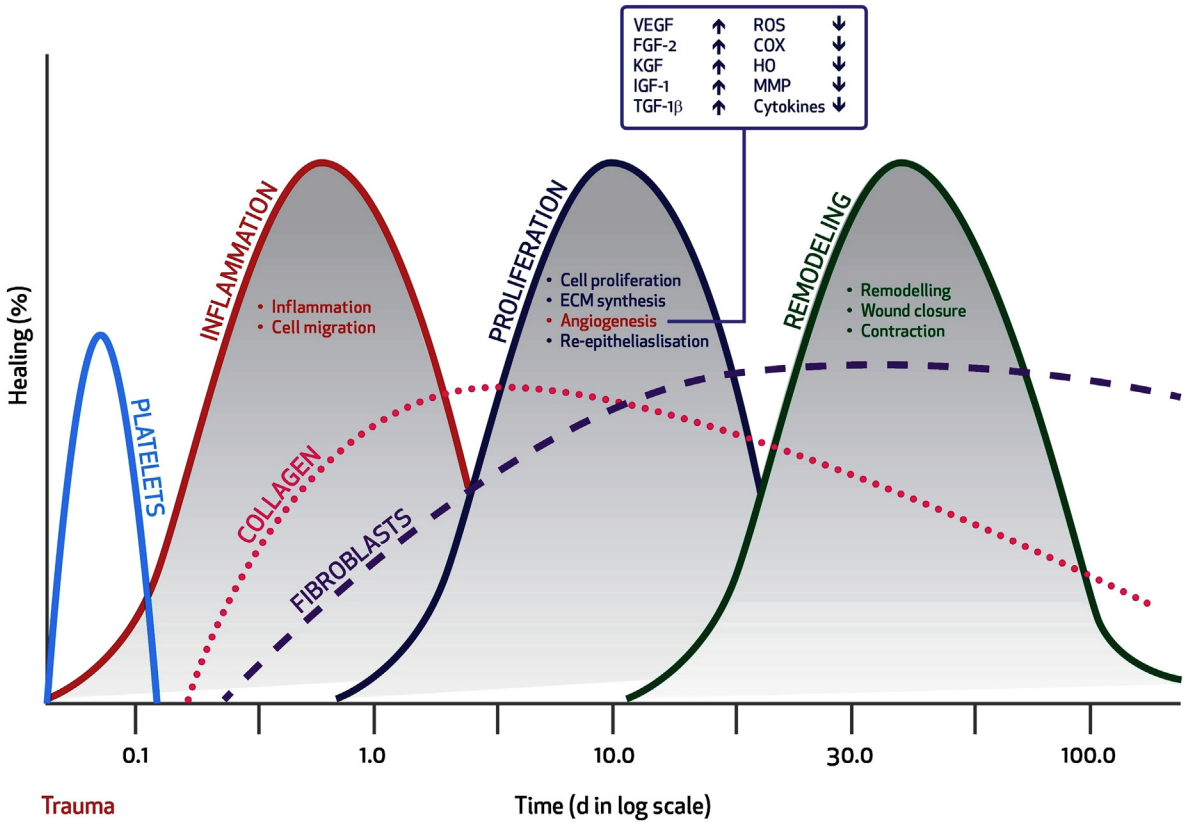


FIGURE 6.1 Different stages of wound healing.

wound healing [10,11]. However, there are many external and internal factors, such as imbalanced diet, alcoholism, smoking, age, sex, stress, obesity, and hormonal status, oxygenation, medications, as well as infections and disease conditions, such as ischemia, abnormal blood pressure, diabetes, etc. which can interfere with this process, leading to abnormal wound healing.

6.2.1 Hemostasis Phase

The normal wound healing process starts immediately after tissue injury, which begins with bleeding and outflow of lymphatic fluid, resulting in a flow of coagulation factors to form a fibrin clot. This fibrin clot immediately stops bleeding (hemostasis) and facilitates in releasing chemokines, cytokines, and growth factors such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor, which attracts the inflammatory cells and promotes the inflammatory phase [10,12,13].

6.2.2 Inflammatory Phase

After 8 h of injury, the inflammatory phase commences with the permeation of polymorphonuclear leukocytes (PMNs), neutrophils, macrophages, and lymphocytes at wound sites. PMNs, facilitated by TGF-β, result in the swelling of the wound, whereas neutrophils phagocytize the damaged tissue, cell debris, and microbes and remove them from wound site. Several wound healing factors are secreted by macrophages. In this phase, signaling molecules are also released to initiate the third proliferative phase of wound healing.

6.2.3 Proliferative Phase

Also known as the granulation phase, this phase is characterized by several subphases: fibroplasia, matrix deposition, angiogenesis, epithelialization, and wound contraction [14]. At the end of the inflammatory phase, fibroblasts start migrating and proliferating into the wound, and in 2–3 weeks, they are the predominant cells that lay down the collagen matrix in the wound site [15]. The next subphase is matrix deposition, which leads the deposition of collagen matrix in the wound to increase the strength of the wound and to provide a platform to the cells that play a role in inflammation, angiogenesis, and connective tissue construction. Angiogenesis is an important phase in wound healing, leading to the formation of a network of blood vessels around the wound. Epithelialization subphase leads to the development of a barrier between the wound and the environment with the migration of epithelial cells from the wound edges. The wound contraction subphase minimizes the size of the wound by contraction myofibroblast actins.

6.2.4 Maturation Phase

The maturation phase is also called the tissue remodeling phase because of the constant alterations of the wound during this phase. The crucial step in this phase is that the collagen production and degradation remains in equilibrium, thus maintaining the amount of collagen in the wound. During this phase collagen fibers are organized and aligned along with the cleavage lines of the body to provide tensile strength to the healing wound. After achieving strength, the redness of the wound disappears as the blood vessels disappear.

6.3 ANGIOGENESIS AND ITS ROLE IN WOUND HEALING

Angiogenesis involves the formation of new blood vessels from the preexisting blood vessels that play an important role in various physiological processes, including wound healing and tissue repair. Angiogenesis is an important subphase of the proliferative phase of wound healing. Apart from the most superficial wounds, all wounds require angiogenesis for healing, as new blood vessels are required to provide nutrients and oxygen to promote cell growth and granulation of tissues.

The angiogenesis process begins after 4 days of injury due to the low oxygen tension and lack of nutrients, resulting in release of angiogenic molecules that attract inflammatory mediators and endothelial cells and promote their proliferation. The injured tissue secretes angiogenic factors that bind to the receptors of the endothelial cells of preexisting blood vessels. Through this binding process, several enzymes and proteins are released and dissolve membranes covering the blood vessels. Angiogenesis always proceeds concomitantly with the formation of new tissue and starts with the degradation of the membrane underlying the endothelial cells leading to the formation of blood vessels sprouting from the older capillaries. These vessels further extend into the wound and invade the fibrin-rich clots, which leads to the development of a microvascular network throughout the granulation tissue. The ends of these vessels join through the process of anastomosis and lead to the formation of a closed circuit. Newly formed blood vessels are then structurally supported by smooth muscle cells and finally continuous blood flow begins. This

whole process of angiogenesis is governed by various angiogenic and antiangiogenic factors. Angiogenic factors promote angiogenesis, whereas antiangiogenic factors hinder the process [16,17]. Angiogenesis is controlled by a fine balance between proangiogenic -compounds and antiangiogenic factors (cytokines). If this balance favors angiogenesis, it can lead to diseases such as macular degeneration, rheumatoid arthritis, cancer growth, and metastases. However, if the balance is inclined toward a reduction in angiogenesis, then it can result in cardiovascular disease, peptic ulcers, and impaired wound healing [18]. The inadequate production of angiogenesis growth factors and/or excessive amounts of angiogenesis inhibitors leads to deficient angiogenesis. The release and regulation of these angiogenic factors depends on the type, location, and intensity of the wound, as well as on the sex, age, hormonal status, and condition of the patient. Currently, approximately 20 proangiogenic and more than 30 antiangiogenic factors along with several receptors and signaling partners that play an important role in angiogenesis have been identified and characterized [3]. These angiogenic cascade factors are categorized as follows:

1. *Soluble growth factors* include FGFs such as FGF-1 and FGF-2. These growth factors were previously known as acidic FGF and basic FGF, respectively [19]. They were among the first discovered FGFs and are known to possess angiogenic activity. Both these factors are known to be secreted by injured cells at wound sites, resulting in the initial stimulus for angiogenesis. Another important soluble growth factor is vascular endothelial growth factor (VEGF), which can induce the division and differentiation of cultured endothelial cells, which thus indicates a direct action on these cells [20,21]. Different cell types, such as fibroblasts, endothelial cells, macrophages, and keratinocytes produce VEGF; however, the latter two are responsible for the production of VEGF during wound healing [22]. Lack of oxygen during tissue injuries induces the production of VEGF, which plays an important role in angiogenesis during the proliferative phase [23]. Inhibiting this growth factor is an approved antiangiogenic therapy for many clinical conditions, especially in ophthalmic conditions such as wet macular degeneration.
2. *Inhibiting factors* include TGF-α and angiogenin. These factors inhibit the proliferation and enhance the differentiation of endocrine cells. Angiogenic inhibitors play an important role in maintaining the angiogenic balance, which influences the rate of new blood vessel formation.
3. *Extracellular matrix-bound cytokines*, such as angiostatin, thrombospondin, and endostatin, contribute to the regulation of angiogenesis.

6.4 PLANTS WITH PROANGIOGENIC POTENTIAL

Although wound healing has become a highly sophisticated field in the modern medical sphere, plants have an illustrious history as natural mediators facilitating efficient wound healing. The efficacy of several plants in wound healing is extensively recorded in some of the most ancient healing modalities such as Chinese and African traditional medicine and Ayurveda [24,25]. In addition, tribal folklore from many countries such as Nigeria, Peru, and Mali, among others, describes the use of several plants to treat wounds and burns [26–28]. Approximately 450 plant species have been identified with promising wound healing properties [29]. Plants promote wound healing by multiple mechanisms, usually by promoting angiogenesis. Numerous studies indicate that plants such as *Aloe vera*, ginseng, and *Astragalus membranaceus*, among others, have noteworthy proangiogenic potential. During the wound healing process, most of these plants promote angiogenesis predominately via the upregulation of VEGF expression and/or and activation of the mitogen-activated protein kinases pathway [18]. The chemical constituents responsible for proangiogenic activity are often polyphenols, sterols, and saponins [3]. Thus far, nearly 41 plants and their isolated compounds are reported as having angiogenesis-stimulating properties during the wound healing process (summarized in Table 6.1 and Figure 6.2). In proangiogenic assessment studies, botanical extracts have been generally tested for their influence on the formation of blood vessels in vitro and in vivo, with special attention to the impact on VEGF expression [3]. Many studies have preceded further and isolated active fractions and proangiogenic compounds in an evidence-based ethnopharmacological approach.

6.4.1 *Achyranthes aspera* (Amaranthaceae)

Devil's horsewhip, also called the prickly chaff flower, is an annual herb occurring throughout the tropical region. It has been used as an emmenagogue, purgative, diuretic, antimalarial, antihyperlipidemic, estrogenic, antileprotic, antispasmodic, cardiotonic, antibacterial, and antiviral agent in traditional healing systems [77]. The wound healing efficacy has been reported to be from the methanol leaf extract, which enhances the formation of granulation tissue of burn wounds. It has also been reported that a 5% ointment of *Achyranthes aspera* displayed significant wound healing activity by upregulation of expression of matrix metalloproteinases (MMPs), MMP-2 and MMP-9 [30].

TABLE 6.1 Proangiogenic Activity of Plants

Species	Active extract/fraction/active compounds	Mechanism of action	References
Achyranthes aspera	Methanol leaf extract	Upregulation of MMP-2 and MMP-9 expression	[30]
Aloe barbadensis	β-Sitosterol	Increased the production of angiogenic factors and/or the expression of their respective receptors	[31]
	Acemannan	Induction of fibroblast proliferation and stimulation of KGF-1, VEGF; and type I collagen expressions	[32]
Alternanthera brasiliana	Methanol extract of leaves	–	[33]
Anadenanthera colubrina	Alcohol extract	–	[34]
Andrographis paniculata	Aqueous extract of leaves	–	[35]
Angelica sinensis	Polysaccharide extract	Enhancement of VEGF expression and stimulation JNK 1/2 and p38 phosphorylation	[36]
Astilbe thunbergii	Eucryphin	Increased VEGF, TGF-βl, and HIF-1α expression	[37]
Astragalus membranaceus	Ethyl acetate extract of root	Activation of VEGF-KDR/Flk and PI3K-Akt-eNOS pathways	[38]
	Polysaccharide	Upregulation of the expression of angiopoietin 1 (Ang1)	[39]
	Astragaloside IV	Enhancement of VEGF and KDR/Flk-1/VEGFR2 expression; activation of Akt	[40]
	Formononetin	Increased VEGF and VEGFR-2/Flk-1 expression	[41]
Blechnum orientale	Water extract	–	[42]
Calendula officinalis	Ethanol extract	Induction of neovascularization	[43]
Camellia sinensis	Methanol leaf extract	–	[44]
Centella asiatica	Asiaticoside	Increased MCP-1 expression stimulating VEGF expression	[45]
Cinnamomum cassia	Ethanol extract and cinnamic acid	Upregulation of VEGF and Flk-1/KDR receptor expression	[46]
Clematis burgensis	Methanol leaf extract	–	[47]
Clematis longicauda	Methanol leaf extract	–	[47]
Euphorbia caducifolia	Latex	–	[48]
Ficus deltoidea	Aqueous extract whole plant	–	[49]
Gynura procumbens	Ethanol extract of leaves	–	[50]
Hippophae rhamnoides	Aqueous leaf extract	Upregulation of VEGF expression	[51]
Justicia flava	Methanol leaf extract	–	[52]
Lannea welwitschii	Methanol leaf extract	–	[52]
Lantana camara	Ethanol extract of leaves	–	[53]
Martynia annua	Ethanol extract of leaves	–	[54]
	Flavonoid-rich fraction and luteolin	–	[55]

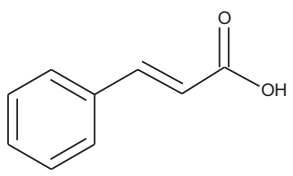
Continued

TABLE 6.1 Proangiogenic Activity of Plants—cont'd

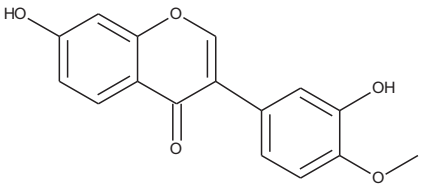
Species	Active extract/fraction/active compounds	Mechanism of action	References
Nicotiana tabacum	Nicotine	Stimulation of nicotinic acetylcholine receptors	[56]
Opuntia dillenii	Aqueous and ethanolic extract	Upregulation of VEGF	[57]
Otostegia persica	Methanol extract	–	[58]
Panax ginseng	Korean red ginseng water extract	Activation of the PI3K/Akt-dependent ERK1/2 and eNOS signaling pathways	[59]
	Ginsenoside Rb_1	Stimulation of VEGF and f IL-1β production	[60]
Panax notoginseng	Saponin extract	Enhancement of VEGF and KDR/Flk-1 expression; activation of PI3K-Akt-eNOS signaling pathway	[61]
Patrinia villosa	Ethyl acetate fraction	Activation of FAK signaling pathway	[62]
Phyllanthus niruri	Leaves extract	–	[63]
Picrorhiza kurroa	Picroliv from root	Upregulation of VEGF expression and receptors Flt-1 and KDR	[64]
Pistacia atlantica	Resin extract	Increasing the concentration of growth factor PDGF and FGF	[65]
Plagiochila beddomei	Methanol extract of thallus	Enhancement of VEGFA expression	[66]
Puerariae flos	Ethanol extract	Activation of MEK/ERK-, phosphatidylinositol 3-kinase/Akt/eNOS-, and Src/FAKdependent pathways	[67]
Rehmannia glutinosa	Aqueous extract of root	–	[68]
	Norviburtinal	–	[69]
Rosmarinus officinalis	Essential oil of the aerial parts	–	[70]
Salvia miltiorrhiza	Crude root extract and salvianolic acid B	Upregulation of VEGF and VEGF receptors genes expression	[71]
Stewartia koreana	Methanol extract of leaves	Stimulation of ERK phosphorylation and Akt kinases	[72]
Strobilanthes crispus	Ethanol extract of leaves	–	[73]
Tephrosia purpurea	Flavonoid-rich fraction and pongamol	–	[74]
Terminalia chebula	Fruit tannin extract	Modulated VEGF gene expression	[75]
Uncaria rhynchophylla	Ethanol extract of root	Increased VEGF, and bFGF gene expression and protein secretion	[76]

6.4.2 *Aloe barbadensis* (Xanthorrhoeaceae)

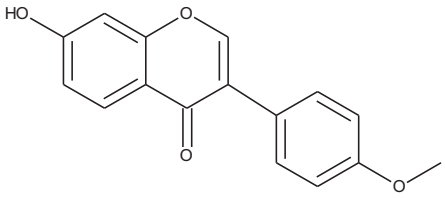
Aloe barbadensis (syn *A. vera*) is a perennial succulent plant used to treat several dermatological conditions. The healing properties of *A. vera* have been ascribed to the copious amounts of leaf gel, which is rich in polysaccharides [78]. *Aloe vera* is effective in wound healing both by oral and topical routes [79]. It has been extensively reported that *A. vera* gel promotes wound healing through various mechanisms such as keeping the wound moist, increasing epithelial cell migration, enhancing rapid maturation of collagen, and reducing inflammation [78,80]. The angiogenic stimulating potential of *A. vera* is well established. *Aloe vera* gel extracts and isolated molecules (e.g., β-sitosterol) have been reported to stimulate angiogenesis in chorioallantoic membrane assays of chick embryos. The induction of angiogenesis was observed in 64% of the eggs at 10 μg concentration of β-sitosterol [31]. It has been found that in the presence of heparin, β-sitosterol stimulated neovascularization in the mouse Matrigel plug



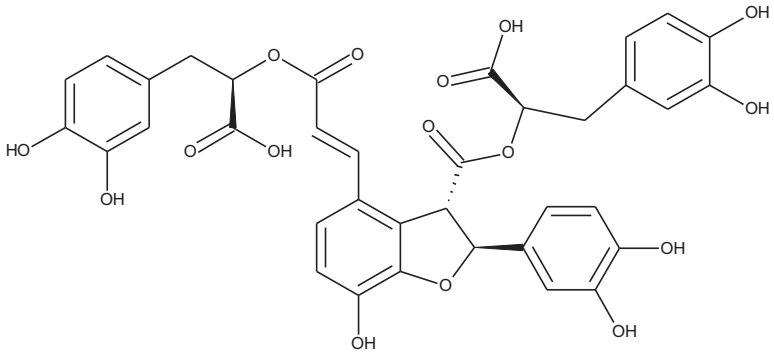
Cinnamic acid



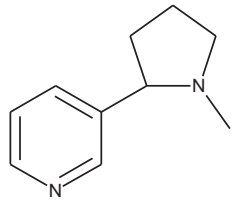
Calycosin



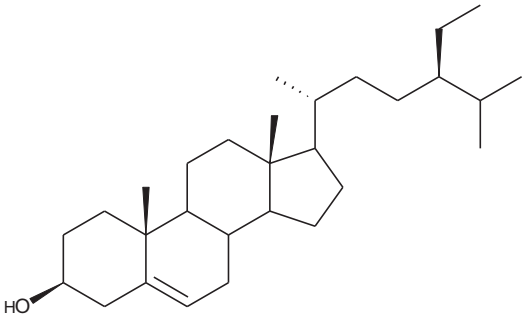
Formononetin



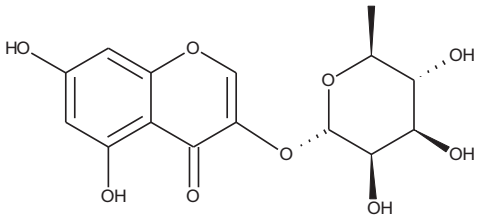
Salvianolic acid B



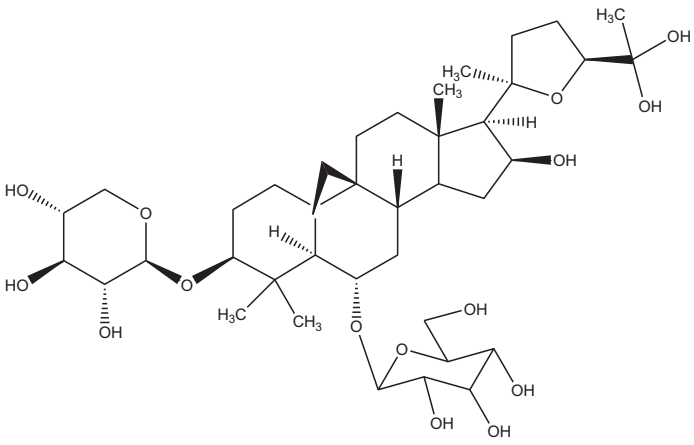
Nicotine



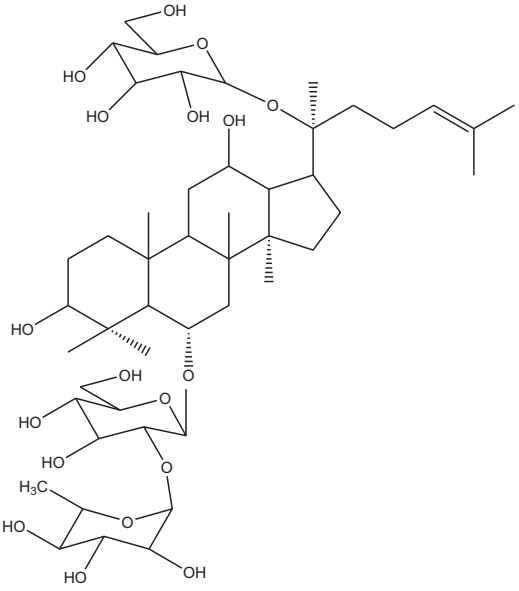
β-Sitosterol



Eucryphin

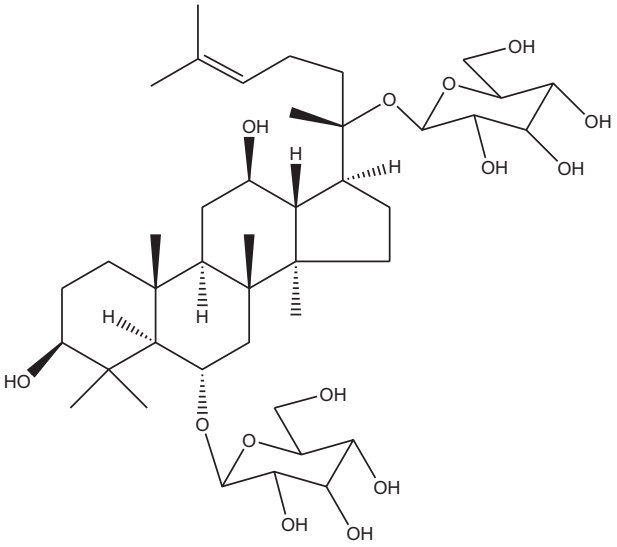


Astragaloside IV

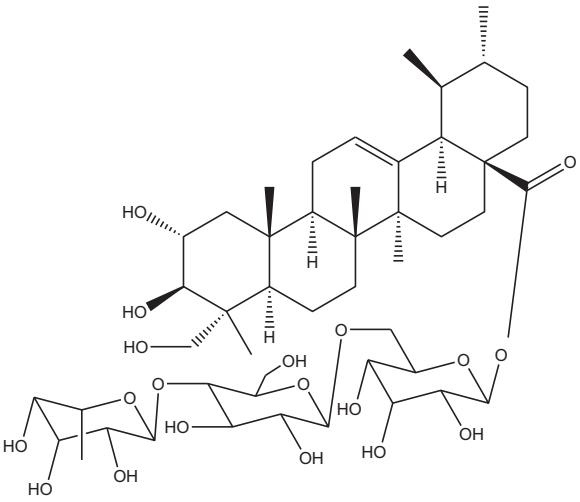


Ginsenoside Re

FIGURE 6.2 Proangiogenic compounds isolated from plants.



Ginsenoside Rg1



Asiaticoside

FIGURE 6.2 cont'd.

assay and the motility of human umbilical vein endothelial cells (HUVECs) in an in vitro wound migration assay. The most active fraction from a dichloromethane extract of *A. vera* gel promoted angiogenesis by enhancing mRNA expression of a urokinase-type plasminogen activator, MMP-2, and membrane-type MMP in calf pulmonary artery endothelial cells [81]. Delayed wound healing is one of the complications of diabetes mellitus, which is caused by downregulation of VEGF expression. The oral administration of *A. vera* (30 mg/head) to diabetic rats has been found to be useful in the upregulation of VEGF gene expression on day 2 postwounding [82]. In addition, it has also been reported that a polyherbal formulation containing the aqueous lyophilized leaf extracts of *Hippophae rhamnoides* L. and *A. vera* and the ethanol rhizome extract of *Curcuma longa* L., in an optimized ratio (1:7:1), accelerates normal and impaired diabetic wound healing by promoting angiogenesis in vivo through upregulation of VEGF expression [83].

6.4.3 *Angelica sinensis* (Apiaceae)

Dang Gui/female ginseng is a herb indigenous to China, where it is widely used in Chinese traditional medicine for the treatment of various ailments, including wound healing and especially for gynecologic disorders [84]. Its pharmacological properties are mainly associated with phthalides, polysaccharides, organic acids, and their esters [84]. Despite some controversial reports, *Angelica sinensis* has proven to be efficacious in wound healing by simulating angiogenesis. The proangiogenic effects of an *An. sinensis* extract has been studied on HUVEC in vitro and also by using the zebrafish model [36]. An aqueous extract of *A. sinensis* root promotes angiogenesis through enhancing VEGF expression and stimulating c-Jun N-terminal kinases (JNK1 and JNK-2) and p38 phosphorylation. The wound healing potential has also been reported for the polysaccharide-rich extract, ferulic acid, and aqueous isolated compound (SBD.4) of *A. sinensis* [84–86].

6.4.4 *Astilbe thunbergii* (Saxifragaceae)

Astilbe is a genus composed of 18 rhizomatous flowering species native to Asia and North America. In China, the dried rhizomes of *Astilbe thunbergii* (Sieb. et Zucc) Miq have been used for the treatment of sword cuts, bite wounds caused by animals, frostbite, burns, suppurative dermatitis, and cutaneous inflammatory diseases [87]. Recent pharmacological studies have reported that the topical application of the ethyl-acetate-soluble fraction of 70% rhizome ethanol extract (0.5% and 1.0% ointment) promotes the healing of burn wounds. Three burnwound-healing-promoting effectors, eucryphin (ED_{50}: 4 μg/wound), bergenin (ED_{50}: 190 μg/wound), and astilbin (ED_{50}: 64 μg/wound), were isolated from this active ethyl-acetate-soluble fraction. Among these three compounds, eucryphin was found to promote burn wound healing most significantly [87]. Eucryphin (100 μg/wound) improves burn wound healing by promoting angiogenesis as a result of stimulating VEGF and TGF-β1 production caused by the increase in hypoxia-inducible factor-1 alpha expression in keratinocytes [37].

6.4.5 *Astragalus membranaceus* (Fabaceae)

"Huáng qí" or "yellow leader" ranks among the 50 most fundamental herbs used in traditional Chinese medicine [3]. The dried root of *Astragalus membranaceus* (Fisch. ex Link) Bunge is commonly referred to as *Astragali Radix* and is well known for its tonic property and has been widely used to enhance the repair and recovery of tissues and organs such as the lung, heart, and neurons [88]. The main constituents of *Astragali Radix* include polysaccharides, saponins (astragaloside I, II, and IV), flavonoids (calycosin), amino acids, and several trace elements [89]. The topical application of a boiled water extract of *Astragali Radix* directly to wounds once daily for 11 consecutive days was found to be beneficial in the early stages of wound healing using a rat model [90]. In humans, *Astragali Radix* accelerates diabetic foot wound healing through the actions of tissue regeneration, angiogenesis, and antiinflammation [91]. *Astragali Radix* promoted angiogenesis in HUVEC in vitro. It stimulates HUVEC to proliferate and enhances their motility in the wound healing migration assay via enhancing VEGF mRNA expression and activation of the phosphatidylinositol 3-kinase-Akt-endothelial nitric oxide synthase (PI3K-Akt-eNOS) pathway [38]. In addition, Astragaloside IV, the main component of *Astragali Radix*, has been reported to exert potential proangiogenic effects in vitro and in vivo, and its proangiogenic activity most likely involves both VEGF- and Akt-dependent signaling pathways. The isoflavone calycosin is the most potent proangiogenic agent among all chemical constituents reported in *Astragali Radix* that induce angiogenesis in HUVEC and zebrafish embryos via the upregulation of VEGF, VEGFR1, and VEGFR2 mRNA expression [40]. Formononetin is another important component isolated from *Astragali Radix*. Oral administration to rats at a dose of 20 μg/kg or 200 μg/kg displayed a significant increase in the number of vessels and the expression of VEGF and VEGF receptor 2 (VEGFR-2/flk-1) in the early stage of chondrogenesis. However, the higher doses of formononetin had no significant effect on the expression of VEGF and VEGFR-2/Flk-1 [41].

6.4.6 *Calendula officinalis* (Asteraceae)

Calendula officinalis L. is an annual herb of Mediterranean origin, commonly known as pot marigold, ruddles, common marigold, garden marigold, English marigold, or Scottish marigold. The flowers of *C. officinalis* have been used extensively as an antiinflammatory agent, for the treatment of wounds, first-degree burns, contusions, and skin rashes. Several pharmacological studies have reported and confirmed the wound healing potential claim of *C. officinalis* [92–94]. It is further believed that the wound healing potential of *C. officinalis* is associated with angiogenesis-promoting activity. Several experimental models suggest that the angiogenesis-promoting activity of *C. officinalis* is not directly related to the expression of VEGF and could possibly be associated with other proangiogenic factors [43]. *Calendula officinalis* has demonstrated positive effects on angiogenesis through induction of neovascularization and other proangiogenic factors such as FGF, TGF-β, and angiogenic cytokines such as interleukin-8 and tumor necrosis factor-α [43].

6.4.7 *Centella asiatica* (Mackinlayaceae)

Centella asiatica (pennywort) is a herbaceous, annual plant, boasting a long history of use in South East Asia and India for treating skin and vascular disease. It is a well-documented medicinal plant in Ayurvedic medicine, traditional African medicine, and traditional Chinese medicine [95]. *Centella asiatica* is reported to have a wide range of preventive and therapeutic effects. The modulation of collagen production and deposition in wound healing is of primary importance [96]. Active components isolated from the leaves include triterpenoid compounds such as two glycosides (asiaticoside and madecassoside) and their corresponding aglycones (asiatic acid and madecassic acid) [97]. Wound healing properties have been reported for various extracts of *C. asiatica* using the incision and partial-thickness burn wound models of rats. Asiatic acid (from the ethyl acetate extract) was found to be the most active component involved in wound healing [98]. Asiaticoside, is the other pharmacologically active component of *C. asiatica*, which displays angiogenesis-stimulating potential though induction of the bFGF expression, as bFGF is a strong angiogenic factor and a mitogen for endothelial cells [99]. It has also been observed that asiaticoside can promote angiogenesis via stimulation of VEGF production caused by the increase in monocyte chemoattractant protein-1 expression in keratinocytes and an increase in interleukin-1β expression in macrophages [45]. Madecassoside, a major triterpene in *C. asiatica*, administered orally at a concentration of 24 mg/kg in rats and at 1 mg/kg dose in guinea pigs facilitates complete wound healing by promoting skin angiogenesis [100,101].

6.4.8 *Cinnamomum cassia* (Lauraceae)

"Chinese cassia" or "Chinese cinnamon" is a tall evergreen tree originating from southern China, but widely cultivated in most of Asia. The aromatic bark and buds of *Cinnamomum cassia* are primarily used as a spice. Several pharmacological properties have also

ascribed to the bark, which mainly include antioxidant, antimicrobial, antiinflammation, antidiabetic, wound healing, and antitumor activity [46,102]. Wound healing potential has been observed in ethanol extracts of *Ci. cassia*, and its active compound, cinnamic acid, has been found to be associated with angiogenesis-stimulating potential. Researchers have also established that both the ethanol extract and cinnamic acid of *Ci. cassia* increase the production of VEGF by upregulating the VEGF and receptor Flk-1/KDR gene expression in endothelial cells [46].

6.4.9 *Hippophae rhamnoides* (Elaeagnaceae)

Sea buckthorn is native to Europe and Asia and has been used for many years in various parts of the world for medicinal and nutritional purposes. All plant parts are used for the treatment of flu, cardiovascular diseases, mucosal injuries, and skin disorders [103,104]. Ripe fruit is the richest source of vitamins (A, C, E, and K), carotenoids, flavonoids, and organic acids, whereas the leaves predominantly contain flavonoids, tannins, and triterpenes [104]. Oils extracted from the fruit and seed have generally been used for treating scalds, burns of different etiology, skin radiation lesions, and gastric and duodenal ulcers [105]. Several pharmacological studies have revealed that the leaf extract and seed oil has potent application in wound healing, especially in the treatment of burn and ulcerative wounds [51,106]. It has been reported that the tropical application of aqueous leaf extracts of sea buckthorn (5.0%, w/w) on experimental burn wounds in rats enhances the wound healing process by influencing the different phases of wound repair, especially in promoting angiogenesis via upregulating VEGF expression [51].

6.4.10 *Nicotiana tabacum* (Solanaceae)

The genus *Nicotiana* includes several herbaceous plants and shrubs native to the Americas, Australia, South West Africa, and the South Pacific. Different *Nicotiana* species are commonly known as tobacco plants. *Nicotiana tabacum* is cultivated globally for the production of tobacco leaf, which is generally used in the preparation of cigarettes and other tobacco-containing products. The potent stimulant drug, nicotine, is a parasympathomimetic alkaloid found in the leaves (0.6–3.0% of the dry weight). However, high levels of this stimulant can be fatal. Despite the known adverse effects of smoking on wound healing of the skin, it has been shown that nicotine, at a low concentration, promotes wound healing by accelerating angiogenesis [107]. Recently, an endogenous cholinergic pathway for angiogenesis mediated by endothelial nicotinic acetylcholine receptors was discovered, and it has been demonstrated that nicotine stimulates nicotinic acetylcholine receptors (nAChRs) on the endothelium to induce the angiogenic processes [56]. It has been observed that nicotine exerts a proangiogenesis effect by stimulating alpha7-nonneronal nAChR (alpha7 N-nAChR) in endothelial cells [108].

6.4.11 *Opuntia dillenii* (Cactaceae)

Opuntia dillenii is a succulent shrub growing in semi-desert regions in the tropics and subtropics and is commonly known as pear bush, prickly pear, mal rachette, or tuna [109]. Wound healing effects have been demonstrated for the aqueous and ethanolic extract of wild cacti. At a 25% (w/w) concentration, it promotes wound healing processes through improving angiogenesis by upregulation of VEGF. The combination of aqueous and ethanolic extracts displayed pronounced healing efficacy [57].

6.4.12 *Panax ginseng* (Araliaceae)

Ginseng is a slow-growing perennial plant with fleshy roots, which has been used to treat several diseases, including liver and kidney dysfunction, hypertension, noninsulin-dependent diabetes mellitus, and postmenopausal disorders in China, Korea, and Japan. Ginseng extract is topically applied to cure atopic suppurative dermatitis, wounds, and skin inflammation [110]. Ginseng root contains 2–3% ginsenosides (saponins) with over 40 different structural analogs identified, of which Rb1 and Rg1 are the most abundant [111]. Both *P. ginseng* and *Panax quinquefolium* (American ginseng) have exhibited potent wound healing activity, which has been ascribed to angiogenesis stimulatory active principles present in the roots. The water extract of Korean red ginseng (*P. ginseng*) stimulates angiogenesis by activating the PI3K/Akt-dependent extracellular signal-regulated kinase (ERK)1/2 and nitric oxide synthase (eNOS) pathways in the HUVEC [59]. It has been reported that ginsenosides Re and Rg1 (high in *P. ginseng*) enhance angiogenesis, whereas the ginsenosides Rb1, Rg3, and Rh2 (*P. quinquefolium*) inhibit angiogenesis. Because of this, *P. ginseng* is considered superior in wound healing compared with *P. quinquefolium* [112]. Rg1 found to induce proangiogenic factors—nitric oxide (NO) and VEGF in HUVECs through PI3K/Akt pathways [59]. Moreover, ginsenoside Re helps to enhance proliferation, migration, and tube formation in HUVEC [112]. Similar to *P. ginseng*, proangiogenic activity has been attributed to the total saponin content of *Panax notoginseng*. In HUVEC,

it has been observed that the total saponin extract of *P. notoginseng* stimulates angiogenesis through upregulating VEGF and receptor KDR/Flk-1 mRNA expression [61].

6.4.13 *Patrinia villosa* (Valerianaceae)

The genus *Patrinia* harbors 20 species, mainly distributed in Central and East Asia and the northeast of North America. *Patrinia* species, especially *Patrinia scabiosaefolia* and *Patrinia villosa*, have been used medicinally to treat viral and bacterial infections [113]. *Patrinia villosa* has been used in traditional oriental medicine for inflammation, wound healing, ascetics, and abdominal pain after child birth [114]. The wound healing potential of this species may be linked to its proangiogenic activity. It has been demonstrated that the aqueous extract of whole *P. villosa* induces angiogenesis via activation of focal adhesion kinase (FAK) in cultured HUVEC. Furthermore, it has been reported that the bioactive components of ethyl acetate fraction of aqueous extracts could be responsible for the observed proangiogenic activity.

6.4.14 *Picrorhiza kurroa* (Scrophulariaceae)

Picrorhiza kurroa is a medicinally valuable yet endangered plant occurring in the Himalayan region of India. *Picrorhiza kurroa* is widely used in traditional as well as modern medicine for the treatment of liver disorders, fever, asthma, and jaundice [115]. Picroliv is the active principle of *P. kurroa*, mainly composed of iridoid glycosides, picroside-I and kutkoside. It is a hepatoprotective and a potent antioxidant and also protects from hemorrhage resuscitation injury [64]. Picroliv has been reported to have angiogenesis-promoting potential via upregulating Flt-1 and KDR, as angiogenesis signaling is mediated by VEGF binding to its receptor tyrosine kinases, Flt-1, Flt-4, and KDR, present on endothelial cells. It has been reported that Picroliv appears to enhance the expression of VEGF in the wound tissues on day 4 post-wounding [64].

6.4.15 *Pistacia atlantica* (Anacardiaceae)

Pistacia is a genus of 15 known species, of which *Pistacia atlantica* is one of the species commonly known as Mt Atlas mastic tree and as the Persian turpentine tree native to several countries such as Iran, Iraq, and Turkey. In Iran it is called "baneh" and is generally used for making chewing gum. It also has been used traditionally for the treatment of peptic ulcers and as a breath freshener [65]. The resin of *P. atlantica* was found to be useful in the treatment of burn wounds. It has been reported that *P. atlantica* resin extract has a concentration-dependent effect on the healing of burn wounds after 14 days of treatment through improving the angiogenesis and also by increasing the concentration of growth factors such as PDGF and FGF [65].

6.4.16 *Plagiochila beddomei* (Plagiochilaceae)

This bryophyte is widely distributed in different parts of India and is used ethnomedicinally in the treatment of skin diseases. The wound healing potential of the methanolic and aqueous extracts from *Plagiochila beddomei* thallus was clinically studied in the Paliyar tribe, Madurai district of India. The methanol extract of *P. beddomei* has demonstrated potent wound healing via enhancing VEGFA expression [66].

6.4.17 *Pueraria thunbergiana* (Leguminosae)

Pueraria thunbergiana (Kudzu) is native to eastern Asia, Southeast Asia, and some Pacific Islands. The name *Pueraria thunbergiana* is a synonym for *Pueraria montana* or *Pueraria lobata*. The root (*radix*) and flower (*flos*) of this plant have been used in Chinese herbal medicines to treat several diseases such as liver damage, dementia, and alcohol detoxification [67,116]. Wound healing potential has been observed for the methanolic and ethyl acetate tuber extracts of *Pueraria tuberose* [117]. The angiogenesis-promoting potential of *Pueraria* species is well established. It has been reported that *Puerariae flos* (ethanol extract) increases angiogenic events (in vitro), such as endothelial cell proliferation, migration, and tube formation, as well as in vivo neovascularization, which further leads to the activation of multiple signal modulators, such as ERK, Akt, eNOS, NO production, p38, Src, and FAK, without increasing VEGF expression [67].

6.4.18 *Salvia miltiorrhiza* (Lamiaceae)

Salvia miltiorrhiza, a member of the mint family is indigenous to China and Japan. Its roots are highly valued in traditional Chinese medicine, and generally used to treat hematological abnormalities, heart disease, hepatitis, hemorrhage, menstrual abnormalities, and collagen-induced platelet aggregation [118]. In addition, *S. miltiorrhiza* is known to be beneficial in the treatment of chronic ulcers [119]. Proangiogenic potential has been reported for the crude aqueous and ethanol extracts and also for an isolated compound, salvianolic acid B, in a murine SVR endothelial cell line assay. Both crude and isolated compounds salvianolic acid B promote angiogenesis through upregulation of VEGF and VEGF receptor gene expression [71].

6.4.19 *Stewartia koreana* (Theaceae)

Stewartia koreana is a deciduous tree native to and extensively distributed throughout Korea [120]. Lee and coworkers reported that the extracts of *S. koreana* leaves induced angiogenesis, extracellular matrix remodeling in a mouse model, and stimulated wound healing on punched skin of the back of mice [72]. A phytochemical analysis of the active extract indicated the presence of a variety of compounds, including triterpenoids, alkaloids, and flavonoids.

6.4.20 *Terminalia chebula* (Combretaceae)

Terminalia chebula (yellow myrobalan or chebulic myrobalan) is native to southern Asia and extends from India and Nepal to southwestern China and south to Sri Lanka, Malaysia, and Vietnam. Its fruit is generally used for medicinal purposes and is historically accredited in Ayurvedic literature. In Thai culture, it is considered as a natural remedy for skin diseases, wound healing, and rejuvenation [75]. Recent pharmacological studies have confirmed a variety of biological activities, such as anticancer, antidiabetic, wound healing, and antibacterial properties [121]. The wound healing potential of *T. chebula* has been attributed to its leaves, bark, and fruit [75,122,123]. Tannins extracted from immature fruits of *T. chebula* promote cutaneous wound healing in rats by promoting angiogenesis by upregulating immune-histochemical, transcriptional, and translational levels of VEGFA expression [75].

6.4.21 *Uncaria rhynchophylla* (Rubiaceae)

Uncaria rhynchophylla is a constituent of the crude Chinese drug, Gou-teng, which is mainly used for the treatment of convulsion, hypertension, epilepsy, eclampsia, and cerebral diseases. Most of its pharmacological properties have been predominantly attributed to indole alkaloids [124]. Recent pharmacological evidence supporting the use of *U. rhynchophylla* in the treatment of wound healing and cardiovascular diseases has been found to be associated with its angiogenesis-inducing potential. It has been observed that a 70% ethanolic root extract of *U. rhynchophylla* significantly enhances HUVEC proliferation in a dose-dependent manner. It has also been reported that extracts of Gou-teng promote angiogenesis by increasing VEGF and bFGF gene expression and protein secretion of HUVEC [76].

6.4.22 Other Plants Useful in Wound Healing with Proangiogenic Potential

Besides these, numerous other plants have potent wound healing potential, which linked to their proangiogenic ability. Based on histological analysis of angiogenesis in healed wounds, several other plants have been reported to have wound healing potential, including *Alternanthera brasiliana* (Amaranthaceae), *Anadenanthera colubrina* (Fabaceae), *Andrographis paniculata* (Acanthaceae), *Blechnum orientale* (Blechnaceae), *Camellia sinensis* (Theaceae), *Clematis burgensis* (Ranunculaceae), *Clematis longicauda* (Ranunculaceae), *Euphorbia caducifolia* (Euphorbiaceae), *Ficus deltoidea* (Moraceae), *Gynura procumbens* (Asteraceae), *Justicia flava* (Acanthaceae), *Lannea welwitschii* (Anacardiaceae), *Lantana camara* (Verbenaceae), *Martynia annua* (Martyniaceae), *Otostegia persica* (Lamiaceae), *Phyllanthus niruri* (Phyllanthaceae), *Rehmannia glutinosa* (Scrophulariaceae), *Rosmarinus officinalis* (Lamiaceae), *Strobilanthes crispus* (Acanthaceae), and *Tephrosia purpurea* (Fabaceae). However, the extract mechanism by which these plants promote angiogenesis in wound healing is not clear. Besides single herb, some studies showed that various combinations of different plant extracts i.e., polyherbal preparations may have potent wound healing action; however results are not well validated at clinical trial levels.

6.5 CONCLUSION

Wound healing is not a simple process of tissue growth but is a complex and highly regulated process. Despite the availability of a wide range of modern medicines, wound healing, especially chronic wounds, remains a challenging field of research, and wound healing is even more difficult in such diseases as diabetes and burn conditions. Plants are the oldest known medicines for wound care and management, and they are still promising candidates for novel drugs in this respect. The usefulness of several plants in wound healing has been described in different systems of traditional medicine, such as Ayurveda and Chinese traditional medicine. In addition to individual plant extracts or isolated compounds, polyherbal formulations have also been determined to be effective in wound healing. It has been observed that the wound healing potential of medicinal plants and their products is often linked with angiogenesis-promoting potential, which is a critical step of wound healing. Recently, most of these plants have been studied for their impact on the proangiogenesis in vitro and in vivo, with special attention being focused on the influence on the expression of VEGF and its receptor VEGFR2. In addition, it has been found that certain plants also promote angiogenesis through the activation of the mitogen-activated protein kinases pathway.

References

[1] Thakur R, Jain N, Pathak R, Sandhu SS. Practices in wound healing studies of plants. Evid Based Complement Alternat Med 2011;2011:438056.

[2] Majewska I, Gendaszewska-Darmach E. Proangiogenic activity of plant extracts in accelerating wound healing-a new face of old phytomedicines. Acta Biochim Pol 2011;58:449–60.

[3] Strodtbeck F. Physiology of wound healing. New Inf Nurs Rev 2001;1:43–52.

[4] Li J, Chen J, Kirsner R. Pathophysiology of acute wound healing. Clin Dermatol 2007;25:9–18.

[5] Guo S, Di Pietro LA. Factors affecting wound healing. J Dent Res 2010;89(3):219–29.

[6] Kumar B, Vijayakumar M, Govindarajan R, Pushpangadan P. Ethnopharmacological approaches to wound healing-exploring medicinal plants of India. J Ethnopharmacol 2007; 114:103–13.

[7] Polverini PJ. The pathophysiology of angiogenesis. Crit Rev Oral Biol Med 1995;6(3):230–47.

[8] Norrby K. The pathophysiology of angiogenesis. Crit Rev Oral Biol Med 1995;6(3):230–47.

[9] Pandya NM, Dhalla NS, Santani DD. Angiogenesis-a new target for future therapy. Vascul Pharmacol 2006;44(5):265–74.

[10] Gosain A, Di Pietro LA. Aging and wound healing. World J Surg 2004;28:321–6.

[11] Mathieu D, Linke JC, Wattel F. Non-healing wounds. In: Mathieu DE, editor. Handbook on hyperbaric medicine. Netherlands: Springer; 2006. p. 401–27.

[12] Broughton 2nd G, Janis JE, Attinger CE. The basic science of wound healing. Plast Reconstr Surg 2006;117(7):12S–34S.

[13] Campos AC, Groth AK, Branco AB. Assessment and nutritional aspects of wound healing. Curr Opin Clin Nutr Metab Care 2008; 11(3):281–8.

[14] Cho CY, Lo JS. Dressing the part. Dermatol Clin 1998;16(1): 25–47.

[15] Stadelmann WK, Digenis AG, Tobin GR. Physiology and healing dynamics of chronic cutaneous wounds. Am J Surg 1998;176(2A): 26S–38S.

[16] Coleville-Nash PR, Willoughby DA. Growth factors in angiogenesis: current interest and therapeutic potential. Mol Med Today 1997;3(1):14–23.

[17] Di Pietro LA, Nissen NN. Angiogenic mediators in wound healing. In: Maragoudakis ME, editor. Angiogenesis: models, modulators and clinical applications, vol. 28. New York: Plenum Press; 1998. p. 121–8.

[18] Morgan C, Nigam Y. Naturally derived factors and their role in the promotion of angiogenesis for the healing of chronic wounds. Angiogenesis 2013;16(3):493–502.

[19] Abraham JA, Klagsbrun M. Modulation of wound repair by members of the fibroblast growth factor family. In: Clark RAF, editor. The molecular and cellular biology of wound repair. New York: Plenum Press; 1996. p. 195–248.

[20] Hoeben B, Landuyt MS, Highley H, Wildiers AT, van Oosterom, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. Pharmacol Rev 2004;56(4):549–80.

[21] Burgess WH, Maciag T. The heparin-binding (fibroblast) growth factor family of proteins. Annu Rev Biochem 1989;58: 575–602.

[22] Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen 2008;16(5):585–601.

[23] Andrikopoulou E, Zhang X, Sebastian R, Marti G, Liu L, Milner SM, et al. Current insights into the role of HIF-1 in cutaneous wound healing. Curr Mol Med 2011;11(3):218–35.

[24] Biswas TK, Mukherjee B. Plant medicines of Indian origin for wound healing activity: a review. Int J Low Extrem Wounds 2003;2(1):25–39.

[25] Fan TP, Yeh JC, Leung KW, Yue PY, Wong RN. Angiogenesis: from plants to blood vessels. Trends Pharmacol Sci 2006;27(6):297–309.

[26] Villegas LF, Fernández ID, Maldonado H, Torres R, Zavaleta A, Vaisberg AJ, et al. Evaluation of the wound-healing activity of selected traditional medicinal plants from Perú. J Ethnopharmacol 1997;55(3):193–200.

[27] Diallo D, Sogn C, Samaké FB, Paulsen BS, Michaelsen TE, Keita A. Wound healing plants in Mali, the Bamako region. An ethnobotanical survey and complement fixation of water extracts from selected plants. Pharm Biol 2002;40:117–28.

[28] Chah KF, Eze CA, Emuelosi CE, Esimone CO. Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants. J Ethnopharmacol 2006;104:164–7.

[29] Ghosh PK, Gaba A. Phyto-extracts in wound healing. J Pharm Pharm Sci 2013;16(5):760–820.

[30] Barua CC, Talukdar A, Begum SA, Pathak DC, Sarma DK, Borah RS, et al. In vivo wound-healing efficacy and antioxidant activity of *Achyranthes aspera* in experimental burns. Pharm Biol 2012;50(7):892–9.

[31] Moon EJ, Lee YM, Lee OH, Lee MJ, Lee SK, Chung MH, et al. A novel angiogenic factor derived from *Aloe vera* gel: beta-sitosterol, a plant sterol. Angiogenesis 1999;3(2):117–23.

[32] Jettanacheawchankit S, Sasithanasate S, Sangvanich P, Banlunara W, Thunyakitpisal P. Acemannan stimulates gingival fibroblast proliferation; expressions of keratinocyte growth factor-1, vascular endothelial growth factor, and type I collagen; and wound healing. J Pharmacol Sci 2009;109(4):525–31.

[33] Barua CC, Talukdar A, Begum SA, Sarma DK, Pathak DC, Barua AG, et al. Wound healing activity of methanolic extract of leaves of *Alternanthera brasiliana* Kuntz using in vivo and in vitro model. Indian J Exp Biol 2009;47(12):1001–5.

[34] Pessoa WS, Estevão LR, Simões RS, Barros ME, Mendonça FS, Baratella-Evêncio L, et al. Effects of angico extract (*Anadenanthera colubrina* var. *cebil*) in cutaneous wound healing in rats. Acta Cir Bras 2012;27(10):655–70.

[35] Al-Bayaty FH, Abdulla MA, Abu Hassan MI, Ali HM. Effect of *Andrographis paniculata* leaf extract on wound healing in rats. Nat Prod Res 2012;26(5):423–9.

[36] Lam HW, Lin HC, Lao SC, Gao JL, Hong SJ, Leong CW, et al. The angiogenic effects of *Angelica sinensis* extract on HUVEC in vitro and zebrafish in vivo. J Cell Biochem 2008;103:195–211.

[37] Sumiyoshi M, Kimura Y. Enhancing effects of a chromone glycoside, eucryphin, isolated from *Astilbe* rhizomes on burn wound repair and its mechanism. Phytomedicine 2010;17(10):820–9.

[38] Zhang Y, Hu G, Lin HC, Hong SJ, Deng YH, Tang JY, et al. Radix Astragali extract promotes angiogenesis involving vascular endothelial growth factor receptor-related phosphatidylinositol 3-kinase/Akt-dependent pathway in human endothelial cells. Phytother Res 2009;23(9):1205–13.

[39] Yao C, Gao F, Chen Y, Miao X, Zhou Y, Shao D. Experimental research of astragalus polysaccharides collagen sponge in enhancing angiogenesis and collagen synthesis. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi 2011;25(12):1481–5.

[40] Zhang Y, Hu G, Li S, Li ZH, Lam CO, Hong SJ, et al. Pro-angiogenic activity of astragaloside IV in HUVECs in vitro and zebrafish in vivo. Mol Med Rep 2012;5(3):805–11.

[41] Huh JE, Kwon NH, Baek YH, Lee JD, Choi DY, Jingushi S, et al. Formononetin promotes early fracture healing through stimulating angiogenesis by up-regulating VEGFR-2/Flk-1 in a rat fracture model. Int Immunopharmacol 2009;9(12):1357–65.

[42] Lai HY, Lim YY, Kim KH. Potential dermal wound healing agent in *Blechnum orientale* Linn. BMC Complement Altern Med 2011;11:62.

[43] Parente LM, Andrade MA, Brito LA, Moura VM, Miguel MP, Lino-Júnior Rde S, et al. Angiogenic activity of *Calendula officinalis* flowers L. in rats. Acta Cir Bras 2011;26(1):19–24.
[44] Hajiaghaalipour F, Kanthimathi MS, Abdulla MA, Sanusi J. The effect of *Camellia sinensis* on wound healing potential in an animal model. Evid Based Complement Alternat Med 2013;2013: 386734.
[45] Kimura Y, Sumiyoshi M, Samukawa K, Satake N, Sakanaka M. Facilitating action of asiaticoside at low doses on burn wound repair and its mechanism. Eur J Pharmacol 2008;584(2–3):415–23.
[46] Choi DY, Baek YH, Huh JE, Ko JM, Woo H, Lee JD, et al. Stimulatory effect of *Cinnamomum cassia* and cinnamic acid on angiogenesis through up-regulation of VEGF and Flk-1/KDR expression. Int Immunopharmacol 2009;9(7–8):959–67.
[47] Hawaze S, Deti H, Suleman S. Wound healing activity of the methanol extracts of *Clematis* species indigenous to Ethiopia. Int J Green Pharm 2013;7(4):304–8.
[48] Goyal M, Nagori BP, Sasmal D. Wound healing activity of latex of *Euphorbia caducifolia*. J Ethnopharmacol 2012;144(3):786–90.
[49] Abdulla MA, Abdul-Aziz AK, Abu-Luhoom FM, Muhanid M. Role of *Ficus deltoidea* extract in the enhancement of wound healing in experimental rats. Biomed Res 2010;21(3):241–5.
[50] Zahra AA, Kadir FA, Mahmood AA, Al Hadi AA, Suzy SM, Sabri SZ, et al. Acute toxicity study and wound healing potential of *Gynura procumbens* leaf extract in rats. J. Med. Plants Res 2011; 5(12):2551–8.
[51] Upadhyay NK, Kumar R, Siddiqui MS, Gupta A. Mechanism of wound-healing activity of *Hippophae rhamnoides* L. leaf extract in experimental burns. Evid Based Complement Alternat Med 2011;2011:659705.
[52] Agyare C, Bempah SB, Boakye YD, Ayande PG, Adarkwa-Yiadom M, Mensah KB. Evaluation of antimicrobial and wound healing potential of *Justicia flava* and *Lannea welwitschii*. Evid Based Complement Alternat Med 2013;2013:632927.
[53] Abdulla MA, Hassandarvish P, Ali HM, Noor SM, Mahmoud FH, Bashah NSA, et al. Acceleration of wound healing potential by *Lantana camara* leaf extract in experimental rats. Res J Med Sci 2009;3(2):75–9.
[54] Santram L, Singhai A. Preliminary pharmacological evaluation of *Martynia annua* Linn leaves for wound healing. Asian Pac J Trop Biomed 2011;1(6):421–7.
[55] Lodhi S, Singhai AK. Wound healing effect of flavonoid rich fraction and luteolin isolated from *Martynia annua* Linn. on streptozotocin induced diabetic rats. Asian Pac J Trop Med 2013;6(4): 253–9.
[56] Lee J, Cooke JP. Nicotine and pathological angiogenesis. Life Sci 2012;91(21–22):1058–64.
[57] Chen S, Sun MZ, Wang B, Hao L, Zhang C, Xin Y. Wound healing effects of cactus extracts on second degree superficial burned mice. J. Med. Plants Res 2011;5(6):973–8.
[58] Ganjali A, Sotoudeh A, Jahanshahi A, Takhtfooladi MA, Bazzazan A, Roodbari N, et al. *Otostegia persica* extraction on healing process of burn wounds. Acta Cir Bras 2013;28(6): 407–11.
[59] Kim YM, Namkoong S, Yun YG, Hong HD, Lee YC, Ha KS, et al. Water extract of Korean red ginseng stimulates angiogenesis by activating the PI3K/Akt-dependent ERK1/2 and eNOS pathways in human umbilical vein endothelial cells. Biol Pharm Bull 2007;30:1674–9.
[60] Kimura Y, Sumiyoshi M, Kawahira K, Sakanaka M. Effects of ginseng saponins isolated from red ginseng roots on burn wound healing in mice. Br J Pharmacol 2006;148(6):860–70.
[61] Hong SJ, Wan JB, Zhang Y, Hu G, Lin HC, Seto SW, et al. Angiogenic effect of saponin extract from *Panax notoginseng* on HUVECs in vitro and zebrafish in vivo. Phytother Res 2009;23: 677–86.
[62] Jeon J, Lee J, Kim C, An Y, Choi C. Aqueous extract of the medicinal plant *Patrinia villosa* Juss. induces angiogenesis via activation of focal adhesion kinase. Microvasc Res 2010;80(3):303–9.
[63] Ahmed KA, Abdulla MA, Mahmoud FM. Wound healing potential of *Phyllanthus niruri* leaf extract in experimental rats. Middle East J Sci Res 2012;11(11):1614–8.
[64] Singh AK, Sharma A, Warren J, Madhavan S, Steele K, Rajesh KNV, et al. Picroliv accelerates epithelialization and angiogenesis in rat wounds. Planta Med 2007;73(3):251–6.
[65] Haghdoost F, Baradaran MM, Zandifar A, Sanei MH, Zolfaghari B, Javanmard SH. *Pistacia atlantica* resin has a dose-dependent effect on angiogenesis and skin burn wound healing in rat. Evid Based Complement Alternat Med 2013; 2013:893425.
[66] Manoj GS, Murugan K. Wound healing activity of methanolic and aqueous extracts of *Plagiochila beddomei* Steph. thallus in rat model. Indian J Exp Biol 2012;50(8):551–8.
[67] Chung BH, Cho YL, Kim JD, Jo HS, Won MH, Lee H, et al. Promotion of direct angiogenesis in vitro and in vivo by *Puerariae flos* extract via activation of MEK/ERK-, PI3K/Akt/eNOS-, and Src/FAK-dependent pathways. Phytother Res 2010;24:934–40.
[68] Lau TW, Chan YW, Lau CP, Lau KM. Radix Astragali and Radix Rehmanniae, the principal components of two antidiabetic foot ulcer herbal formulae, elicit viability-promoting effects on primary fibroblasts cultured from diabetic foot ulcer tissues. Phytother Res 2009;23:809–15.
[69] Liu CL, Cheng L, Kwok HF, Ko CH, Lau TW, Koon CM, et al. Bioassay-guided isolation of norviburtinal from the root of *Rehmannia glutinosa*, exhibited angiogenesis effect in zebrafish embryo model. J Ethnopharmacol 2011;137(3):1323–7.
[70] Abu-Al-Basal MA. Healing potential of *Rosmarinus officinalis* L. on full-thickness excision cutaneous wounds in alloxan-induced-diabetic BALB/c mice. J Ethnopharmacol 2010;131(2): 443–50.
[71] Lay IS, Chiu JH, Shiao MS, Lui WY, Wu CW. Crude extract of *Salvia miltiorrhiza* and salvianolic acid B enhance in vitro angiogenesis in murine SVR endothelial cell line. Planta Med 2003; 69:26–32.
[72] Lee TH, Lee GW, Kim CW, Bang MH, Baek NI, Kim SH, et al. *Stewartia koreana* extract stimulates proliferation and migration of human endothelial cells and induces neovascularization in vivo. Phytother Res 2010;24:20–5.
[73] Al-Henhena N, Mahmood AA, Al-Magrami A, Nor Syuhada AB, Zahra AA, Summaya MD, et al. Histological study of wound healing potential by ethanol leaf extract of *Strobilanthes crispus* in rats. J. Med. Plants Res 2011;5(16):3660–6.
[74] Lodhi S, Jain AP, Sharma VK, Singhai AK. Wound-healing effect of flavonoid-rich fraction from *Tephrosia purpurea* Linn. on streptozotocin-induced diabetic rats. J Herbs Spices Med Plants 2013;19(2):191–205.
[75] Li K, Diao Y, Zhang H, Wang S, Zhang Z, Yu B, et al. Tannin extracts from immature fruits of *Terminalia chebula Fructus* Retz. promote cutaneous wound healing in rats. BMC Complement Altern Med 2011;11:86.
[76] Choi DY, Huh JE, Lee JD, Cho EM, Baek YH, Yang HR, et al. *Uncaria rhynchophylla* induces angiogenesis in vitro and in vivo. Biol Pharm Bull 2005;28:2248–52.
[77] Bhosale UA, Yegnanarayan R, Pophale P, Somani R. Effect of aqueous extracts of *Achyranthes aspera* Linn. on experimental animal model for inflammation. Anc Sci Life 2012;31(4):202–6.
[78] Gupta VK, Malhotra S. Pharmacological attribute of *Aloe vera*: revalidation through experimental and clinical studies. Ayu 2012;33(2):193–6.
[79] Davis RH, Leitner MG, Russo JM, Byrne ME. Wound healing: oral and topical activity of *Aloe vera*. J Am Podiatr Med Assoc 1989;79(11):559–62.

[80] Reynolds T, Dweck AC. *Aloe vera* leaf gel: a review update. J Ethnopharmacol 1999;68:3–37.
[81] Lee MJ, Lee OH, Yoon SH, Lee SK, Chung MH, Park YI, et al. In vitro angiogenic activity of *Aloe vera* gel on calf pulmonary artery endothelial (CPAE) cells. Arch Pharm Res 1998;21(3): 260–5.
[82] Atiba A, Ueno H, Uzuka Y. The effect of *Aloe vera* oral administration on cutaneous wound healing in type 2 diabetic rats. J Vet Med Sci 2011;73(5):583–9.
[83] Gupta A, Upadhyay NK, Sawhney RC, Kumar R. A poly-herbal formulation accelerates normal and impaired diabetic wound healing. Wound Repair Regen 2008;16(6):784–90.
[84] Cheng CW, Chang WL, Chang LC, Wu CC, Lin YF, Chen JS. Ferulic acid, an *Angelica sinensis*-derived polyphenol, slows the progression of membranous nephropathy in a mouse model. Evid Based Complement Alternat Med 2012;2012:161235.
[85] Ye YN, So HL, Liu ES, Shin VY, Cho CH. Effect of polysaccharides from *Angelica sinensis* on gastric ulcer healing. Life Sci 2003;72(8):925–32.
[86] Zhao H, Deneau J, Che GO, Li S, Vagnini F, Azadi P, et al. *Angelica sinensis* isolate SBD.4: composition, gene expression profiling, mechanism of action and effect on wounds, in rats and humans. Eur J Dermatol 2012;22(1):58–67.
[87] Kimura Y, Sumiyoshi M, Sakanaka M. Effects of *Astilbe thunbergii* rhizomes on wound healing part 1. Isolation of promotional effectors from *Astilbe thunbergii* rhizomes on burn wound healing. J Ethnopharmacol 2007;109(1):72–7.
[88] Chen X, Peng LH, Li N, Li QM, Li P, Fung KP, et al. The healing and anti-scar effects of astragaloside IV on the wound repair in vitro and in vivo. J Ethnopharmacol 2012;139(3):721–7.
[89] Yu QT, Qi LW, Li P, Yi L, Zhao J, Bi Z. Determination of seventeen main flavonoids and saponins in the medicinal plant Huang-qi (Radix astragali) by HPLC-DAD-ELSD. J Sep Sci 2007;30:1292–9.
[90] Han DO, Lee HJ, Hahm DH. Wound-healing activity of *Astragali radix* in rats. Methods Find Exp Clin Pharmacol 2009;31(2): 95–100.
[91] Lau TW, Lam FF, Lau KM, Chan YW, Lee KM, Sahota DS, et al. Pharmacological investigation on the wound healing effects of Radix Rehmanniae in an animal model of diabetic foot ulcer. J Ethnopharmacol 2009;123(1):155–62.
[92] Patrick KF, Kumar S, Edwardson PA, Hutchinson JJ. Induction of vascularisation by an aqueous extracts of the flowers of *Calendula officinalis* L. the European marigold. Phytomedicine 1996; 3(1):11–8.
[93] Parente LM, Lino Júnior Rde S, Tresvenzol LM, Vinaud MC, de Paula JR, et al. Wound healing and anti-inflammatory effect in animal models of *Calendula officinalis* L. growing in Brazil. Evid Based Complement Alternat Med 2012;2012: 375671.
[94] Tanideh N, Tavakoli P, Saghiri MA, Garcia-Godoy F, Amanat D, Tadbir AA, et al. Healing acceleration in hamsters of oral mucositis induced by 5-fluorouracil with topical *Calendula officinalis*. Oral Surg Oral Med Oral Pathol Oral Radiol 2013; 115(3):332–8.
[95] Ruszymah BH, Chowdhury SR, Manan NA, Fong OS, Adenan MI, Saim AB. Aqueous extract of *Centella asiatica* promotes corneal epithelium wound healing in vitro. J Ethnopharmacol 2012;140(2):333–8.
[96] Belcaro G, Maquart FX, Scoccianti M, Dugall M, Hosoi M, Cesarone MR, et al. TECA (titrated extract of *Centella asiatica*): new microcirculatory, biomolecular, and vascular application in preventive and clinical medicine. A status paper. Panminerva Med 2011;53(3):105–18.
[97] Wu F, Bian D, Xia Y, Gong Z, Tan Q, Chen J, et al. Identification of major active ingredients responsible for burn wound healing of *Centella asiatica* herbs. Evid Based Complement Alternat Med 2012;2012:848093.
[98] Somboonwong J, Kankaisre M, Tantisira B, Tantisira MH. Wound healing activities of different extracts of *Centella asiatica* in incision and burn wound models: an experimental animal study. BMC Complement Altern Med 2012;12:103.
[99] Cheng CL, Guo JS, Luk J, Koo MW. The healing effects of *Centella* extract and asiaticoside on acetic acid induced gastric ulcers in rats. Life Sci 2004;74(18):2237–49.
[100] Shukla A, Rasik AM, Jain GK, Shankar R, Kulshrestha DK, Dhawan BN. In vitro and in vivo wound healing activity of asiaticoside isolated from *Centella asiatica*. J Ethnopharmacol 1999; 65(1):1–11.
[101] Liu M, Dai Y, Li Y, Luo Y, Huang F, Gong Z, et al. Madecassoside isolated from *Centella asiatica* herbs facilitates burn wound healing in mice. Planta Med 2008;74(8):809–15.
[102] Kwon HK, Hwang JS, So JS, Lee CG, Sahoo A, Ryu JH, et al. Cinnamon extract induces tumor cell death through inhibition of NF kappa B and AP1. BMC Cancer 2010;10:392.
[103] Beveridge T, Li TSC, Oomah BD. Sea buckthorn products: manufacture and composition. J Agric Food Chem 1999;47:3480–8.
[104] Gupta A, Kumar R, Pal K, Banerjee PK, Sawhney RC. A preclinical study of the effects of sea buckthorn (*Hippophae rhamnoides* L.) leaf extract on cutaneous wound healing in albino rats. Int J Low Extrem Wounds 2005;4(2):88–92.
[105] Xing J, Yang B, Dong Y, Wang B, Wang J, Kallio HP. Effects of seabuckthorn (*Hippophae rhamnoides* L.) seed and pulp oils on experimental models of gastric ulcer in rats. Fitoterapia 2002;73: 644–50.
[106] Upadhyay NK, Kumar R, Mandotra SK, Meena RN, Siddiqui MS, Sawhney RC, et al. Safety and healing efficacy of sea buckthorn (*Hippophae rhamnoides* L.) seed oil on burn wounds in rats. Food Chem Toxicol 2009;47(6):1146–53.
[107] Morimoto N, Takemoto S, Kawazoe T, Suzuki S. Nicotine at a low concentration promotes wound healing. J Surg Res 2008;145(2): 199–204.
[108] Martin JW, Mousa SS, Shaker O, Mousa SA. The multiple faces of nicotine and its implications in tissue and wound repair. Exp Dermatol 2009;18(6):497–505.
[109] Ahmed MS, El Tanbouly ND, Islam WT, Sleem AA, El Senousy AS. Antiinflammatory flavonoids from *Opuntia dillenii* (Ker-Gawl) Haw. flowers growing in Egypt. Phytother Res 2005;19(9):807–9.
[110] Kimura Y, Sumiyoshi M, Sakanaka M. Effects of ginsenoside Rb_1 on skin changes. J Biomed Biotechnol 2012;2012:946242.
[111] Lu J, Yao Q, Chen C. Ginseng compounds: an update on their molecular mechanisms and medical applications. Curr Vasc Pharmacol 2009;7:293–302.
[112] Chen CF, Chiou WF, Zhang JT. Comparison of the pharmacological effects of *Panax ginseng* and *Panax quinquefolium*. Acta Pharmacol Sin 2008;29(9):1103–8.
[113] Peng J, Fan G, Hong Z, Chai Y, Wu Y. Preparative separation of isovitexin and isoorientin from *Patrinia villosa* Juss by high-speed counter-current chromatography. J Chromatogr A 2005; 1074:111–5.
[114] Li N, Zhao B, Yu YF, Dong XP. Studies on anti-inflammation chemical constitutes of *Patrinia villosa*. Zhong Yao Cai 2008;31: 51–3.
[115] Gahlan P, Singh HR, Shankar R, Sharma N, Kumari A, Chawla V, et al. De novo sequencing and characterization of *Picrorhiza kurroa* transcriptome at two temperatures showed major transcriptome adjustments. BMC Genomics 2012;31(13):126.
[116] Yamazaki T, Yaguchi M, Nakajima Y, Hosono T, Niiho Y, Hibi Y, et al. Effects of an aqueous extract of *Puerariae flos* (Thomsonide) on impairment of passive avoidance behavior in mice. J Ethnopharmacol 2005;100:244–8.

[117] Kambhoja S, Murthy KRK. Wound healing and anti-inflammatory activity of *Pueraria tuberosa* (Roxb Ex wild) DC. Biomed 2007;2(2):229–32.
[118] Bian W, Chen F, Bai L, Zhang P, Qin W. Dihydrotanshinone I inhibits angiogenesis both in vitro and in vivo. Acta Biochim Biophys Sin (Shanghai) 2008;40(1):1–6.
[119] Liang YH, Li P, Huang QF, Zhao JX, Liu X, Dai MK. Salvianolic acid B in vitro inhibited matrix metalloproteinases-1, -2, and -9 activities. Zhong Xi Yi Jie He Xue Bao 2009;7(2):145–50.
[120] Lee TH, Lee SM, Lee DY, Son Y, Chung DK, Baek NI, et al. A glycosidic spinasterol from *Koreana stewartia* promotes procollagen production and inhibits matrix metalloproteinase-1 expression in UVB-irradiated human dermal fibroblasts. Biol Pharm Bull 2011;34(5):768–73.
[121] Das ND, Jung KH, Park JH, Mondol MA, Shin HJ, Lee HS, et al. *Terminalia chebula* extract acts as a potential NF-κB inhibitor in human lymphoblastic T cells. Phytother Res 2011;25(6):927–34.
[122] Suguna L, Singh S, Sivakumar P, Sampath P, Chandrakasan G. Influence of *Terminalia chebula* on dermal wound healing in rats. Phytother Res 2002;16(3):227–31.
[123] Khan AA, Kumar V, Singh BK, Singh R. Evaluation of wound healing property *of Terminalia catappa* on excision wound models in Wistar rats. Drug Res (Stuttg) 2014; 64(5):225–8.
[124] Ndagijimana A, Wang X, Pan G, Zhang F, Feng H, Olaleye O. A review on indole alkaloids isolated from *Uncaria rhynchophylla* and their pharmacological studies. Fitoterapia 2013;86:35–47.

LIST OF ABBREVIATIONS

ERK Extracellular signal-regulated kinase
FAK Focal adhesion kinase
FGF Fibroblast growth factor
HUVEC Human umbilical vein endothelial cells
JNK1 Jun N-terminal kinases
KDR Kinase insert domain receptor
MMP Matrix metalloproteinases
nAChR Nicotinic acetylcholine receptor
PDGF Platelet-derived growth factor
PI3K-Akt-eNOS Phosphatidylinositol 3-kinase-Akt-endothelial nitric oxide synthase
PMNs Polymorphonuclear leukocytes
TGF Transforming growth factor
TGF-α Transforming growth factor-α
VEGF Vascular endothelial growth factor
VEGFR Vascular endothelial growth factor receptor

CHAPTER

7

Pharmacovigilance: Tools in Establishing the Safety and Acceptability of the Natural Health Products—Clinical Evaluation

Luigi Gori[2], *Fabio Firenzuoli*[1,2,*]

[1]Center for Integrative Medicine, Careggi University Hospital of Florence, Florence, Italy; [2]Referring Center for Phytotherapy, Tuscany Region, School of Medicine, University of Florence, Florence, Italy

*Corresponding Author: email: fabio.firenzuoli@unifi.it

OUTLINE

7.1 INTRODUCTION

The general concept, often held in marketing (as obvious), by the media and mostly by common people that everything that is completely natural or organic, especially if for therapeutic purpose, is safe is a big error.

The so-called herbal medicines (HMs) have a relatively low risk if properly used by expert health caregivers, but potential important harms are associated with their incorrect use or misuse.

Potential harms can be due to the intrinsic toxicity of herbs, from their contamination, adulteration, plant misidentification, and by interactions with drugs, especially if the plant is used as a purified and concentrated extract, and the decoction too can be unsafe especially if the plant contains alkaloids.

About safety of HMs it is mandatory to follow the same procedures as synthetic drugs, and not a simplified one, but exactly the contrary. A synthetic drug is composed of a single substance that may be metabolized into a few substances that can be studied in a relatively simple way; however, what about a vegetable that may contain tens or hundreds of substances interacting between them? And which of them could interact with a synthetic drug?

As like synthetic drugs, HMs or better herbal extracts (HEs) can give rise to untoward effects, inappropriate

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00007-6

use, which is more frequent than commonly believed, can give rise to adverse effects. Moreover, pharmacokinetic and pharmacodynamic interactions between botanical medicines and synthetic drugs can also be the cause of clinically relevant effects; so a better knowledge in this particular field may be very helpful in avoiding adverse effects, but it remains a very complex scientific area. Uncovering adverse reactions to herbs is much more challenging than uncovering adverse reactions to drugs.

7.2 BRIEF HISTORICAL REVIEW

As a paradigmatic example we can take *Hypericum perforatum* extracts. The first studies on its antibiotic properties were published in the 1950s [1], and the first clinical study on six women was published in 1984 by Muldner while testing a commercial product [2]. In 1999, the first letter about toxicity of *Hypericum* [3] and in 2000, the first report of serious adverse reactions that led to rejection of heart transplant [4] were published; later, the biochemical mechanism was linked to interference with cytochrome 3A4 [5]. In 2003, the definitive scientific acknowledgment that *H. perforatum* extracts could give rise to adverse reactions and in particular to important interference with commonly used synthetic drugs like cyclosporine was published. Why all this time from 1984? Simply because it is not so easy to identify adverse reactions, and probably because then it was considered an HE, safe and having just a placebo effect. Now it is an extract registered as a commercial drug for treatment of psychiatric depression.

7.3 ADVERSE REACTIONS

In general, the probability of finding an adverse reaction before the definitive approval of a synthetic drug is really low. If "only" 10,000 patients participate in a large pharmaceutical trial for 1 year, although all patients are controlled, a severe drug reaction with a 1:20,000 incidence can be missed. But when it is marketed and used by millions of patients, for many years, especially if they are "true patients" [6], it is much more likely to identify adverse effects.

Randomized clinical trials (RCTs) have other important limitations with regard to adverse drug reaction (ADR) detection. RCTs have limited study population and generally duration of study; especially the necessary selective recruitment of patients can result in limited heterogeneity of patients, not only for a disease, but also for age, gender, as well as cultural and mental preparedness [6]. The generalizability of clinical findings from RCTs to clinical practice, including ADRs, is often partially inadequate and can have limitations: the selection of patients for RCTs is generally not representative of the general, normal, population and patients who will receive the treatment may be less vulnerable to ADRs, for example, just because they are much more clinically controlled. If they have persistent nausea and vomiting, they will be removed from the trial and thus will never develop a gastric ulcer. In addition, the inclusion criteria for RCT patients are frequently not completely reported; even in patients with relevant disease, the severity and staging of the disease as well as comorbidities in RCTs may not reflect those found in routine clinical care [6]. Populations such as adolescents and very old patients are highly underrepresented in RCTs, leading to very limited premarketing drug safety information. This issue is still more important for HMs where RCTs often are with few patients and of shorter duration.

Building valid basis for a real clinical knowledge and toxicity in HMs requires balancing two aspects of scientific validity: internal and external validity [7]. Internal validity means that the research must reliably test hypothesized relationships between an intervention and an outcome under controlled conditions. Internally valid research will typically try to answer a focused research question that is salient within the vocabulary and methods of the scientific community at the time the research is conducted. External validity refers to the practical applicability of the research results to a target population outside the experimental conditions of the research study [7].

It is very important to realize that pharmacovigilance systems are almost always based on passive and not active mechanisms, so it is generally estimated that less that 10% of severe adverse effects are reported and reliably described. Thus clinicians become fully aware of adverse reactions only when several case reports are published, unfortunately a slow process.

A further complication is that in many countries, and the Western countries too, the majority of herbal remedies are self-prescribed or counseled by not really expert personal, generally simple shoppers who have very little knowledge of herb toxicological problems. Availability of information on clinical issues of HMs has been poor despite extensive scientific literature.

Furthermore, there are some very simple yet significant problems:

1. Medicinal plants are almost always sold as dietary supplements in most countries, worsening the efforts to get a safe use of HMs.
2. International merchandising regulation of HMs is not even minimally harmonized, because there are meaningful different regulations in European Union, China, India, Japan, Asia, and North and South American countries; as an obvious consequence, lack of rules gives poor guarantee of safety.

7.4 PHARMACOVIGILANCE

Pharmacovigilance has been defined by the World Health Organization (WHO) as the science and activities related to the detection, assessment, understanding, and prevention of adverse effects or any other medicine-related problem. In Europe, Article 106 of Directive 2001/83/EC specifically requires the European Commission in consultation with the European Medicines Agency, member states, and interested parties to draw up guidance on the collection, verification, and presentation of adverse reaction reports to facilitate the exchange of information.

About herbal pharmacovigilance, the WHO, European Union, United States, People's Republic of China, and other countries are enforcing their systems with exchange of information; yet the situation relative to this particular toxicological issue requires particular knowledge, recognition, and monitoring of adverse reactions through pharmacovigilance activities and real clinical herbal, phytochemical, botanical, and agricultural expertise.

7.5 ADVERSE EFFECTS AND HERBAL ADVERSE EFFECTS

The first step is to define as clearly as possible what is an ADR and second how an adverse reaction to a herb can develop. According to the WHO, the definition of an ADR, which has been in use for many decades, is "a response to a drug that is noxious and unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function" [8]; this definition is too simple and does not fit HMs because it does not take into regard sufficiently the problem of different standardization, a small problem in synthetic drugs. There can be important variations in the identity and quantity of the substance used, sometimes in the same preparation or in the same batch; very often they are used without any expert medical advice or at definitive established therapeutic doses. The same botanical species can be used for completely different purposes evidently in different diseases and different patients: *Curcuma longa* can be used as a spice, the extract can be titrated in curcuminoids as an antiinflammatory in Crohn's disease, and it can be used to treat uveitis, diabetic nephropathy, and other conditions, [9].

Herbs like all vegetal substances used for any purpose have still more proper specific problems very different from synthetic drugs: time of harvesting, bioclimatic problems of growth, storage, growth of other plants and life or insects on plants, etc.

We prefer the definition proposed by Edwards and Aronson [10] for adverse reactions "An appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of a medicinal product, which predicts hazard from future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product."

There are tens of thousands of plant species used in this world as medicines and there is an enormous potential for the development of novel products in the world that is hungry for new drugs. This requires the establishment of important and definite regulatory international choices.

There are many studies on several plant species following substantially simple pharmacological models at a single dose; we end up with an enormous amount of experimental biological, and chemical information, but very little clinical data that are generally based on small samples of patients. Often these data are too small for evidence-based clinical decision, highly based on the simple common use of a plant extract, whose only toxicological expertise stems from the fact that it has been used from centuries. A controversial toxicological issue has been established by WHO too [11]. The use of a plant from centuries, regarded as a proof of its safety, is a very poor technical tool, because if a plant decoction or a spice is used by native from the beginning of times without any adverse reaction ever reported (who could report an ADR?), the extraction, purification and titration of some particular substance from the same plant may yield a completely different substance, with a new medical story to write.

WHO more recently stated that "Currently, the majority of adverse events related to the use of herbal products and HMs that are reported are attributable either to poor product quality or to improper use." This can be misleading because there are new extracts and new interference or new evidence that have nothing to do with product quality or improper use.

What is really needed is a clear strategy about what should be considered as active in terms of the required dose and to address from the very beginning adverse effects and toxicity.

7.5.1 The Adverse Reaction—An In-depth View

The terms "adverse reaction" and "adverse effect" may seem interchangeable, but they are not: an adverse effect is from the point of view of the toxicological history of the drug, and may be well known from experimental toxicological file and papers; an adverse reaction is from the point of view of the patient and may be unexpected, new and simple nocebo effect sine materia. Both must be distinguished from an "adverse event," which is an adverse outcome that occurs contemporary to the use of a substance [10] or more substances (synthetic, natural, or placebo), but is not

expected, not necessarily attributable to it, and may be said to be "hydiosyncratic." This is the scientific path that must be followed to identify an adverse event and to definitely link it to a particular substance.

Besides, the adverse reaction to a drug can be classified as:

- dose and non-dose related,
- time related (chronic and delayed),
- withdrawal reactions
- allergic, and
- pharmacological interference.

The reaction can be:

- unexpected/expected;
- of serious, moderate, or mild severity; and
- can give rise to cancer or birth defect.

The accepted international terminology for reporting ADRs is based on WHO's Adverse Reaction Terminology (WHO-ART) [12]. This terminology is hierarchical and links system or organ classes to three types of general terms: broad "high-level" terms, more specific and disease-related or symptom-related "preferred" terms, and finally, the frequently reported alternative "included" term and true synonyms. This terminology is intended for use alongside the general disease terminology—the International Classification of Diseases (ICD) [10]. Work is being undertaken to link these classifications, so that WHO-ART will become a subset of ICD [10].

Another initiative is the medical terminology for drug regulatory authorities (MedDRA) [13]. This terminology includes historical terms from WHO-ART, ICD9, and Coding Symbols for a Thesaurus of Adverse Reaction Terms, used in the past by the US Food and Drug Administration. MedDRA is currently being promoted commercially and is accepted by the European Union, the United States, and Japan [10]. However, compatibility with the ICD and WHO-ART is no longer complete, since MedDRA has more terms and another level in its hierarchy, so that the links between terms may be different from those in WHO-ART, even when there is correspondence in individual terms [10].

These are only the main issues related to the definition of an adverse reaction to a drug and can be used for HMs too.

7.5.2 Causality Assessment

The other fundamental issue is the attribution of causality that may be particularly challenging because herbs have peculiar problems.

- You can have the name of a plant but you can have completely different extracts (from titrated and purified extract to only dehydrated raw material.
- One may have the name of the plant but it can contain leaves, roots, essential oil, and oleoresin; each with a different type of extraction (from supercritical CO_2 to hydroalcoholic extraction) and different substances.
- It can be mixed with other extracts.
- On the batch the name of a product may be written but it may contain a different one.
- The extract can contain contaminants (natural toxins, pesticides, heavy metals).
- The extract may not contain the substance.
- The extract can be substituted or mixed with synthetic substances.
- The extract can be used in a completely different way and in different doses (*H. perforatum* titrated extract can be used as antidepressant; the oleolith is used for healing purpose).
- The extract may contain nothing but only the excipient.

Sometimes to exactly understand what has been used can be impossible. So the attribution of causality of an adverse reaction generally based on classical classification, such as certain, probable, possible, unlikely, and unassessable, can be unreliable. So herbal toxicological expert should be a group of experts with deep botanical, phytochemical, pharmaceutical, toxicological, clinical and traditional medicine, and ethnobotanical expertise.

7.5.3 Laboratory Investigations

Laboratory investigations (blood samples, plasma concentration of phytochemicals, biopsies, and so on) establish baselines for organ function (e.g., liver, kidney, electrocardiogram, thyroid function), can aid diagnosis, but mostly provide a means for clinical toxicological diagnosis and monitoring what happen after a substance has been stopped or a rescue therapy is given to a patient after the onset a major adverse event. Nevertheless laboratory investigations are the most important step to establish a definitive clinical-toxicological diagnosis, they are rarely the key to understand what is the toxic substance. Occasionally they can help to establish baseline functions at the start of suspected herbal therapy. It is become important to rechallenge with an HE and this should be fully considered, particularly if the patient is likely to benefit directly from the knowledge gained.

Especially during rechallenge it is possible to definitely attribute causality; obviously this is unethical, but when it is done it can be dramatically helpful, although rare [14].

7.6 PHARMACOVIGILANCE AND HERBAL PHARMACOVIGILANCE

Pharmacovigilance is the science and activities related and correlated to the investigation, identification,

assessment, understanding, and possibly prevention of adverse effects or any other possible problem in therapeutics (15) : can be synthetic drugs, HEs, vaccination, hemoderivatives or other therapeutic devices.

The fundamental aim of pharmacovigilance is to extend monitoring and investigate drug adverse events that have previously been unrecognized despite evaluation in clinical trials, and this objective is particularly important in the field of HM where clinical trials are scarce and sometimes completely absent.

Spontaneous reporting of ADRs has played a major role in the detection of unsuspected, serious, and unusual ADRs previously undetected during the clinical trial phases. This has led to the withdrawal in the recent past of not only many drugs but also HEs: kava kava, *Senecio scandens*, *Ephedra sinensis*.

The contribution of health professionals is enormous in the complete success of a pharmacovigilance program. It is found that only 6–10% of all ADRs are reported in the Western countries [16,17], and probably much less in many other countries, although data contrast the number of calls to poison centers [18]. A high rate of underreporting is obviously a matter of great concern, which is the most important obstacle to detection of serious ADRs with a major impact on the public health.

7.6.1 Pharmacovigilance Pathways

Pharmacovigilance is based on six main pathways:

- Consumer signal
- Health caregiver signal
- Poison centers
- Manufacturer signal
- Evaluation of clinical studies (meta-analysis, cohort, case reports, and case cohort studies)
- Population statistics

Consumers are frequent callers to poison centers, but very poor callers to authorities or health caregivers, especially medical doctors and especially if they are using herbs, because they usually do that by themselves as self-therapy and not rarely against medical advice.

Health caregivers should be the mainstay of the system but underreporting for synthetic drugs has also been widely attributed to them.

Inman [19] has summarized these factors as the "seven deadly sins." His description of the "sins" include: attitudes relating to professional activities (financial incentives: rewards for reporting; legal aspects: fear of litigation or inquiry into prescribing costs;) and problems associated with ADR-related knowledge and attitudes (complacency: the belief that very serious ADRs are well documented by the time a drug is marketed; diffidence: the belief that reporting an ADR would only be done if there was certainty that it was related to the use of a particular drug; indifference: the belief that the single case an individual doctor might observe could not contribute to medical knowledge; and ignorance: the belief that it is only necessary to report serious or unexpected ADRs), and excuses made by professionals (lethargy: the procrastination and disinterestedness in reporting or lack of time to find a report card and other excuses). Probably about herb the main reason in our opinion should be an eighth sin: scarce knowledge of HM toxicology.

In general to stimulate a reporting culture, pharmacovigilance program recommends reporting of all suspected, even nonserious and common ADRs related to drugs, including Over The Counter products, although this can be misleading with sometimes incorrect overreporting.

In almost all parts of the world poison centers are active, and in Europe and the United States, they are informed about herbal toxicity. The problem is that almost always poison centers are or are alleged to be the frontline for emergencies, while they are not useful for medium- and long-term toxicities. Cases often are just telephone call and unless there are severe or numerous adverse reactions, they often lack important details about products, time course, and dosage of the preparation.

Manufacturers may not be so interested in adverse events, and unless important toxicological problems come up, it is difficult to get too much information, especially in case of HM where many products are marketed easily with respect to synthetic drugs.

7.6.2 Methodological Problems

The association between significant results and publication is well known: studies that report positive or significant results ($P < 0.05$) are more likely to be published, and outcomes that are statistically significant have higher odds of being fully reported than those that are not significant (range of odds ratios: 2.2–4.7) [20]. An analysis of studies that compared trial publications with protocols found that 40–62% of trials changed, introduced, or omitted at least one primary outcome [20].

In a paper review [21] evaluating outcomes of systematic reviews, more than half (157/283, 55%) did not include full data for the review primary outcome of interest from all eligible trials. The median amount of review outcome data missing for any reason was 10%, whereas 50% or more of the potential data were missing in 70 (25%) reviews. It was clear from the publications

examined and classified that 155 (6%) of the 2486 trials for which the researchers had measured and analyzed the review primary outcome did not report or only partially reported the results [21]. For reports that did not mention the review primary outcome, the nine-point classification regarding the presence of outcome reporting bias was shown to have a sensitivity of 88% (95% confidence interval (CI) 65–100%) and specificity of 80% (95% CI 69–90%) on the basis of responses from 62 trialists [20]. One-third of Cochrane reviews (96/283 (34%)) contained at least one trial with high suspicion of outcome reporting bias for the review primary outcome [21]. In a sensitivity analysis undertaken for 81 reviews with a single meta-analysis of the primary outcome of interest, the treatment effect estimate was reduced by 20% or more in 19 (23%). Of the 42 meta-analyses with a statistically significant result, only 8 (19%) became nonsignificant after adjustment for outcome reporting bias and 11 (26%) would have overestimated the treatment effect by 20% or more [21].

Outcome reporting bias is an underrecognized problem that affects the conclusions in a substantial proportion of Cochrane reviews. This obviously has very important effect on the estimation of ADRs.

Population statistics is very important, but it is very expensive and most of all available when it is too late. This is particularly difficult in HM often used from centuries, considered herb and thus automatically safe, and used by patients almost always without any medical advice. Premarketing trials are almost absent, although they are generally able to provide information only about the advantages of single drugs and rarely to establish a safety profile, especially in HM. When a synthetic or herbal drug is used in clinical practice in large unselected populations, epidemiological postmarketing studies find their major confirmation in recalling all the events that occur during monitoring, with estimates of incidence of ADRs that cannot be obtained by spontaneous reports. In these studies, a significant role can be played by all the actors of sanitary systems: family doctors, hospitals databases, pharmacists, dentists, nurses, and so on, but only if there are well-known active pharmacovigilance projects.

The main concern of pharmacovigilance is the lateness in detection of ADRs especially if they are new or unexpected because of their proper clinical nature, severity and/or frequency, and contemporary use of other pharmacological substances. The base of this process is the scientific acumen of the herbal pharmacovigilance domain expert who should be a true expert of HM, or at least, as first-line, a health caregiver prepared at least partially in HM toxicological problems. Moreover, the development of an easily accessible database with screening tools to assist human reviewers in identifying associations worthy of further investigation (i.e., signals) is essential, while it may be counterproductive to use simplified databases relying on background "noise" containing reports sometimes of no substantial public health significance [22]. Data mining algorithms are essential and need to be developed, tested, and/or used by health authorities, pharmaceutical companies, and academic researchers especially to realize an efficient herbal pharmacovigilance.

7.7 TOXICOLOGICOLOGIST'S TOOLKITS

In the literature, there are tens of methods proposed to asses the causality between a drug and an adverse reaction. They can be divided into three categories: expert judgment, operational algorithms, and probabilistic approaches [23–25].

Expert judgment, or global introspection, should rely on clinical expertise and deep knowledge of herbal phytochemistry and toxicology and ethnopharmacology of HM to assess the likelihood of a drug causing an adverse event. It apparently mimics the clinical diagnosis process but if well done can be little subjective, although can suffer from poor intra- and interrater reproducibility when carried out by not true experts of the field. We prefer this because in HM there too many variables to consider and actually a valid algorithmic tool does not exist [26,27].

Algorithms consist of assessing successive causality criteria combined by means of scores or a decision tree developed to standardize causality assessment reasoning. The final result is expressed as an x degree score [28]. Although this approach is easy to use and tends to minimize the inter- and intraobserver variability, the final assessment depends highly on the relative weighting of each criterion, which is often fixed more or less arbitrarily by the author(s) of the method [28–30]. Probabilistic approaches are derived mainly from Bayes' theorem that can be seen as a way of understanding how the probability that a theory is true is affected by a new piece of evidence. It is used to try to clarify the relationship between theory and real evidence of facts on the basis of mathematical rules of probability. This presents the important advantage of providing a mathematical causal assessment and results directly in the form of a probability, that is in a number. So it can be applied if you have a reliable amount of data. In toxicology, it can be used to understand how probable a suspected fact is, or better to evaluate epidemiological data, and understand the meaning of signals, while parametric statistics are probably more useful to study laboratory and experimental data.

7.7.1 Scales and Algorithms

The algorithms published in the literature are based on questions of alternative etiological causes with a simple final question: yes or no. So it is not possible to evaluate in detail the adequacy of inquiries, and this may lead to gross errors. Like the positive toxicological evaluation of a substance added to many other substances and in completely different preparates both in herbal and conventional medicine is a non sense and give to rise to wrong conclusions [22,31–33].

The main validated algorithm base scales are:

WHO-Uppsala Monitoring Centre (UMC)
Council for International Organizations of Medical Sciences (CIOMS)/Roussel Uclaf Causality Assessment Method (RUCAM) scale
Naranjo

The causality assessment system proposed by the WHO Collaborating Centre for International Drug Monitoring, the WHO-UMC, and the Naranjo Probability Scale are the generally accepted and the most widely used methods for ADR causality assessment in common practice because they have a simple methodology.

WHO-UMC

We think this algorithm, although apparently too simple and subjective, if completed can comply acceptably with the problems of HM. However, it does not take into considerable account the possibility of pharmacological interference, which can be a very important toxicological problem especially in HM; in fact often herbs are used contemporarily with other substances. We think the major defect is that it can be used only by experts, and cannot be easily and reliably used by every health caregiver.

CIOMS/RUCAM

This scale is almost complete and can be easily used, but it is substantially dedicated only to liver injuries. Nevertheless, it has some limits such as the age (why the age $\geq$ of 55 y.o. chosen as a risk factor?) and alcohol consumption; it does not take into consideration the importance of knowing the real content of the extract and possible herb–drug interference and does not give in our opinion definitive importance to previously published reports. There are disagreements in calculating the time to onset and in the definition of hepatocellular, cholestatic, and mixed reactions, which may be misleading.

Naranjo

We criticize this scale because we think it is an oversimplification and partially contradictory especially for HEs. The question whether the event appear after administration of the drug is obvious. Rechallenge, which normally is the best evidence of adverse reaction, has only two points. The question whether the adverse event is confirmed by objective evidence is contrary to the entire scale because it is substantially a clinical diagnosis and not an algorithm-based diagnosis. Specifically regarding HM there is no question about the real concentration of the active principle in the batch considered. Also, paradoxically it gives scarce importance to the content of any substance in the blood, which is, however, very difficult to detect especially for HEs. The main limitation of the scale is that it can be used to assess the likelihood of an adverse drug event associated with only one drug.

Unfortunately, the difficulties in assessing drug causality are huge especially in HM because you generally have many substances, sometimes indefinite. Herbal ADRs are complex clinical problems that involve three key variables: the patient, the botanical substance, synthetic drugs and the event; each variable has different problems, which should be wisely evaluated in 360°:

Patient: There are multitudes of variables to consider: allergies; metabolic risk factors; age; sex; severity of illness; comorbid features; cardiac, renal, or liver function; etc.

Drug: Dosage, duration of use, known toxicity, timing of administration, and other variables must be considered. If you are dealing with a new drug, clinical trials may not have been robust enough to detect rare ADRs.

Event: Previous reports, timing of onset, disease exacerbation, and new medical problems often cloud the picture.

Then there is background noise that may confound the diagnosis. In fact, the clinical picture in pediatric, elderly, cancer, or intensive care patients is often so complex, with the presence of multiple disorders and multiple drugs, that possible drug-related effects are not easily sorted out.

7.7.2 Predictive Toxicology

For many reasons, the eukaryotic budding (*Saccharomyces cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeasts are ideal models to conduct functional toxicological studies [34]. Numerous metabolic and signaling pathways, along with basic cellular processes, are conserved in more complex organisms such as humans [34]. Human homologs have been identified for a large number of yeast genes, with several hundreds of the conserved genes linked to disease in humans [35,36]. A long history of genetic manipulation in yeasts confers the ability to selectively target and examine conserved

genes and pathways throughout their genomes, facilitating functional analyses. The ease of culture and availability of software resources, molecular protocols, and genetic and physical interaction data collectively bolster the value of yeasts in toxicology.

Genome-wide ribonucleic acid (RNA) interference (RNAi) screens have become an important tool in drug discovery [37] and can be readily applied to functional toxicological studies. RNAi methods exploit existing cellular machinery to destroy the messenger RNA (mRNA) of a target gene, thus preventing translation and effectively "knocking down" the function of the target gene. RNAi screens in cell lines can be useful to functional toxicology but are experimentally complex, as incomplete knockdown of target genes and off-target effects can complicate execution and analysis [38].

For a variety of reasons, functional toxicology has its limitations in the range of organisms used. Less complex organisms such as yeasts, other fungi, and bacteria possess P450 enzymes, but their role in toxicant metabolism is limited [37]. To better understand chemical toxicity to humans, toxicant activation or deactivation may be catalyzed in these experiments by adding S-9 human liver microsomes. Additionally, in unicellular organisms, cell lines, and less complex eukaryotes, one is unable to examine a toxicant's target organs or systemic effects. The discovery of human disease models through orthologous phenotypes will address these issues [37].

In whole organisms, throughput is the major barrier to progress in functional toxicology. In yeasts and bacteria, bar-coded systems allow for the high-throughput examination of thousands of deletion mutants in parallel [39,40]. The lack of such methods in whole organisms such as zebrafish or rodent models diminishes throughput, and complicates systematic identification of genes required for resistance to a toxicant. Until high-throughput screens are devised in whole organisms, actually such systems are invaluable in extending or confirming results gathered in yeasts or bacteria.

The current gold standard for evaluation of the carcinogenic potential of newly developed drugs and other chemical compounds is the classical 2-year chronic rodent bioassay. There is a mechanistic distinction between genotoxic carcinogens, which form deoxyribonucleic acid (DNA) adducts and cause direct DNA damage, as opposed to nongenotoxic carcinogens, for which a wide variety of alternative hepatocarcinogenic mechanisms have been described [41]. While genotoxic carcinogens can be identified early by means of in vitro genotoxicity assays (e.g., Ames test), no short-term assay exists for the detection of nongenotoxic carcinogens. Several groups have reported the application of toxicogenomic methods for prediction of the outcome of chronic bioassays based on gene expression profiles compiled from short-term in vivo studies. Most studies published in this field focused on mRNA expression profiling and employed machine learning algorithms or statistical methods to predict the carcinogenic class of compounds based on characteristic expression patterns, called signatures [42,43]

The classification outcomes may then be used to prioritize environmental and/or industrial chemicals for further exploration in chronic carcinogenicity bioassays [44]. Furthermore, the toxicogenomics approach can deliver complementary mechanistic insights, as specific molecular profiles can be associated with toxicological phenotypes and adverse effects observed in animal studies [43].

Building on published toxicogenomics studies that mostly focused on mRNA expression and used individual genes as predictive features, two novel concepts have been introduced: first, the integration of omics data across platforms that interrogate different biological layers (mRNA, microRNA, and protein expression) and second, the abstraction from individual signature genes to higher order levels, such as pathway enrichments or molecular interactions [45].

7.8 CONCLUSIONS

The safety and efficacy of so-called "natural" products have not been thoroughly investigated. And on this topic it is important to improve communication with the public, at least with respect to safety. Natural products are promoted to the public as equally or more effective and less toxic than conventional drugs, although some herbs are also well known to have adverse effects. The awareness of the need for monitoring the safety of natural health products is steadily albeit slowly stimulating the creation by the health authorities of each country systems reporting suspected adverse reactions. For example, in Italy, this system has been established and shared between the Italian National Institute of Health, the Ministry of Health, and the Italian Medicines Agency [46], in order to encourage voluntary reporting, which can help improve awareness among health care professionals and patients about the benefit–harm profile of these remedies. The reports must then be evaluated by a multidisciplinary group of experts to address and solve the many problems specific to each case.

We hope that similar initiatives will become "normal" in every country.

References

[1] Vincent D, Segonzag G. Antibiotic effect of *Hypericum*. Toulouse Med 1951;52:779–80.
[2] Müldner H, Zöller M. Antidepressive effect of a *Hypericum* extract standardized to an active hypericine complex. Biochemical and clinical studies. Arzneimittelforschung 1984;4:918–20.
[3] Firenzuoli F, Gori L. Toxicity of *Hypericum perforatum*. Forsch Komplementarmed 1999;6:271.

[4] Ruschitzka F, Meier PJ, Turina M, Lüscher TF, Noll G. Acute heart transplant rejection due to Saint John's wort. Lancet 2000;355: 548–9.
[5] Roby CA, Anderson GD, Kantor E, Dryer DA, Burstein AH. St John's Wort: effect on CYP3A4 activity. Clin Pharmacol Ther 2000;67:451–7.
[6] Ioannidis JPA. Why most published research findings are false. PLoS Med 2005;2:e124.
[7] Linde K, Jonas WB. Evaluating complementary and alternative medicine: the balance of rigor and relevance. In: Jonas WB, Levin JS, editors. Essentials of complementary and alternative medicine. Baltimore: Lippincott Williams & Wilkins; 1999. p. 57–71.
[8] World Health Organization. International drug monitoring: the role of national centres. Report No: 498. Geneva, Switzerland: World Health Organization; 1972.
[9] Gupta SC, Patchva S, Aggarwal BB. Therapeutic roles of curcumin: lessons learned from clinical trials. AAPS J 2013;15: 195–218.
[10] Edwards IR, Aronson JK. Adverse drug reactions: definitions, diagnosis, and management. Lancet 2000;356:1255–9.
[11] WHO. Annex II. Guidelines for the assessment of herbal medicines (WHO Technical Report Series No. 863), Geneva. 1996.
[12] WHO. International monitoring of adverse reactions to drugs: adverse reaction terminology. Uppsala, Sweden: WHO Collaborating Centre for International Drug Monitoring; 1992.
[13] Wood KL, On behalf of the MEDDRA Working Party. The medical dictionary for drug regulatory affairs (MEDDRA). Pharmacoepidemiol Drug Saf 1994;3:7–13.
[14] Gori L, Galluzzi P, Mascherini V, Gallo E, Lapi F, Mennini-Ippolito F, et al. Two contemporary cases of hepatitis associated with *Teucrium chamaedrys* L. decoction use: case reports and review of literature. Basic Clin Pharmacol Toxicol 2011;109:521–6.
[15] World Health Organization Collaborating Centre for International Drug Monitoring. The importance of pharmacovigilance. Safety monitoring of medicinal products. Geneva: World Health Organization; 2002.
[16] Smith CC, Bennett PM, Pearce HM, Harrison PI, Reynolds DJ, Aronson JK, et al. Adverse drug reaction in a hospital general medical unit meriting notification to the committee on safety of medicines. Br J Clin Pharmacol 1996;42:423–42.
[17] Feely J, Moriarty S, O'Connor P. Stimulating reporting of adverse drug reaction by using a fee. Br Med J 1990;300:22–3.
[18] Litovitz TL, Klein-Schwartz W, Rodgers Jr GC, Cobaugh DJ, Youniss J, Omslaer JC, et al. 2001 Annual report of the American Association of Poison Control Centers Toxic Exposure Surveillance System. Am J Emerg Med 2002;20:391–452.
[19] Inman WH. Attitudes to adverse drug-reaction reporting. Br J Clin Pharmacol 1996;41:433–5.
[20] Dwan K, Altman DG, Arnaiz JA, Bloom J, Chan A, Cronin E, et al. Systematic review of 6 the empirical evidence of study publication bias and outcome reporting bias. PloS ONE 2008;3:e3081.
[21] Kirkham JJ, Dwan KM, Altman DG, Gamble C, Dodd S, Smyth R, et al. The impact of outcome reporting bias in randomised controlled trials on a cohort of systematic reviews. BMJ 2010; 340:c365.
[22] Firenzuoli F, Gori L, Roberti di Sarsina P. Black cohosh hepatic safety: follow-up of 107 patients consuming a special *Cimicifuga racemosa* rhizome herbal extract and review of literature. Evid Based Complement Alternat Med 2011;2011:821392.
[23] Agbabiaka TB, Savović J, Ernst E. Methods for causality assessment of adverse drug reactions: a systematic review. Drug Saf 2008;31(1):21–37. Review.
[24] Meyboom RH, Hekster YA, Egberts AC, Gribnau FW, Edwards IR. Causal or casual? The role of causality assessment in pharmacovigilance. Drug Saf Dec 1997;17(6):374–89.
[25] Stephens MD. The diagnosis of adverse medical events associated with drug treatment. Adverse Drug React Acute Poisoning Rev 1987;6(1):1–35.
[26] Macedo AF, Marques FB, Ribeiro CF, Teixeira F. Causality assessment of adverse drug reactions: comparison of the results obtained from published decisional algorithms and from the evaluations of an expert panel, according to different levels of imputability. J Clin Pharm Ther 2003;28:137–43.
[27] Théophile H, Arimone Y, Miremont-Salamé G, Moore N, Fourrier-Réglat A, Haramburu F, et al. Comparison of three methods (consensual expert judgement, algorithmic and probabilistic approaches) of causality assessment of adverse drug reactions: an assessment using reports made to a French pharmacovigilance centre. Drug Saf 2010;33:1045–54.
[28] Théophile H, André M, Arimone Y, Haramburu F, Miremont-Salamé G, Bégaud B. An updated method improved the assessment of adverse drug reaction in routine pharmacovigilance. J Clin Epidemiol 2012;65:1069–77.
[29] Auriche M. Bayesian approach to the imputability of undesirable phenomena to drugs. Therapie 1985;40(5):301–6.
[30] Péré JC, Godin MH, Bégaud B, Haramburu F, Albin H. Sensitivity and specificity of imputability criteria. Study and comparison of these efficacy indices for 7 methods. Therapie 1985 Sep-Oct; 40(5):307–12.
[31] Firenzuoli F, Gori L, Di Simone L, Morsuillo M. Important bias in the *Astragalus* meta-analysis. J Clin Oncol 2006;24:3215–6. author reply 3216–7.
[32] Firenzuoli F, Gori L, Mugelli A, Vannacci A. Current issues and perspectives in herbal hepatotoxicity: a hidden epidemic. Intern Emerg Med 2013;8:3–5.
[33] Gori L, Gallo E, Mascherini V, Mugelli A, Vannacci A, Firenzuoli F. Can estragole in fennel seed decoctions really be considered a danger for human health? A fennel safety update. Evid Based Complement Alternat Med 2012;2012:860542.
[34] Gaytán BD, Vulpe CD. Functional toxicology: tools to advance the future of toxicity testing. Front Genet 2014;5:110.
[35] Steinmetz LM, Scharfe C, Deutschbauer AM, Mokranjac D, Herman ZS, Jones T, et al. Systematic screen for human disease genes in yeast. Nat Genet 2002;31:400–4.
[36] Wood V, Gwilliam R, Rajandream MA, Lyne M, Lyne R, Stewart A, et al. The genome sequence of *Schizosaccharomyces pombe*. Nature 2002;415:871–80.
[37] Kiefer J, Yin HH, Que QQ, Mousses S. High-throughput siRNA screening as a method of perturbation of biological systems and identification of targeted pathways coupled with compound screening. Methods Mol Biol 2009;563:275–87.
[38] North M, Vulpe CD. Functional toxicogenomics: mechanism-centered toxicology. Int J Mol Sci 2010;11:4796–813.
[39] Oh J, Fung E, Price MN, Dehal PS, Davis RW, Giaever G, et al. A universal TagModule collection for parallel genetic analysis of microorganisms. Nucleic Acids Res 2010;38:e146.
[40] Giaever G, Chu AM, Ni L, Connelly C, Riles L, Véronneau S. Functional profiling of the *Saccharomyces cerevisiae* genome. Nature 2002;418:387–91.
[41] Römer M, Eichner J, Metzger U, Templin MF, Plummer S, Ellinger-Ziegelbauer H, et al. Cross-platform toxicogenomics for the prediction of non-genotoxic hepatocarcinogenesis in rat. PLoS One 2014;9:e97640.
[42] Waters MD, Jackson M, Lea I. Characterizing and predicting carcinogenicity and mode of action using conventional and toxicogenomics methods. Mutat Res 2010;705:184–200.
[43] Afshari CA, Hamadeh HK, Bushel PR. The evolution of bioinformatics in toxicology: advancing toxicogenomics. Toxicol Sci 2011; 120:S225–37.
[44] Auerbach SS, Shah RR, Mav D, Smith CS, Walker NJ, Vallant MK, et al. Predicting the hepatocarcinogenic potential of

alkenylbenzene flavoring agents using toxicogenomics and machine learning. Toxicol Appl Pharmacol 2010;243:300–14.
[45] Khan SR, Baghdasarian A, Fahlman RP, Michail K, Siraki AG. Current status and future prospects of toxicogenomics in drug discovery. Drug Discov Today 2014;19:562–78.
[46] Menniti-Ippolito F, Mazzanti G, Santuccio C, Moro PA, Calapai G, Firenzuoli F, et al. Surveillance of suspected adverse reactions to natural health products in Italy. Pharmacoepidemiol Drug Saf 2008;17:626–35.

LIST OF ABBREVIATIONS

HM Herbal medicine
HE Herbal extract
ADR Adverse drug reaction
RCT Randomized clinical trial

CHAPTER

8

Validation of Antiviral Potential of Herbal Ethnomedicine

Debprasad Chattopadhyay[1], *Durbadal Ojha*[1], *Supriya Mondal*[1], *Debayan Goswami*[1,2]

[1]ICMR Virus Unit, I.D. & B.G. Hospital, Beliaghata, Kolkata, India; [2]School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

OUTLINE

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00008-8

8.1 INTRODUCTION

Viruses are ultramicroscopic, acellular, metabolically inert nucleoprotein particles of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), with or without a lipid envelop [1,2]. They are obligate intracellular parasites, utilize the host's cell machineries to propagate, and cause ailments as benign as a common wart, as irritating as a cold, or as deadly as the bloody African fever [2]. Entry of a virus into the specific host cell depends on the precise attachment or fitting of the viral surface molecules with the molecules of the host cell. Each viral strain is unique in its surface antigenic structure, host cell receptors, and life cycle, and thus adopts various invasion strategies to infect every form of life. Due to their genetic variation, mode of transmission, effective replication, and the ability to persist within the host, viruses can cause widespread diseases from bacteria to humans [3–6].

Thus, the development of antiviral drugs requires great efforts. Several reports on the discovery of new drugs from traditional medicines [7–12] indicated the use of medicinal plants as a potential source for antiviral drug development [1,7–9]. Several reports indicated that a wide variety of phytochemicals, including alkaloids, flavonoids, terpenoids, polyphenolics, coumarins, lignans, have therapeutic applications [4–12] due to their interferences on viral entry or replication, along with their antioxidative properties [6,9,13]. Different in vitro and in vivo bioassays on phytochemicals lead to the identification of potential antiviral molecules. In this chapter, we will summarize the scientific approaches used to validate potential "antiviral leads" against selected genetically and functionally diverse viral families, from crude extracts to pure compounds. In-depth studies on some common in vitro antiviral assays with testing of efficacies, modes, and molecular mechanisms of action will be described here with in vivo methods against a few selected viruses.

Viral infection control strategies include (1) public health measures to minimize the risk of infection, (2) vaccination to the exposed individuals, and (3) antiinfective drugs for infected individuals. Public health measures such as safe drinking water can prevent Polio and Rota virus infections, while pest control can prevent arthropod and rodent-borne yellow fever, dengue, encephalitis, and hanta and arena viruses. However, to provide these benefits to the people of underdeveloped and developing world is a challenge. A second challenge is to discover means to control airborne respiratory infections. As public health measures are never totally effective, vaccination is a second strategy. Although vaccines dramatically decreased the incidence of polio, yellow fever, rabies, measles, mumps, rubella, and hepatitis B, they are not yet effective in human immunodeficiency virus (HIV), herpes simplex virus (HSV), hepatitis C virus (HCV), influenza, including common cold, caused by 100^{+} strains of Rhinoviruses. Developing a polyvalent vaccine against all these viruses is also a challenge. Moreover, Alpha and Filovirus (Ebola) cause devastating epidemics with sporadic outbreaks, and it is difficult to vaccinate the entire population to eradicate them. Thus, infection can and will occur despite the best effort of humankind. Hence, for antiviral drug development, scientists are looking into the features of an ideal antiviral drug that (1) effectively inhibits some essential viral processes, (2) prevents the development of drug-resistant viruses, (3) has broad activity (single drug against any of the 100^{+} common cold viruses), and (4) has minimum or no effect on the host system. The first feature is obvious while the remaining depend on the viral process being targeted.

8.2 RATIONALE FOR ANTIVIRAL DRUG DEVELOPMENT

Till 2014, only 37 antivirals are approved by the US Food and Drug Administration, although many viral diseases have no effective drug(s). Moreover, several viral diseases require long-term treatment that usually leads to the development of drug-resistant viruses. The existing antiviral *nucleoside analogs* act as antimetabolites in DNA synthesis and prevent viral replication in infected cells, and the related *nucleotide analogs* are used against hepatitis B virus (HBV), HCV, HSV, and HIV. In infected cells, these analogs are incorporated into the replicating DNA strand and phosphorylated as a chain terminator to stop viral DNA polymerase activity, and affect the mitochondrial DNA, leading to adverse effects such as bone marrow suppression. Another family of synthetic nucleoside analog reverse-transcriptase (RT) inhibitors help to design drugs with preferential activity, while some nucleoside analogs can act as nucleoside analog reverse-transcriptase inhibitors and polymerase inhibitors for HBV. Thus, antiviral *nucleotides analogs* repress viral reproduction by interfering with viral nucleic acid replication, compete with natural deoxynucleotide triphosphates (dNTP)/NTP substrates, and incorporate into the nascent viral nucleic acid leading to chain termination or mutagenesis (Table 8.1).

Extensive long-term clinical use of antivirals, such as acyclovir, the reference treatment for herpes viruses [14], results in the emergence of drug-resistant strains [15], due to mutations in viral *thymidine kinase* and/or DNA *polymerase* [16], along with renal impairment [17,18]. As antiviral drugs are virus specific and target oriented, they require appropriate diagnosis before therapeutic intervention. Another factor is the availability of suitable vaccines and their degree of effectiveness against a particular virus, as vaccination is the most effective approach for the prevention of a disease. However, in

TABLE 8.1 Some Nucleoside and Nucleotide Analogs and Their Antiviral Properties

Nucleotide	Analog	Incorporated to viral	Mechanism of viral inhibition
Vidarabine triphosphate	ATP	DNA polymerase	Unknown
Ribavirin triphosphate	GTP	RNA polymerase	Mutagenesis of genome replication
Acyclovir triphosphate	GTP	DNA polymerase	Chain termination of DNA synthesis
Zidoduvine triphosphate	TTP	Reverse transcriptase	CT of cDNA synthesis
Stavudine triphosphate	TTP	Reverse transcriptase	Do
Lamivudine triphosphate	CTP	Reverse transcriptase	Do
Nucleoside	**Analog**	**Incorporated to viral**	**Mechanism of inhibition**
Didanosine	Deoxyadenosine	HIV-RT	CT of viral cDNA synthesis
Abacavir, Entecavir	Deoxyguanosine	HIV-RT, HBV	Do
Telbivudine, zidovudine	Deoxythymidine	HBV, HIV-RT	Do
Emtrici- and Zalci-tabine	Deoxycytidine	HIV-RT and HBV	Do
Idoxuridine Trifluridine	Deoxyuridine	HIV-RT	Do

ATP, Adenosine triphosphate; GTP, Guanosine triphosphate; TTP, Thymidine triphosphate; CTP, Cytidine triphosphate; CT, Chain termination, (Dai L, Huang Q, Boeke JD. (2011). Effect of reverse transcriptase inhibitors on Line-1 and Ty1 reverse transcriptase activities and on LINE-1 retrotransposition. *BMC Biochemistry* **12**:18–28).

diseases such as hepatitis B, infected patients provide a large market for chemotherapy. Therefore, there is an unmatched need for readily available antiviral drugs at affordable prices with minimal side effects. Hence, traditional medicines are explored as novel antiviral agents, as many of these ancient medicaments, containing diverse plant metabolites, have potent antiviral activities [10–12,19,20].

8.3 DEVELOPMENT OF EFFECTIVE ANTIVIRAL DRUGS

Despite continuous advancement in medical science, viral diseases are a major cause of death around the globe. The development of an antiviral drug depends on the unique features of host–virus interactions, as viruses are acellular and host- and route-specific, and infect only by their nucleic acid(s) that regulate the host cell DNA for multiplication. Being an obligate intracellular parasite, viral replication depends on the metabolic pathways of the host. Thus, designing an effective antiviral drug to target virus-specific enzymes or replication steps without affecting the host cell is very difficult [20]. Advancement in molecular biology and genetics helps to design a rational approach for developing antiviral agents by targeting specific sites/steps of the viral life cycle, including (1) inactivation of the extracellular virus particles, (2) prevention of viral attachment or fusion, (3) entry or penetration, (4) replication of viral genome, (5) synthesis of viral-specific proteins or intermediates, and (6) assembly or release of new virions. However, the most desirable target is the inhibition of major viral enzymes essential for replication and spread of the disease. Most of the well-studied inhibitors of HIV, HSV, or Influenza viruses target the host cell binding (T-20, betulinic acid), uncoating of capsid (amantadine, pleconaril), viral replication (RT inhibitors: zidovudine, abacavir, nevirapine), viral enzymes integrase or DNA/RNA polymerase (acyclovir, cidofovir, ribavirin), proteinases of polyprotein precursors, and assembly or maturation (indinavir, ritonavir, rimantadine). On the basis of these strategies, several compounds were tested on different viruses, but only 40 licensed antivirals are approved till 2014 [1,5]. Moreover, studies on viral genomics, gene functions, and regulations led to the rational designing of gene-based drugs that induce protective antiviral immunity, interference with viral replication, gene expression, or viral messenger RNAs with limited delivery to the sites of replication and nuclease degradation [13].

8.4 MEDICINAL PLANTS AS A SOURCE OF ANTIVIRAL DRUGS: AN OVERVIEW

The history of human civilization revealed that almost all ancient cultures rely upon medicinal plants for treating ailments of the body and mind, and about 80% of the world population uses traditional medicine for primary health care [4]. The discovery of synthetic drugs with better efficacy and quick recovery in many diseases leads to several adverse effects during long-term clinical use, particularly in chronic or "difficult-to-treat" diseases. Thus, there has been a huge upsurge in the use of natural products for preventing and

treating serious viral diseases. However, most herbal products have no quality control, are unable to provide consistent results, and even produce undesirable effects. About 40% of the modern-day anticancer and antimicrobial drugs have their roots in traditional medicine [4,5,20], but till 2014, we have barely analyzed the tip of iceberg to design more drugs from plants. Hence, it is of utmost importance to search for phytochemicals with antiviral potential from unexplored plants used in ethnomedicine, before we lose our herbal resources due to rapid anthropogenic activities including industrialization and urbanization [1,8].

Many viruses have unique features in their structure or replication cycle that may act as the potential target for designing new antivirals, as evident with acyclovir (acycloguanosine), which blocks certain enzymes of herpes viruses responsible for triggering disease. Due to the structural diversity and broad range of bioactivities, ethnomedicines can serve as a source of complementary antivirals by inhibiting some specific enzymes or steps of viral replication or cellular factors (Table 8.2) of many DNA/RNA viruses [5].

Ayurveda and traditional Chinese medicine, in particular, use several medicinal plants to reduce disease severity, and many of them have antiviral activity [1,5,7,8,21]. 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 [4,5]. During the past 40 years, numerous broad-based screening programs for the antiviral activity of medicinal plants have been initiated using in vitro and in vivo assays. Out of 100 British Colombian medicinal plants, only a few showed activities against corona viruses, respiratory syncytial virus (RSV), para influenza, and HSV [22], while the marine algae *Spirulina* showed antimutagenic, immunomodulatory, and antiviral activities [23,24]. Interestingly, Cyanovirin N, an 11-kDa protein of blue green algae, inactivates HIV-1 by binding with its glycoprotein120 [25], while sulfated polysaccharides of seaweeds and alga showed anti-HIV and anti-HSV activities [26]. A list of selected medicinal plants and their compounds having antiviral activities against common viral diseases is presented in Table 8.3, and the structures of some important compounds are depicted in Figure 8.1.

TABLE 8.2 The Viruses and Their Viral and Cellular Targets for Antiviral Drug Development

Virus	Viral target	Cellular target
Parvo, Polyoma, Papiloma viruses	DNA polymerase	–
Adenovirus	DNA polymerase	Cellular factors
Herpesviruses	DNA pol, kinase, helicase	–
Poxvirus	DNA and RNA polymerase	4 Enzymes*
Hepadna virus	DNA polymerase (RT)	–
Picornavirus	Capsid RNA polymerase	–
Flavivirus	RNA polymerase	–
Arenavirus	RNA polymerase	4 Enzymes*
Bunya, Toga, Rhabdo Filovirus	RNA polymerase	4 Enzymes*
Hepacivirus	-Do-, helicase, protease	–
Orthomyxovirus	Matrix neuraminidase	–
Paramuxovirus	Fusion polypeptides	4 Enzymes*
Coronavirus	RNA poly, helicase, protease	–
Rheovirus	–	4 Enzymes*
Retrovirus	Protease, integrase, TAT	Integration-transcription factors

** Inosine 5′ monophosphate dehydrogenase, S-adenosylomocysteine hydrolase, Oritidine 5′-phosphate decarboxylase, Cytosine 5′-triphosphate synthetase.*

8.5 METHODS FOR THE VALIDATION OF ANTIVIRAL ACTIVITY OF PLANTS

In vitro assays are the preliminary step toward the identification of antiviral activity of a plant extract or phytocompounds. These assays measure the ability of a virus to infect and replicate in specific cell lines and the response of a particular extract toward the relevant virus infection. The cell culture system provides a rapid and less cumbersome method for the growth of viruses at higher titers, testing of cytotoxicity and antiviral efficacy, maintenance of cultures, and genetic manipulations. The cell lines characterized and routinely used for common viruses along with their respective methods are given in Table 8.4.

8.5.1 In vitro Assay

8.5.1.1 Indirect Assays

Indirect assays are the first step for screening a large number of extracts or phytocompounds at a time, as virus infection and multiplication result in a cytopathic effect (CPE) due to the release of infectious virion or induction of apoptosis. Inhibition of CPE in the presence of a test extract or phytocompound may be due to the inhibition of viral replication. Till 2014, various indirect assays have been developed, validated, and modified for individual viruses.

8.5.1.2 Visual Observation of CPE

Visual observation of CPE under an inverted microscope is usually carried out by infecting a semiconfluent cell monolayer with a susceptible virus in

TABLE 8.3 Partial List of Viruses Inhibited by Plants Used in Traditional Medicines

Virus	Medicinal plant	Family name	Isolated molecule	Antiviral effect	References
HSV	*Tanacetum vulgare*	Asteraceae	Parthenolide	Anti-HSV-1 and -HSV-2	[27]
	Ophiorrhiza nicobarica	Rubiaceae	Harmaline	Inhibit IE gene synthesis	[10,11]
	Rhus javanica	Anacardiaceae	Moronic acid	Anti-HSV-1 and -HSV-2	[28]
	Achyranthes aspera	Amaranthaceae	Oleanolic acid	Anti-HSV-1 and -HSV-2	[12]
	Odina wodier	Anacardiaceae	Chlorogenic acid	Prevent attachment	[29]
	Mallotus peltatus	Euphorbiaceae	Ursolic acid	HSV replication	[30]
	Terminalia chebula	Combretaceae	Chebulagic acid, punicalagin	Inhibit entry and cell spread	[31]
	Ilex asprella	Aquifoliaceae	Asprellanoside A, Oblonganoside H	Anti-HSV-1	[32]
	Azadirachta indica	Meliaceae	Sulfonoquinovosyldiacylglyceride	Anti-HSV-1 and -HSV-2	[33]
	Ficus benjamina	Moraceae	Quercetin 3-O-rutinoside, Kaempferol 3-O-robinobioside	Anti-HSV-1 and -HSV-2	[34]
	Artocarpus lakoocha	Moraceae	Oxyresveratrol	Inhibit viral replication	[35]
HIV	*Mimusops elengi*	Sapotaceae	Gallocatechin, Epigallocatechin	HIV-1 integrase activity	[36]
	Schisandra sphenanthera	Schisandraceae	Schisphendilactones A, B	Anti-HIV-1 activity	[37]
	Olea europaea	Oleaceae	Maslinic acid	Anti-HIV	[38]
	Stellera chamaejasme	Thymelaeaceae	Stelleralides A	Anti-HIV	[39]
	Artemisia annua	Asteraceae	Artemisinin	Anti-HIV activity	[40]
	Sargassum fusiforme	Sargassaceae	Palmitic acid	Inhibits HIV entry	[41]
	Melochia odorata	Sterculiaceae	Waltherione A	Inhibition of HIV P24	[42]
	Emblica officinalis	Phyllanthaceae	Curcumin	Anti-HIV activity	[43]
	Pelargonium sidoides	Geraniaceae	–	Blocks attachment of HIV-1, and prevent entry	[44]
Influenza virus	*Syzygium aromaticum*	Myrtaceae	Eugenol	Inhibit the activation of extracellular signal-regulated kinase, p38-mitogen-activated protein kinase, IκB kinase (IKK)/NF-κB signal pathways	[45]
	Silybum marianum L.	Asteraceae	23-(S)-2-Amino-3-phenyl-propanoyl-silybin	activate ERK/p38 MAPK and IKK pathways	[46]
	Ribes nigrum folium	Saxifragaceae	–	Antiinfluenza A	[47]
	Taraxacum officinale	Asteraceae	–	Antiinfluenza (H1N1)	[48]
	Caesalpinia sappan	Fabaceae	3-Deoxysappanchalcone	Antiinfluenza, apoptosis, and antiinflammation	[49]

Continued

TABLE 8.3 Partial List of Viruses Inhibited by Plants Used in Traditional Medicines—cont'd

Virus	Medicinal plant	Family name	Isolated molecule	Antiviral effect	References
	Vaccinium angustifolium, *Vitis vinifera* L, *Cinnamomum cassia*	Ericaceae, Vitaceae, Lauraceae	Procyanidin	Antiinfluenza A by inhibiting the formation of Atg5-Atg12/Atg16 heterotrimer	[50]
	Adenium obesum	Apocynaceae	Oleandrigenin-β-D-glucosyl l (1→4)-β-D-digitalose	Antiinfluenza A/PR/8/34 (H1N1)	[51]
	Angelica keiskei	Apiaceae	Xanthokeistal A	Neuraminidase inhibitor	[52]
	Melaleuca alternifolia	Myrtaceae	Terpinen-4-ol, terpinolene	Anti-A/PR/8 virus (H1N1)	[53]
Human RSV	*Rosmarinus officinalis*	Lamiaceae	Carnosic acid	Inhibit replication of RSV	[54]
	Gentiana lutea	Gentianaceae	–	Anti-RSV	[55]
	Cimicifuga foetida L.	Ranunculaceae	Cimicifugin	Inhibit viral attachment and internalization	[56]
HBV	*Artemisia capillaris*	Asteraceae	8-(Z)-Decene-4, 6-diyne-1, 3, 10-triol (1), 1, 3S, 8S-trihydro xydec-9-en-4, 6-yne (2)	Inhibit HBV DNA replication	[57]
	Aster tataricus L.	Asteraceae	Astataricusones, astataricusol A	Inhibit HBV DNA replication	[58]
	Swertia macrosperma	Gentianaceae	Swermacrolactones, luteolin	Inhibit secretion of HBV surface antigen	[59]
	Piper longum Linn.	Piperaceae	Piperine	Inhibit the secretion of HBV surface antigen	[60]
	Phyllanthus niruri L.	Phyllanthaceae	Nirtetralin A	Anti-HBV activities	[61]
HCV	*Morinda citrifolia*	Rubiaceae	Pyropheophorbide, pheophorbide	Inhibit entry and postentry steps of HCV	[62]
	Syncephalastrum racemosum	Syncephalastraceae	Ursolic acid	Anti-HCV activity	[63]
	Marrubium peregrinum L	Lamiaceae	Ladanein	HCV entry inhibitor	[64]
	Acacia nilotica	Fabaceae	–	Anti-HCV activity	[65]
	Vaccinium virgatum Aiton	Ericaceae	Proanthocyanidin	Inhibit HCV replication	[66]

PV	*Coffea arabica*	Rubiaceae	N-methyl-pyridinium formate	Anti-PV	[67]
	Baccharis gaudichaudiana	Asteraceae/ Compositae	Apigenin	Anti-PV type 2	[68]
	Heteropteris aphrodisiaca	Malpighiaceae	Aliphatic nitro compound	Inhibit PV type 1	[69]
	Dianella longifolia	Xanthorrhoeaceae	Chrysophanic acid	Inhibit PV 2 and PV 3 replication	[70]
	Pterocaulon sphacelatum	Asteraceae	Chrysosplenol C	Inhibit PV	[71]
VHSV	*Rhus verniciflua*	Anacardiaceae	Fisetin	Anti-VHSV	[72]
	Olea europaea L.	Oleaceae	Oleuropein	Inhibit VHSV replication	[73]
SARS-CoV	*Lycoris radiata*	Amaryllidaceae	Lycorine	Anti-SARS-CoV	[74]
	Glycyrrhiza glabra	Fabaceae	Glycyrrhizin	Anti-SARS-CoV	[75]
VSV	*Glycyrrhiza glabra*	Fabaceae	Glycyrrhizin	Inhibit phosphorylation enzymes and latency of VSV	[75]
	Calendula arvensis	Asteraceae	Oleanolic acid	Inhibit VSV multiplication	[76]
	Trichilia glabra L.	Meliaceae	–	Leaf extract inhibit VSV	[77]
Human ADV	*Glycine max*	Fabaceae	–	Inhibit ADV-1, Coxsackie B1	[78]
	Gentiana lutea	Gentianaceae	–	Against ADV-5	[55]
	Astragalus membranaceus	Fabaceae	Astragaloside IV	Inhibit ADV-3 replication	[79]
	Ficus carica	Moraceae	–	Inhibit replication	[80]
DEN	*Aedes aegypti*	Culicidae	Triptamine	Larvicidal	[81]
	Scutellaria baicalensis	Lamiaceae	Baicalein	Virucidal against DEN-2	[82]
	Azadirachta indica Juss.	Meliaceae	–	Leaf extract inhibit DEN-2	[83]

ADV, adenovirus; DEN, Dengue virus; PV, Poliovirus; SARS-CoV, severe acute respiratory syndrome coronavirus; VHSV, viral hemorrhagic septicemia virus; ERK, extracellular signal-regulated kinase, MAPK, p38-mitogen-activated protein kinase; IKK, IκB kinase.

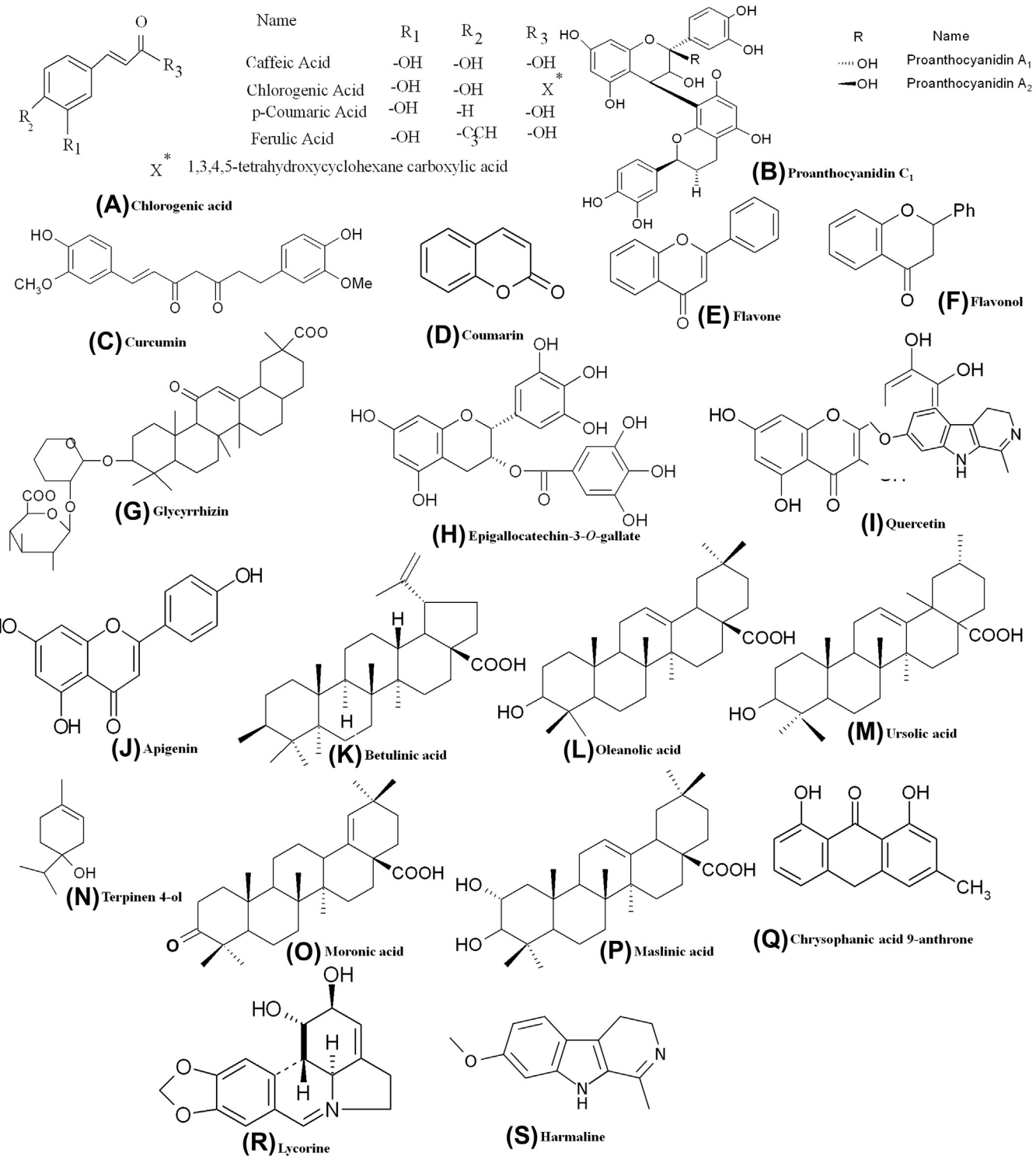


FIGURE 8.1 Structures of some important compounds.

the presence or absence of a test drug, in a microtiter plate (Figure 8.2).

This initial evaluation is usually done at a twofold concentration (with a 10-fold difference, e.g., 1 and 10 ng/ml or μg/ml). An 18 h culture (80–90% confluency) of an appropriate cell line should be added with the test drug or placebo, immediately after the virus infection. When the drug effect was tested as pretreatment, cells need to be treated with the test drug for 15–180 min before virus infection, and incubated

TABLE 8.4 In vitro and In vivo Models for Antiviral Assays Against Common Viruses

			In vitro assay			
Virus	**Disease**	**In vitro assay (Cell line)**	**Antiviral activity**	**Mechanistic study**	**In vivo model**	**In vivo methods**
HSV	Herpes	Vero, MRC-5, HFF, BHK, HepG2cells	MTT assay, plaque reduction assay, CPE reduction, $TCID_{50}$ [10,11,29,30]	IFA, ELISA, quantitative RT-PCR, Western blot, electrophoretic mobility shift assay (EMSA), Attachment assay, penetration assay [10,11,29,30]	Mouse, rat, rabbit guinea pig	Skin irritation test, cutaneous lesion assay, viral vaginitis assay, ocular HSV study, latency and reactivation study, footpad/ dorsal root ganglia model [10,11,29,30]
Influenza	Flu	MDCK, A549 cell lines	MTT, hemagglutination, and plaque assay [47]	Virus adsorption assay, Q-Rt PCR, Western blot, flow cytometry, IFA [47]	Mice, ferrets, Chicken	Treatment with extract/ compound, mouse monitoring, histology and immunohistology [47]
Polio virus	Paralysis, aseptic meningitis	Hela, Vero, Human Rhabdomyosarcoma cells	MTT, plaque reduction And luciferase assay [125]	EMSA, Q-RT PCR, Western blot, siRNA transfection [125]	Mouse, rat	Prophylactic and therapeutic efficacy, infectivity titers in brain/ spinal cord [111]
HBV	Liver inflammation, Jaundice	HepG2, HepG2.2.15, COS-7 cells	MTT, ELISA [126,127].	Q-Rt PCR, Western blot [126,127]	Mice, Ducklings	Infection and treatment, detection of duck hepatitis B virus-DNA, Histopathology of duck liver [126,127]
HCV	Liver cirrhosis	Huh7, Ava5 cells	Full-replication, replicon, and RdRp assay, $TCID_{50}$ [89,90]	Pseudoparticle infection assay, ELISA, Western blot [90].	Chimpanzee, Cynomolgus monkey	10 and 18-Day treatment schedule [90]
HIV	Acquired immunodeficiency syndrome	TZM-bl, MT-4 cells, Vk2/E6E7, lymphocytic reporter cell line	CPE reduction, TZM-bl and CEM-GFP cell-based assay [44, 85]	Quantitative RT-PCR, Virus infectivity assays, pulse-chase analysis, Western blot, siRNA transfection [85]	Transgenic mice, rabbits	Transgenic rat model, vaginal irritation study [112]
Rabies	Hydrophobia	McCoy cells	MTT assay, CPE reduction assay [128].	Confocal fluorescence Microscopy, Q-Rt PCR, Western blot [129]	Mice	Protection assay [113], determination of mortality rate [114]
DENV-2	Dengue fever	C6/36 mosquito and Vero cells	Foci forming unit reduction and prophylactic activity assay [130,131]	Quantitative RT-PCR, extracellular virucidal activity [130,131]	Mice	Virus inhibition assay [83]

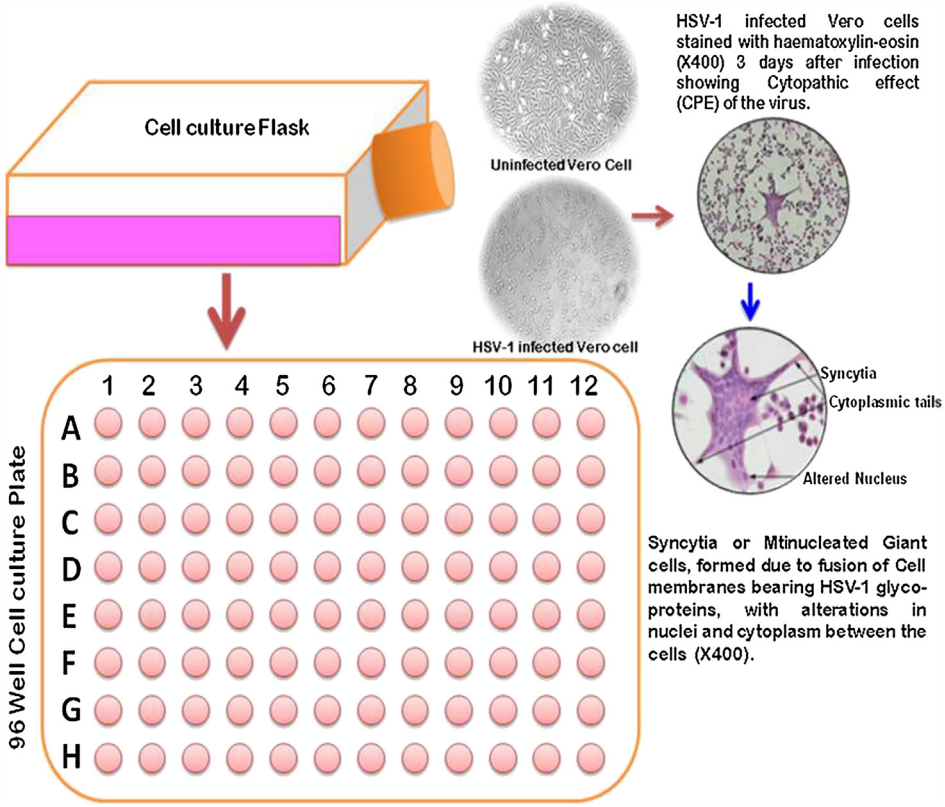


FIGURE 8.2 HSV-1 infected Vero (African green monkey kidney) cells showing CPE as the formation of Syncytia (multinucleated giant cell). This assay can be used to detect the antiviral activity of the test drug and cytopathicity can be expressed microscopically as 0 = No CPE; 1 = 0–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100%.

for a standard time to induce viral CPE. After every 24 h until the end of the experiment, the plate needs to be visualized microscopically for changes in cell morphology, compared to that for the control cells. Here, the uninfected cell monolayer treated with the test drug will provide an idea of the maximum dose of drug causing minimum cell toxicity, as enlargement, granularity, rounding off, detachment, etc., and the degree of cytotoxicity as T (100% toxic), PVH (partially very heavy toxic, 80%), PH (partially heavy toxic, 60%), P (partial toxic 40%), PS (partial slightly toxic, 20%), or 0 (nontoxic, 0%). A 50% cell inhibitory or cytotoxic concentration (CC_{50}) and 50% end point of virus inhibitory or effective concentration (EC_{50}) can be determined from a graph plotted with the concentration versus cellular effect due to virus infection, while the selectivity or therapeutic index (SI or TI) is calculated as CC_{50}/EC_{50}.

8.5.1.3 MTT or MTS Assay

Another rapid and sensitive in vitro assay for the evaluation of antiviral agents is based on the spectrophotometric assessment of the viability of virus-infected or mock-infected cells, via the in situ reduction of a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS); this has an equal sensitivity to that of the plaque reduction assay [10,11,84]. The mechanism is based on the conversion of a yellow water-soluble dye MTT or MTS to a purple colored insoluble formazan crystal by the mitochondrial enzymes of the viable cells. The quantitation of the amount of formazan produced in each well is then determined spectrophotometrically at 490 nm, and the same is subtracted from the background absorption of the plate at 650 nm. The cytotoxicity of the test drug needs to be evaluated in the same plate, and for data analysis, a statistical software program is used to determine the efficacy (EC_{50}), cell toxicity (CC_{50}), and SI or TI of the test drug. This method allowed the screening of a larger number of extract/compounds at the same time with a simplified procedure.

Protocol: Cell Monolayer (100–200 µl) cultured in 10 Columns (C2–11) of a 96-well flat-bottom plate ($\sim 2 \times 10^4$ cells/ml) needs to be added at a twofold dilution of the test drug, after 24 h (eight concentrations in serum-free medium), the medium is removed and

added with the virus (0.5–5 multiplicity of infection (MOI)) in six wells of C3–11 (Rows 3–8). Then, C-2 (uninfected Control) and Rows 1–2 of C3–11 are added with the media and after adsorption (45–60 min) at 37 °C, unabsorbed viruses are removed by washing with fresh media. The infected cells should then be mixed with the test drug (0—highest concentration) in all wells of C3–11, and incubated (37 °C in a CO_2 incubator) for 24–48 h. After removing the drug-containing media, 50 µl of MTT solution (5 mg/ml in phosphate buffered saline (PBS)) should be added with the fresh media (200 µl) to all wells in C1–11, and incubated for 3–4 h at 37 °C. Finally, the medium should be replaced with dimethyl sulfoxide (DMSO; 200 µl) to dissolve the formazan in C1–11, and after 15 min, glycine buffer (25 µl) should be mixed in all wells (pH 10.5) and the absorbance should be read (570 nm). Usually, the results are calculated by plotting a graph with absorbance (*Y*-axis) versus drug concentration (*X*-axis). Here, Column 2 with no virus serves as the cell viability control and wells in Column 3 with virus without the drug serve as the virus-induced loss of cell viability. Rows 1–2 of Columns 3–11 with an increasing concentration of the drug without virus provide IC_{50} (50% cell inhibitory concentration) of the drug, while EC_{50} or EC_{90} can be deduced by plotting absorbance of virus-infected wells (Column 3, rows 3–8) versus concentration of the drug showing an increased absorbance by 50 or 90% over the virus alone [29].

8.5.1.4 TZM-bl Cell-Based Assay

This neutralizing antibody assay, used for HIV-1, simian immunodeficiency virus (SIV), and simian-HIV, is done in TZM-bl cells as it reflects the reduction in Tat-induced luciferase (Luc) reporter gene expression after a single round of virus infection. TZM-bl cells are HeLa cell clones, engineered to express cluster of differentiation 4 and C–C chemokine receptor type 5, and contain integrated reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under the control of an HIV-1 long terminal repeat. Thus, they are highly sensitive and prone to infection by immunodeficiency viruses including primary HIV-1 isolates and cloned Env-pseudotyped viruses. Here, diethylaminoethyl (DEAE) dextran is used to enhance infectivity during neutralization. Soon after infection, reporter gene expression is induced by a viral transactivator protein *Tat*. Luciferase activity can be quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inoculum [85]. This high-throughput assay requires a 96-well plate, and clonal cells provide enhanced precision and uniformity. This has been validated for a single-round infection with either uncloned viruses grown in human lymphocytes or molecularly cloned Env-pseudotyped viruses produced by transfection in 293T/17 cells [85].

Protocol: TZM-bl cells (4×10^4/well) are usually seeded in a 24-well plate and incubated overnight. In a separate vial, HIV-1NL4.3 (MOI 0.05) virion is mixed with the test drug or vehicle for 1 h at 37 °C, and then added to TZM-bl cells, and incubated for 4 h. After washing the cells (with cold PBS), fresh media with the test drug should be added and cultured for 48 h, using untreated HIV-1 infected cells (negative) and azidothymidine (AZT)-treated cells (positive) as a control. The cells then need to be washed twice with PBS, lysed with 1X lysis buffer, and the supernatant added with the substrate can be analyzed for luciferase activity in an optiplate using a fluorimeter. The results are expressed as percentage inhibition as luminescence in the experimental group (test drug or AZT)/luminescence of infected cells without the drug x 100; and percent inhibition can be calculated by subtracting the above value from 100 [44,85].

8.5.1.5 CEM-Green Fluorescent Protein Cell-Based Assay

CEM-green fluorescent protein (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, the cells ($2.0x10^5$/well) should be incubated with the test drug up to 8 days, using AZT and solvents (used to prepare the test drug) as control(s). The virus-infected cells are then lysed with 1x Promega cell lysis buffer (150 µl), and transferred to culture plate to read the absorbance at 485 nm (excitation) and 520 nm (emission) by means of a fluorimeter. The results can be expressed as percentage inhibition: GFP fluorescence in the experimental group/fluorescence in infected cells without the test drug X 100. Percent inhibition should be calculated by subtracting the above value from 100 [85].

8.5.1.6 Virus Yield Reduction Assay

This is generally used to confirm the results of the CPE reduction or inhibition assay with the freshly prepared test drug that showed activity in the initial experiments. After 3–5 days of the test, the cells are lysed by a freeze–thaw cycle, to elute the virion, centrifuged ($10{,}000 \times g$), and the resulting supernatants with increasing drug concentration are titrated for infectious virion and subjected to a fresh CPE inhibition test. Briefly, cells plated in a 96-well plate with susceptible cell lines need to be incubated for 24 h, and then added with the serially diluted virus preparations from drug-treated cells. Development of CPE indicates the presence of infectious virus, and 90% effective concentration (EC_{90}) of the test drug is determined as the concentration that inhibits virus yield by one $\log_{10}$. Infectious virus yield assay, pretreated or posttreated with the test

drug (reduced virus infectivity by 90% or $TCID_{50}$/ml) can be calculated from this assay.

Secondary testing of potential antiviral compounds: The antiviral activity of the test compound is expressed as a TI or SI, determined by dividing CC_{50} by EC_{50}. In general, an SI of $\geq$10 is considered as a potential antiviral agent, although a low SI for the positive control needs to be considered. Compounds having SI values of $\geq$10 need to be evaluated against additional virus strains to establish the full spectrum of activity, and the potential antiviral drug needs to be screened further by direct assays to measure viral replication or titer inhibition [1].

8.5.1.7 Direct Assays

Direct assays can measure $\geq$50% reduction in the viral titer in the presence of the test drug, compared to the untreated cells. CPE inhibition can be determined by end point titration [86], which evaluates the virucidal activity after preincubation with the virus plus test compound [87,88]. Fifty percent end point titration is done on confluent monolayers (10^4 cells/well) infected with serial 10-fold dilutions (10^7 $TCID_{50}$/ml) of the virus suspension in 96-well plate. After adsorption for 1 h at 37 °C, the test drug (serial twofold dilutions) is added (to the maintenance medium with 2% fetal bovine serum (FBS) plus antibiotics), and incubated at 37 °C to record the viral CPE under a light microscope after 4–5 days with virus control, uninfected drug-treated and untreated cell controls. Cytotoxicity of the test agent is the concentration that killed cell monolayers when no virus titer can be determined, while the antiviral activity is the inhibition of the virus titer at the maximum nontoxic dose of the highest concentration of the test drug without affecting cell morphology. Antiviral activity needs to be present in at least two subsequent dilutions of the test agent; otherwise, the activity is only virucidal or is due to the toxicity of the test drug. Extracellular virucidal activity can also be determined by titration of residual infectious virus particles after incubation of the test drug with virus suspension (10^6 $TCID_{50}$/ml) for 1 h at 37 °C.

8.5.1.8 Immunofluorescence Assay

This assay is used for the quantitative estimation of viruses against which antibodies are available, either commercially or in-house, but it is unable to differentiate between infectious and noninfectious viral particles. Briefly, the untreated or drug-treated cells are infected with a known amount of virus (MOI 5–10). After adsorption (45–60 min), the unabsorbed virus particle is removed by washing, and then added with the fresh media to incubate for 24–36 h (50% of time required to achieve 3^+ CPE). Then, the cells need to be rewashed with PBS, fixed with 3–4% paraformaldehyde, washed, and permeabilized with acetone or 0.5% triton X-100. After washing, the cells should be blocked with 1% bovine serum albumin (BSA) in PBS for 30 min followed by incubation with mouse or rabbit antibody against a specific viral protein for 1–4 h at 37 °C. After repeated washing, the cells need to be incubated with a fluorescent-tagged secondary antibody for 1 h, washed, and visualized under a fluorescent microscope compared to the fluorescence of untreated and drug-treated cells. Alternatively, for quantitation, the cells are trypsinized after treatment, fixed with 4% paraformaldehyde, washed, permeabilized, and then labeled with a fluorescent-tagged antibody, followed by propidium iodide (PI: 50 μg/ml in PBS). The counting of cells is done in a fluorescent-activated cell counter to quantitate the fluorescence percentage. Here, the measure of PI will indirectly measure cytotoxicity caused by drug treatment or virus infection [10,11,30].

8.5.1.9 Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) is a solid-phase rapid, sensitive, and specific assay used for the qualitative detection of the viral antigen or antibody and gross quantitation of the virus. Absolute quantitation is done by a series of predetermined viral titers in ELISA matched with unknown samples that estimate the quantity of the virus (untreated or drug-treated). As this method cannot differentiate between infectious and noninfectious virus particles, the drug affecting at the earlier or later stage of viral replication or maturation will not be differentiated. Briefly, the untreated or drug-treated cells are infected with a known amount of virus, adsorbed for 1 h, washed and incubated (2–4 days) for CPE. The virus stock harvested after freeze thawing needs to be centrifuged, and diluted for ELISA. Each well of a plate coated with virus-specific antibody needs to be mixed with 100 μl of controls or test drug and incubated (1 h, at room temperature) with Horseradish peroxidase conjugate (100 μl), alkaline phosphatase, or β-D-galactosidase-labeled virus-specific antibody. After washing five times, the substrate (100 μl) is added and reincubated in the dark for 10 min. The reaction is then stopped with a stop solution (5% H_2SO_4) and the absorbance is read (optical density $(OD)_{450}$). Alternatively, a quadruplicate cell monolayer in 96-well plates is overlaid with a $\log_{10}$ dilution of the test drug followed by infection with the virus. After 16–20 h of incubation at 37 °C, monolayers are fixed with 0.05% glutaraldehyde and assayed for virus-specific protein(s) on the cell surface. An ELISA is performed with monoclonal antibody to the specific protein of the corresponding virus and protein-A horseradish peroxidase conjugate, and the OD is measured at 450 nm. The results are expressed as a percentage of virus-infected cells (virus control), and the concentration causing 50% reduction in OD

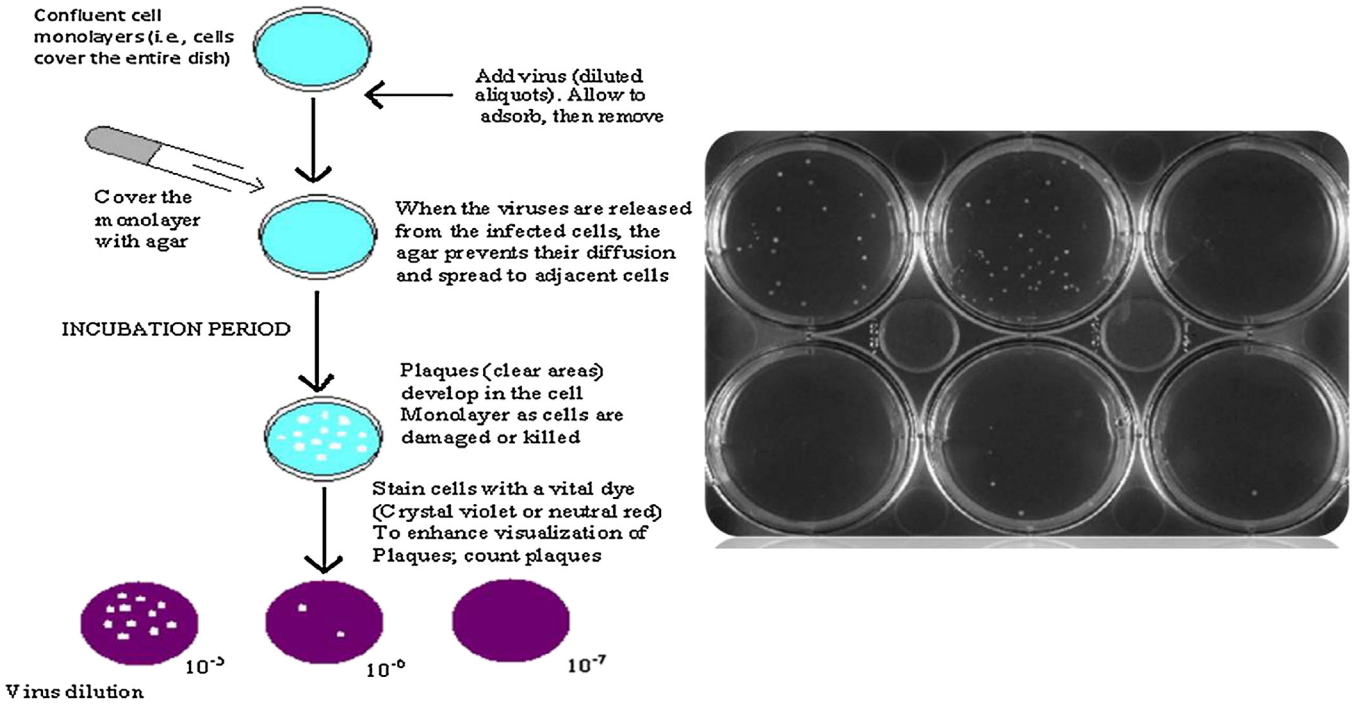


FIGURE 8.3 Schematic representation and pictures of viral plaque assay.

values (EC_{50}) should be calculated from graphic plots, by the determination of SI (ratio of CC_{50}:EC_{50}) [1,10,29].

8.5.1.10 Plaque Reduction Assay

The plaque reduction assay, a phenotypic assay, can be used to determine the IC_{50} values of drugs and to determine inhibitor sensitivity (e.g., neuraminidase). In this assay, each infectious virus particle multiplies to form a localized area of infected cells or "plaque." The plaques are revealed either as areas of dead/destroyed cells detected by cellular stains or as areas of infected cells by immunostaining (Figure 8.3).

Protocol: Here, the confluent (80–90%) cell monolayer (1×10^5 cells/cm^2) is infected with a $\log_{10}$ dilution of viral plaque-forming unit (PFU) in the presence or absence of the test drug, allowed to adsorb (1 h at 37 °C in 5% CO_2), and then the cells are washed twice with prewarm minimum essential medium (MEM). Drug dilutions prepared in the overlay medium are then overlaid on the infected culture, without the test drug. Overlay medium I comprises MEM/Dulbecco's modified Eagle's medium (DMEM) with trypsin (10 μg/ml), 1% low melting agarose, without serum and the test drug. Overlay medium II comprises: 100 ml 10x MEM, supplemented with glutamine (10 ml), antibiotics (10 ml), bicarbonate (40 ml), and FBS (20 ml). Usually, 45 ml of carboxy methyl cellulose is added to 9 ml of the medium, and the plates are incubated (37 °C in 5% CO_2) for 3–5 days and then fixed with 10% formalin or 4% formaldehyde for 30 min. The cells are then stained with methylene blue (1 ml/well) or 1% crystal violet (w/v), and rinsed with tap water, allowed to dry overnight, and the plaques (dark areas) are counted by low power magnification of a binocular microscope. The antiviral effect is usually measured as the percentage inhibition of plaque formation: [(mean number of plaques in control)−(mean number of plaques in sample)] × 100/Mean number of plaque in control. The concentration of the test drug required to inhibit up to 50% of virus growth (IC_{50} or EC_{50}), as compared to the virus control, is estimated from the graphical plot as dose–response curves by regression analysis.

8.5.1.11 Hemagglutination Assay to Measure Viral Titer

The hemagglutination activation (HA) assay is a quantitation of the viral surface or envelop hemagglutination protein of some viral families that can agglutinate (stick to) human or animal red blood cells (RBCs) and bind to its *N*-acetylneuraminic acid to form a lattice. In contrast to the plaque assay, HA cannot measure of viral infectivity, because no virus replication is required in this assay. It is an easy, simple, and rapid method for large samples, and the conditions depend on the type of virus, as some viruses bind RBCs only at certain pH values, others are at certain ionic strengths. If a test drug inhibits virus replication, it will also affect the viral titer and will thus reduce the HA value. Briefly, the virus dilution (1:4–1:512) is applied to an RBC dilution (0.1–0.7% for

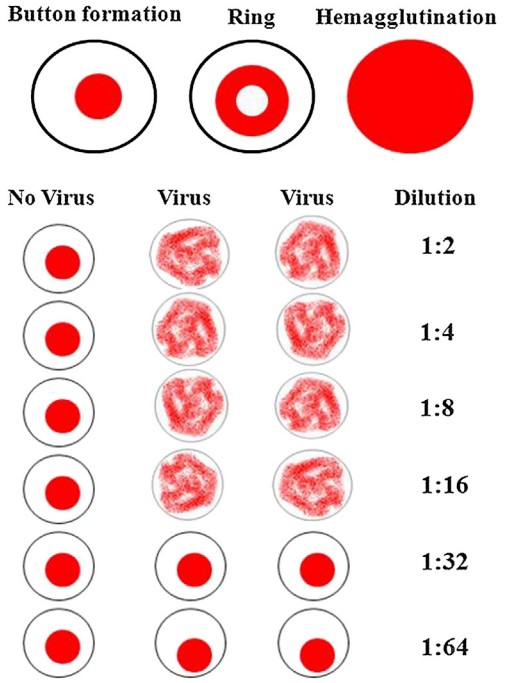


FIGURE 8.4 HA showing viral HA units. Lanes 2–3 has 1: 16 HA units.

30 min at 4 °C, because the viruses with neuraminidase activity will detach from the RBCs, and then the lattice-forming parts will be counted to calculate the titer as Virus concentration/ml = 10^7 X HA titer.

Protocol: The RBCs separated from blood is collected in Alseiver's solution (20.5 g dextrose, 8.0 g sodium citrate dihydrate, 4.2 g sodium chloride, and 0.55 g citric acid per liter, pH 6.1) and kept overnight at 4 °C. The cells are washed with PBS (2X), and the quantity of the cell pack (2.0 ml blood yields 0.5–1.0 ml pack) is measured in 10% suspension with PBS. The RBC solution (0.75%) in PBS (0.75 ml 10% RBCs in 10 ml PBS, pH 7.2) is added to each well of a 96-well plate, except for the first well of each row, along with the antigen (culture fluid containing virus from drug-treated and untreated controls) to the first two wells of each row. A twofold dilution (transferring 50 µl from the second well of each column A2-K2 to A3-K3) is mixed with 0.75% RBCs (50 ml) in all wells, and incubated for 60 min at room temperature. The cell control needs to be checked for the complete settling of the RBCs, and the results are recorded in the HA sheet. The RBCs form a button or ring at the bottom of the wells that is recorded as "O," while hemagglutination (RBCs remain in suspension) is recorded as a + symbol. The highest dilution of virus that causes complete hemagglutination is considered as the end point. The HA titer is the reciprocal of the dilution of the virus in the last well with complete hemagglutination (Figure 8.4).

Inhibition of virus hemagglutination activity (HA): Viruses having surface HA proteins, such as influenza, are able to agglutinate RBCs, which can be visualized by mixing virus dilutions with RBC, and can thus, be used to investigate the inhibitory effect of any drug onto the HA. Briefly, the 10-fold serially diluted (1–1000X) test drug, along with the diluted virus stocks (1:4 to 1:128), is used. The virus dilution (50 µl/well) is added to drug-containing wells, preincubated for 45 min, and need to be mixed with RBC (1/20 in PBS) solution. Here, up to a certain dilution, the viral particles may lose their ability to agglutinate RBCs, which indicates an interaction of the drug with the viral HA.

8.5.1.12 Virus Inactivation Assay

The virus (10^4 PFU/ml)–test drug mix is usually incubated for 1 h at 37 °C, and then diluted 100-fold (100 PFU/well) with media containing 2% FBS to get a subtherapeutic concentration of the test drug. Then, the monolayer, seeded in the 12-well plate, is mixed with the virus inoculums. For comparison, virus–test drug mix diluted 100-fold (no incubation period), are added with the respective cells for infection. The 100-fold dilution helps to titrate the test agent below its effective doses and prevent meaningful interactions with the host cell surface. After adsorption for 1 h at 37 °C, the diluted inoculums are discarded, and the cells washed with PBS, should be added with an overlay medium (with 2% FBS), and incubated at 37 °C for 72 h before being subjected to the plaque assay, and the viral plaque numbers obtained from infections set in the presence of the test drug are compared with the control [29].

8.5.1.13 Attachment Assay

Viral attachment to the host cell surface can be assayed at 4 °C, as it allows binding but prevents viral entry, by ELISA [31]. Briefly, 96-well plates seeded with susceptible cells (2×10^4 cells/well) are grown overnight, and the cell monolayers chilled at 4 °C for 1 h, are challenged with the virus (MOI 5) in the presence of the test drug using heparin as the control, for 3 h at 4 °C. The wells are then washed with ice-cold PBS, fixed with prechilled 4% paraformaldehyde in PBS for 1 h on ice, and blocked with 5% BSA at 4 °C. To detect bound virus, the samples are incubated at 37 °C for 1 h with a primary antibody in PBST (PBS with 0.05% Tween 20) plus 5% BSA. The wells are washed twice with PBST plus 5% BSA and twice with PBST only, each at 5-min interval on a shaker, and mixed with secondary antibody in PBST with 5% BSA. After incubation (37 °C for 1 h), the wells are washed and developed with a 3,3′,5,5′-tetramethyl-benzidine two-component microwell peroxidase substrate for 20 min and the reaction is stopped with 1 M phosphoric acid. The absorbance is read immediately at 450 nm, and the

values are expressed as the fold change of absorbance relative to the mock infection control [11,31].

8.5.1.14 Penetration Assay

Cell monolayers grown in 12-well plates are chilled at 4 °C for 1 h and incubated with the virus (100 PFU/well) for 3 h at 4 °C. The infected cell monolayer is then reincubated with the test drug, or heparin (100 μg/ml), for 20 min at 37 °C to facilitate penetration. Then, the extracellular virus is inactivated by citrate buffer (pH 3.0) for 1 min, and washed with PBS before being overlaid with DMEM containing 2% FBS. After 48 h of incubation at 37 °C, viral plaques are stained and counted [31].

8.5.1.15 Virus Adsorption Assay

The plated cells (0.8×10^5 cells/well for a 12-well plate) grown overnight at 30% confluence are added (300 μl) with virus dilution, and DEAE dextran at a final concentration of 20 μg/ml. After adsorption (2 h at 37 °C in CO_2 incubator), the plates are placed in a rocker, to prevent the cells from drying, and fresh medium (1−2 ml) containing the test drug is added to each well and incubated for 40−48 h in 5% CO_2 at 37 °C, for subconfluent growth. After removing the media, fixing solution (1−2 ml) is added to each well and incubated for 5 min at room temperature (β-galactosidase activity decreases dramatically if the fixing solution is left for >10 min). Then on discarding the fixing solution, the cells are washed twice with PBS, stained, and incubated at 37 °C for 50 min. Finally, the plates are stained to count the number of blue syncytia, and the titration values are expressed as the number of stained cells multiplied by the viral dilution.

8.5.1.16 Replicon Assay

Cells harboring viral subgenomic replicon are usually maintained with 0.25 mg/mL G418, and the viral replicon cells should be seeded in a 96-well plate and incubated at 37 °C in 5% CO_2. After 24 h, the culture medium needs to be replaced with a medium containing the serially diluted test drug with 2% FBS and 1% DMSO and incubated for 72 h. Then, the total RNAs are extracted, and the RNA levels are quantified by a quantitative real-time polymerase chain reaction (qRTPCR), using a real-time PCR system with specific primers, and glyceraldehyde-3-phosphate dehydrogenase gene as control. Antiviral activity can be determined by RNA levels in drug-treated cells as compared to that in mock-treated cells [89].

8.5.1.17 Pseudoparticle Infection Assay

This assay requires HIV-1 based pseudoparticles containing HCV E1E2 or vesicular stomatitis virus (VSV) glycoprotein G. Pseudotyped viruses added to Huh7.5.1 cells are incubated for 3 h at 37 °C, and mixed with the test agent 15 min prior to virus addition. After removing the supernatant, the cells are incubated at 37 °C for 72 h and measured for luciferase activity [90].

8.5.2 Studies on the Mechanism of Action

The mechanism of action of a test drug can be predicted from the studies of addition and removal of the drug along with inhibition of preinfection, coinfection, and postinfection, as depicted in Figure 8.5.

Based on the time when the test drug yielded maximum inhibition, a mechanistic study can be done by targeting each and every step (surface glycoproteins for fusion and entry, different proteins, transcriptional and translational events for gene expression) separately, using several modern methods.

8.5.2.1 Confocal Fluorescence Microscopy

Cells adsorbed on a microscopy-adapted slide for 6 days will be infected with the virus in the presence or absence of the test drug for 3 h. The cells are then washed and incubated with or without polyclonal anti-gp160 (50 μg/ml) antibodies at 4 °C for 30 min, rewashed with PBS, 0.01% azide, 0.5% BSA, and labeled with polyclonal mouse antihuman immunoglobulin G−fluorescein isothiocyanate (FITC), and then fixed with 1% paraformaldehyde. Cover slides need to be mounted in Mowiol to be observed by sequential acquisition using a microscope with planapochromat ×63, 1.4 numerical aperture oil immersion objectives, and optical sections at an image resolution of 512 × 512 pixels [85].

8.5.2.2 Quantitative Real-Time PCR

Real-time PCR is widely accepted for viral load determination due to its rapidity, low interassay and intraassay variabilities, sensitivity, reproducibility, and reduced risk of carryover contamination. The method involves the direct measurement of the amount of PCR product obtained, even during the reaction. Mathematical analysis of the data, and comparison to control reactions containing known amounts of template, allows the calculation of the input DNA amount in the initial reaction. The severity of some diseases can be correlated well with the viral load, making real-time PCR quantitation useful, not only the presence of a virus but also the viral reactivation or persistence in disease progression [91−93]. This property is successfully adapted to screen potential antiviral compounds in vitro, where the fold decrease in viral genetic material in the presence of drug will measure its antiviral activity.

8.5.2.3 Quantitative Reverse Transcription-PCR

Briefly, cDNA prepared from the respective RNA virus by an RT kit is subjected to quantitative real-time

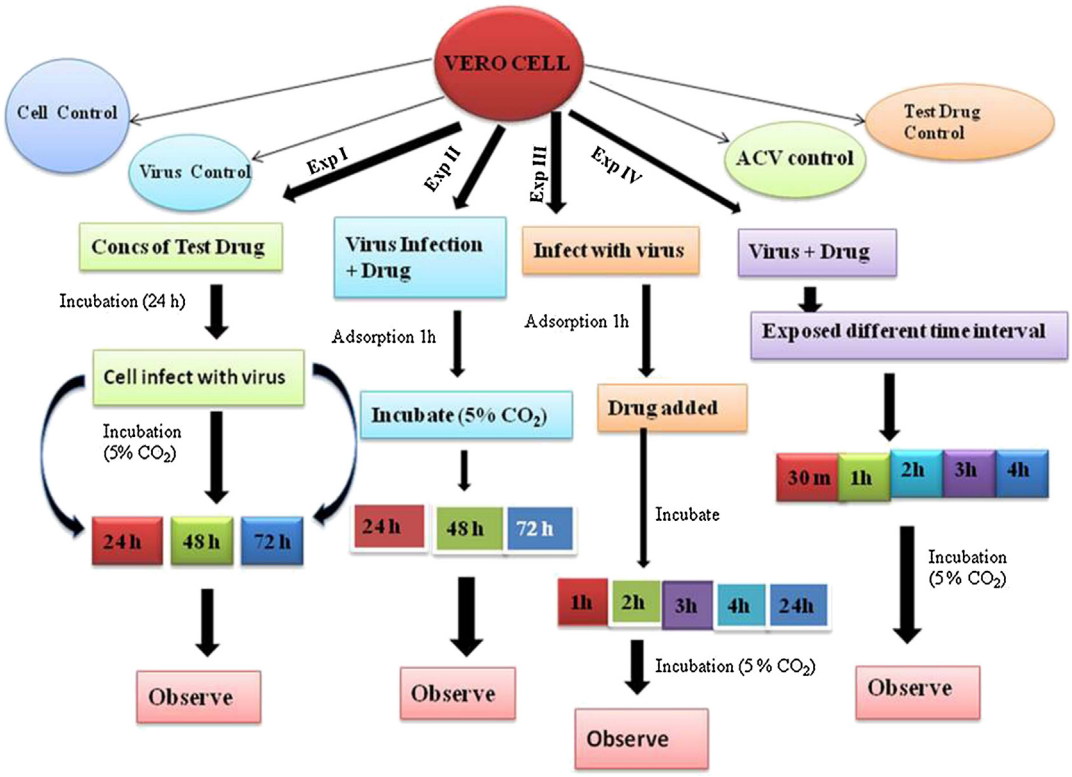


FIGURE 8.5 Schematic protocol of time of addition and removal along with preinfection, coinfection, and postinfection assays of drugs against viruses grown in Vero cells (e.g., Herpes simplex virus, Dengue).

PCR using cDNA (5 µl) in a volume of 25 µl with a TaqMan1 PCR Core Reagent Kit. Primers and probe are used at the optimum concentration. For PCR amplifications, carried out as per the manufacturer's instructions, RNA/DNA of the known virus serves as the positive control, and water serves as the negative control. Amplification of target DNA and detection of PCR products are performed with a GeneAmp1 5700 Sequence Detection System. Amplification of the target sequence is detected by an increase in fluorescence above a baseline with no or little change in fluorescence. To analyze the data, the reporter fluorescence is automatically normalized to a passive reference to avoid the measurement of non-PCR-related fluorescence [94]. A threshold is set above the baseline to get a threshold cycle value (Ct), which is the cycle number at which the fluorescence passes the fixed threshold with a statistically significant increase in fluorescence. The qPCR amplification standard curve for the test agent is designed from Ct values versus the log of standard concentrations [95–97].

Calculation and interpretation of antiviral effect: A higher Ct value corresponding to the drug-treated sample, compared with the untreated one, is the antiviral effect of the drug when the viral load ($\times 10^6$ particles or fold decrease) calculated from the standard plot of Ct values is less in the drug-treated sample than in the untreated one. From these data, IC_{50} or IC_{90} (50 or 90% reduction) of viral particles or of fold change may also be calculated.

8.5.2.4 Western Blot Analysis

The corresponding cell lines (1×10^6 cells/well) at an 80–90% confluence are infected with a five concentrations of $\log_{10}$ dilution of PFU of the respective virus, in the absence or presence of a test drug. The infected cells were incubated for 24 h at 37 °C in 5% CO_2, and equal amounts of protein (40 µg/sample) extract from whole cells are harvested in buffer (200 µl/well) containing 20 mM Tris (pH 7 ± 0.5), 50 mM NaCl, 5% nonyl phenoxypolyethoxylethanol-40 (NP-40%), and 0.05% deoxycholate (DOC), added with 2X sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1:1) and heated to 100 °C for 5 min. The gel washed with SDS-PAGE running buffer is then loaded with the above sample and run at 100 V through the stacking part at 200 V, after the proteins move through the stack and migrate through the resolving gel (until the blue dye front reaches the end of the glass plates). After soaking a PVDF membrane in methanol (30 second) and distilled water the membrane will be placed in between two fiber pads and four Whatmann

papers (precut) in a shallow tray, fitted with a Transfer buffer for a few minutes. After cutting off the stacking gel and soaking in the transfer buffer (few min), the gel is transferred in gel cassettes to prepare the transfer sandwich, cover it, and insert the gel cassette into the electrode module and the bioice cooling unit into the buffer chamber (filled with the transfer buffer), and allow 1−2 h at 4 °C at 100 V. The gel is then stained with 1X Pongee S for 1 min, destained with ddH_2O, rinsed with PBS, and the membrane is incubated with blocking buffer on a shaker for 1−2 h at 37° or at 4 °C overnight. The membrane is then incubated with the diluted primary antibody (in dilution buffer) on a shaker for 1 h at 37 °C or overnight at 4 °C, washed four times with washing buffer (5−10 min). The membrane is then incubated with the diluted secondary antibody (in blocking buffer) on a shaker for 1 h at 37 °C or overnight at 4 °C, washed four times with washing buffer for 5−10 min, and visualized using the commercial enhanced chemiluminescence Western blot detection kit.

8.5.2.5 Pulse-Chase Analysis

This examines a time-dependent cellular process by successive exposure of the cells to a labeled compound (pulse) and then in an unlabeled form (chase), by radioactivity. Corresponding cell lines (1×10^6 cells/well) cultured in a six-well plate at an 80−90% confluency is infected with five concentrations of $\log_{10}$ dilution of viral PFUs, either in the absence or presence of a test drug. The infected cells are incubated for 24 h at 37 °C in 5% CO_2, and then equal amounts of protein (40 μg/sample) extract from whole cells are harvested in buffer (200 μl/well) containing 20 mM Tris (pH 7 ± 0.5), 50 mM NaCl, 5% NP-40%, and 0.05% DOC. Synthesized protein labeled with Click-iT Metabolic Labeling Reagents and 293T cells transfected with pNL4-3 are used. At 3 h posttransfection, the cell supernatant is replaced with fresh medium with or without 10 μM fangchinoline. After 24 h, the cells need to be labeled with azidohomoalanine (AHA; 50 μM) for 1 h in methionine-free medium with or without fangchinoline. The supernatant is then replaced with complete medium in the presence or absence of fangchinoline, and the cells should be chased for the indicated time, after being lysed in lysis buffer (50 mM Tris HCl pH 8.0, 1% SDS, protease inhibitor). The AHA-incorporated protein is then biotinylated by the Click-instant Protein reaction buffer Kit and Biotin Alkyne. After precipitation and dissolution, the biotinylated protein needs to be collected with Dynabeads MyOne Streptavidin T1, and the purified nascent protein will be eluted into the SDS-PAGE buffer by boiling for Western blot analysis [85].

8.5.2.6 Flow Cytometry

The cells (10^6/well) were first dissociated by a nonenzymatic cell dissociation buffer, and then infected with the virus (MOI 1) in the presence or absence of the test drug for 1 h at 37 °C, using DMSO (0.1%) as control. The cells are washed twice with ice-cold fluorescence-activated cell sorting (FACS) buffer (1X PBS, 2% fetal calf serum (FCS), and 0.1% sodium azide), need to be blocked with 5% FCS for 30 min on ice, and then stained with an FITC-conjugated antibody, washed with FACS buffer, and then fixed with 1% paraformaldehyde before being subjected to standard flow cytometry analysis. Normal rabbit serum can serve as the isotype control, while the data acquisition and flow Cytometry can be performed on a Cyan flow cytometer [31].

8.5.2.7 Electrophoretic Mobility Shift Assay

The mobility shift electrophoresis is a gel or band shift retardation assay, which uses the common affinity electrophoresis technique for studying protein−DNA or protein−RNA interactions to determine whether a protein or mixture of proteins is capable of binding to a given DNA or RNA sequence, and to indicate whether more than one protein molecule is involved in the binding complex. Gel shift assays are often performed to study transcription initiation, DNA replication, DNA repair, or RNA processing and maturation. Here, an oligonucleotide sequence (5′-GCATGCTAATGATATTCTTTG-3′) of the promoter gene of a virus (e.g., ICP0 of HSV) is biotinylated by a Biotin 3′ end DNA labeling kit. The nuclear extracts of virus-infected cells treated with the test drug for the indicated time are mixed with reaction mixtures (20 μl) containing 3 μg of nuclear extracts, 20 fmol of Biotin 3′ end-labeled probe, 50 ng/μl of poly (dI-dC), 2.5% glycerol, 0.05% NP-40 (1%), 5 mM $MgCl_2$, and 1X binding buffer. After incubation for 20 min at room temperature, reaction mixtures are applied to 4% polyacrylamide gels in 0.5X Tris-borate-ethylenediaminetetraacetic acid buffer at 4 °C, and the gels are transferred to Nylon membranes using a Semi Dry Transfer Cell (Bio-Rad, USA). The transferred oligos are then immobilized by ultraviolet crosslinking for 10 min. For detection of bound oligos, membranes are blocked with blocking buffer, followed by the addition of Streptavidin−Horseradish Peroxidase conjugate and developed according to the manufacturer's instructions. For supershift assays, nuclear extracts are preincubated with host cell factor-1 polyclonal antibodies for 30 min on ice [9,10].

8.5.2.8 Small Interfering RNA Transfection

Small interfering RNA (siRNA or silencing RNA) is a short (21−23 bp), double-stranded RNA nucleotide, and its transfection is "transference," involved in the

silencing of genes. The siRNA is extremely valuable in silencing gene expression and studying gene functions. Usually, the corresponding cells are treated with siRNA, specific for specific viral gene, and grown in 6/12-well plates at a 70–80% confluency, and the medium is removed. The cells are washed twice with PBS, and the transfection mixture [71.5 pmol of duplex siRNA ($siRNA^{specific\ gene}$ or $siRNA^{lamin\ A/C}$) and 0.25% Lipofectamine 2000 in OptiMEM] is incubated for 30 min at room temperature before adding to each well. The cells are then incubated with the transfection mixture for 4–5 h at 37 °C in 5% CO_2. The transfection medium is then replaced with fresh MEM with 10% FBS, repeated at 15 h posttransfection, and the transfected cells are infected with specific viruses at 36 hour post-transfection (hpt) to perform the immunofluorescence assays as mentioned above.

8.5.3 In vivo Assays

The in vitro antiviral activity of the test drug can be validated by in vivo testing in specific models. On the basis of the target site of infection and disease presentation, various animal models, namely, mouse, guinea pigs, ferrets, rabbit, and primates, can be used for different viruses.

8.5.3.1 Herpes Simplex Virus

HSV is an enveloped double-stranded DNA virus, classified into two types HSV-1 and HSV-2. Usually, HSV-1 is associated with orolabialis, keratitis, and encephalitis, whereas HSV-2 is associated with genital herpes [98], and both are transmitted through close personal contact [99]. HSV can hide from the immune attack of the host by latency and reactivate frequently to cause recurrent episodes [100]. Moreover, HSV-2 is a high risk factor for acquisition of HIV infection [101,102]. A recent report showed that HSV-suppressive therapy can greatly reduce HIV-1 load in persons coinfected with HSV [103]. Moreover, the determinants of effective immunity against HSV infection are not yet identified [104,105], and therapeutic vaccines failed to provide protection from infection or recurrences [104]. Hence, broad spectrum antiviral(s) of natural origin is an important strategy for the management of HSV infection. Thus, any agent that showed anti-HSV activity is usually tested for toxicity along with a skin irritation test to determine its acceptability for topical application, while in vivo efficacy studies are performed by developing cutaneous, vaginal, ocular (Figure 8.6), and latency associated infection models, when required.

8.5.3.1.1 Skin Irritation Test

The dermal hypersensitivity and related allergic manifestation of any anti-HSV agent alone or in ointment dosage form can be tested in batches (3) of mice. Specific doses of test drug/ointment (0.5% w/w) or ointment base are applied on the shaved and cleaned dorsal area (100- to 150-mm^2 area) of each animal. After 4 h, the residual ointment is removed, washed, and blotted dry to observe for any signs of inflammation, redness, flash, flare, and wheel corresponding to hypersensitivity. For further confirmation of dermal toxicity, the dorsal hair of female mice is shaved (with hair remover cream, cleaned with luke warm water, and dried) and the naked skin (100- to 150-mm^2 area) is abraded with a dermal (Seven-Star) needle to apply 0.1 g of ointment (desired %) to the abraded area of cohorts of animals. After 24 h, the ointment is removed, washed with warm water, and the animals are examined for erythema and edema within another 1 h. The animals are also observed up to the next 72 h for additional confirmation of toxicity.

8.5.3.1.2 Cutaneous Lesion Assay

To study the drug potency on HSV-1-induced cutaneous lesion, the dorsal skin of mice/Guinea pigs can be prepared and abraded (as above), and the abraded area is divided into four quadrants, and each is infected with HSV-1 (30 µl of 10-fold dilution), and observed up to 10 days for herpes lesion. On the basis of the initial result, stock virus (usually 150 µl of 10^8 PFU) is used to infect a 42-cm^2 area for consistent lesion development. Once the lesion develops, animal cohorts ($n = 15$) are infected and treated with the (1) test drug or drug-based ointment, (2) 3–5% (wt/wt) acyclovir, or (3) ointment base (1.5 g per dose) on the infected area with sterile cotton swabs, twice daily for 6 days (Figures 8.7 and 8.8).

The extent of the lesion needs to be scored daily as 1.0–1.6, lesions on one-fourth of the infected area; 1.7–2.4, lesions on one-half of the infected area; 2.5–3.2, lesions on three-fourths of the infected area; and 3.3–4.0, lesions on the entire infected area [106].

8.5.3.1.3 Reduction in Viral Vaginitis in Mice or Cotton Rats

To test the in vivo efficacy of test drug/formulation against HSV-2, the genital herpes model with random breed BALB/c female mice (Figure 8.9) or *Sigmodon hispidus* (cotton) rats [107] is developed by intravaginal inoculation of the virus in anesthetized 6-week-old animals.

After one-week of acclimatization (23 ± 3 °C), the animals ($n = 10$ for each dilution) will be inoculated with the virus (30 µl of 10^{-3} stock) into the vagina by a needle (size 12) and observed for 12 days for vaginitis or lethality (LD_{50}). To test the efficacy, fresh batches of animals are infected with 10 LD_{50} of the virus (10^5 PFU). Following inoculation, a vaginal cotton swab sample is collected from each animal, transferred to 0.5 ml of PBS, and stored at −20 °C. The animals are divided

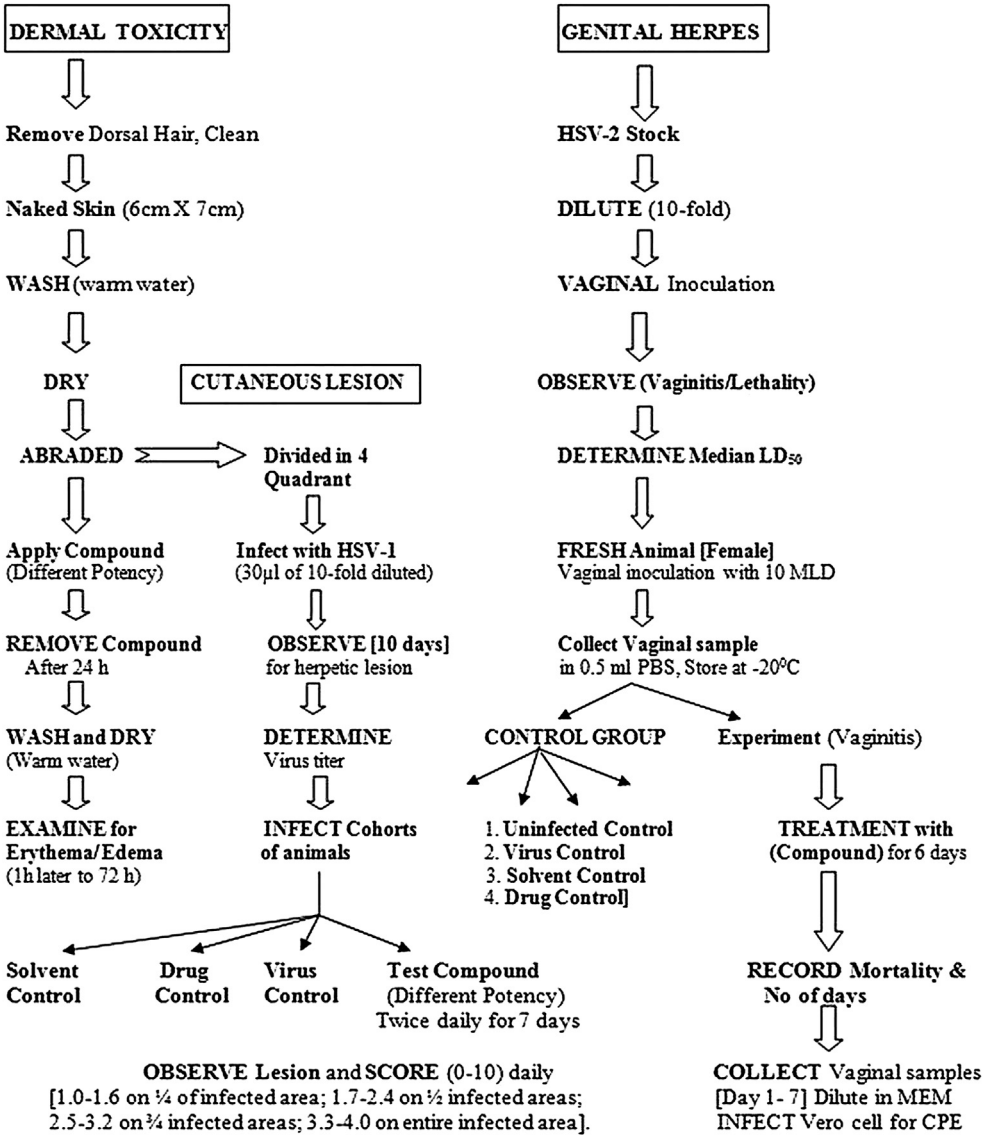


FIGURE 8.6 Dermal toxicity, cutaneous and vaginal infection model flowchart for herpes simplex virus.

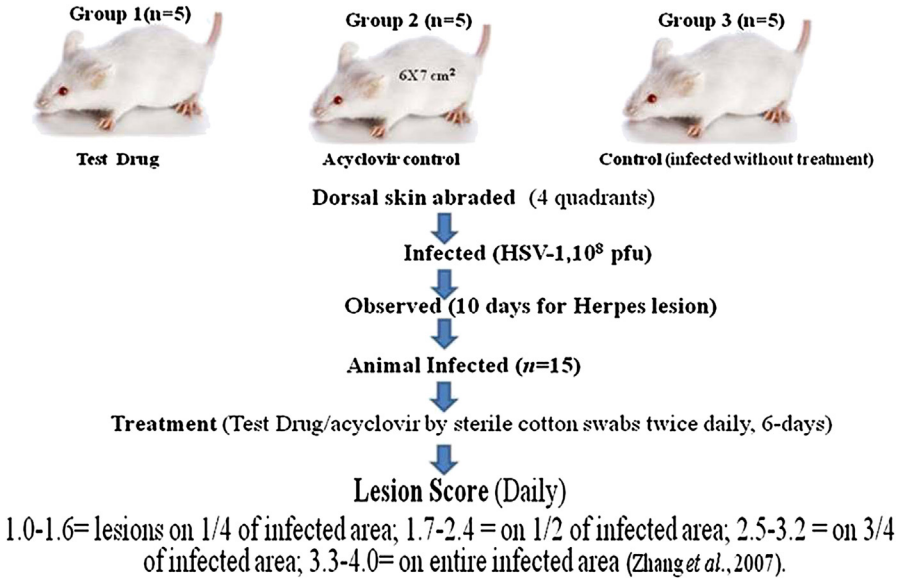


FIGURE 8.7 Cutaneous model development (outline).

BALB/c mice (n=10)

Cutaneously infected with HSV-1 ($1x10^6$ pfu)

Administration of Test Drug or ACV orally or topically

Twice daily for 7 days (Oral) | Thrice for 7 days (Topical)

Score: skin lesions, severity and death three times daily

(0, no lesion; 2, vesicle in local region; 4, erosion/ulceration; 6, mild zosteriform lesion; 8, moderate zosteriform lesion; 10, severe lesion and death).

Removal of brain & skin (whole lesions around inoculation area) under ether anaesthesia on days 2, 4, 6 or 8 after infection

Homogenized (PBS) and Centrifuged (3000 rpm for 15 min)

Determination of virus yield (supernatant) by Plaque assay

Observation of infected animals for 30 days to record mortality rates

FIGURE 8.8 Efficacy test protocol on cutaneously infected mice (schematic).

into test groups (different potency), positive control (acyclovir), negative control (solvent or ointment base), and no treatment (virus control) groups along with an additional uninfected control group. Symptoms of viral vaginitis (topical edema of the vaginal tract with turbid secretions) will be observed on the third day of infection. Treatment begins on day 3 postinfection, by applying the test agent or formulations to the vaginal tract with cotton swabs (2 mg per mouse) twice daily for 6 days [106]. Mortality and the number of days for mortality to occur are recorded. The vaginal swab samples are collected from day 1 following the completion of treatment, as well as from the deceased animals immediately following their death (Figure 8.5). The vaginal samples are then diluted five times in MEM and used to infect Vero cells. Samples that gave a positive CPE are considered positive for HSV-2 [106].

Female BALB/c mice
(7 weeks old, 18-20gm)

Depo-medoxyprogesterone acetate (Sigma) at 2mg/mouse 5-7 days before infection

Swabbed vaginal area with calcium alginate

Inoculated intravaginally with HSV-2 (9×10^4 pfu) by a blunt ended tips

After 3-4 h

Treated with HM-7 (0-0.5 mg/kg) or its ointment or ACV (5 mg/kg bw)

Observed 12 days for vaginitis/lethality to determine the MLD

Monitored and scored genital pathology daily

No infection (0); slight redness of external vagina (1); swelling & redness of external vagina (2); severe swelling & redness of vagina & surrounding tissue with hair loss (3); hind-limb paralysis (4); Death (5).

Collection of infected Vaginal tissues at Stage 4 for Histopathology
Vaginal washes for assessment of Viral Shedding by Plaque assay

FIGURE 8.9 Viral virginities model with HSV-2.

8.5.3.1.4 Vaginal Inoculation of HSV in Guinea Pigs

Vaginal inoculation of female Guinea pigs with HSV-1 or HSV-2 results in obvious primary infection. Following recovery, survivors of primary infection periodically display vesicular recrudescence in the vaginal area from which infectious virus and/or viral DNA can be recovered. Although reactivation cannot be reliably induced, the HSV-2 spontaneously reactivates with a much higher frequency than HSV-1, making it a very attractive model for comparative analysis of the influence of viral genes on reactivation and HSV recombinant viruses. This model helps to investigate and identify important features in this difference. The value of guinea pigs in studying drug or vaccine efficiency and experimental pathogenesis makes this an extremely valuable and promising system.

8.5.3.1.5 Ocular Herpes Virus Model

A fast, simple reactivation model to study ocular herpes virus infection and latency is developed by Gordon et al. [108] in New Zealand female rabbits (1.5–2.0 kg). Following topical anesthesia (0.5% proparacaine HC1 eye drops), each unscarified rabbit eye will be inoculated with a thymidine kinase-positive HSV-1W (5×10^4 pfu/eye) into the lower fornix, following topical anesthesia with eye drops. The virus establishes latency and reactivates [109]. Successful inoculation (100%) of eyes on day 7 will produce herpetic dendritic ulcers with significant HSV-1 titer (10^4 pfu/ml) and viral shedding, determined by a neutralization test. After satisfactory anesthesia, the globe is proptosed with a wooden cotton applicator and an operating microscope for all surgical manipulations. The intrastromal injection (0.25-ml tuberculin syringe with a no. 30 short bevel needle) is given into the central corneal stroma. The first group is given deionized sterile endotoxin-free water; the second group is given 100 µl air, while the 3rd group is not given any injection. For all three groups, the needle should be carefully withdrawn with gentle pressure so that the proptosed globe gently returns to the orbit. The anterior chamber, injected with deionized sterile water, is made at the limbus, inserted into the anterior chamber parallel to the iris plane. The needle is carefully withdrawn, and the insertion site is pressed with a cotton swab for 30 s, to avoid aqueous loss, and return of the proptosed globe to its proper place in the orbit. For topical administration, 100 ml deionized sterile water is administered onto the cornea of the proptosed globe

by means of a pipette, and the globe returns to the orbit by gentle digital pressure. Viral Shedding (latent HSV-1 after reactivation and induced shedding into the tear film) is determined by swabbing the eyes 2 days before treatment and for seven consecutive days after the treatment. Each eye swab is mixed with 0.3 ml MEM (Eagle's medium with Earle's salt, 10% newborn calf serum, 1% penicillin–streptomycin, 1% Fungizone), vortexed, and the eluant is plated onto a Vero cell monolayer. After a 1-h adsorption, 1.5 ml of medium is added to the well, and the plate is examined daily for 7 days for the CPE of HSV-1. Random HSV-1 isolates can be confirmed by neutralization [108,110].

8.5.3.1.6 Latency and Reactivation in Rabbits

Infection of rabbit eyes leads to a latent infection in which virus can be recovered from the trigeminal nerve ganglia following explantation and cocultivation with indicator cells. In addition, virus can be sporadically recovered from the eye following latency. In fact, reactivation can be efficiently induced by the iontophoresis of epinephrine into the eye, and this model can help to establish the latency associated transcript (LAT) expression for efficient reactivation.

8.5.3.1.6 The Mouse Eye or Trigeminal Ganglia Model

A second murine model for HSV-1 and HSV-2 latency involves the infection of the cornea followed by latency in the trigeminal ganglia. As in the footpad model, latent HSV genomes express LAT in a portion of those neurons maintaining them, and the virus can be recovered by cocultivation of the explanted ganglia. Interestingly, this method is similar to an in vivo method. Here, latently infected mice are transiently exposed to hyperthermia, and then trigeminal ganglia are excised, sectioned, and assayed for the presence of observable virus by immunohistochemistry or genetic engineering (when recombinant virus with an expressible marker in the genome is used).

8.5.3.2 Influenza Virus

Inbred female Balb/c or C57Bl/6 mice of six to eight weeks old are anesthetized by an intraperitoneal injection [150 µl of ketamine rompun-solution (2%-rompun and 10% ketamine solution are mixed at 1:10 with PBS)] and infected intranasally (i.n.) with 1×10^2 pfu/50 µl of Influenza A virus. For infection of Influenza A virus, preincubated with the specific drug, 102/25 µl, 103/25, or 104/25 µl of virus is incubated with 25 µl drug (1 mg/ml) or with 25 µl PBS for 30 min at room temperature [68].

8.5.3.2.1 Treatment of Balb/C Mice with the Test Drug

Eight-week-old BALB/c mice are anesthetized using an intraperitoneal (i.p.) injection of ketamine (200 µl of 10% ketamine solution) and xylazin (2% xylazin solution) at 1:10 with PBS. Mice are treated with the test drug using the COAALA Mouse Aerosol Application System. BALB/c mice are placed in the tube cylinder and exposed to 1.5 ml (per mouse), 2 bars of an aerosolized test agent for 10 min twice a day for 3 (lung titer) or 5 days (body weight), along with a control group (H_2O). Mice are infected 10 min after the first exposure via the intranasal route with the virus A/FPV/Bratislava/79 (H7N7) (10^3 pfu) or the recombinant A/Puerto Rico/8/34 (H1N1rec) (4×10^2 pfu) in a 50 µl volume to determine the viral replication in the lung and monitor the body weight. The health status of the animals is controlled twice a day, and their body weight is measured every day. The animals are sacrificed upon a body weight loss of 20%. For the determination of lung virus titer, mouse lungs are collected in PBS (3 ml) on day 3 post-infection (p.i.) and homogenized using a FastPrep24 homogenizator with Lysing Matrix D, centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatants are subjected to the plaque assay.

8.5.3.2.2 Mouse Monitoring

Body temperature and gross motor activity of the animals are monitored with suitable software (Vital View®) and hardware (Mini Mitter, USA) for data acquisition on physiological parameters. The hardware includes a transmitter (E-Mitter)/receiver system that collects data on temperature and motor activity. The vitality or gross motor activity provides a basic index of the movement of mice with implanted E-Mitter. As the mouse moves, the movement of the implanted E-Mitter results in subtle changes in the transmitted signal detected by a receiver and registered by a computer. The Vital View software recorded an index of movement every 5 min to produce a longitudinal record of the activity, and for implantation of E-Mitters the mice are anesthetized with an i.p. injection of 150 µl ketamine/rompun. The shaved ventral surface of the abdomen with a midline abdominal skin incision 0.5–1 cm below the diaphragm with a 2-cm length. The abdomen is opened with a 2-cm incision along the linea alba to position the E-Mitter in the abdominal cavity. Then the closure of the incision is achieved with wound clips (autoclip 9 mm; Becton & Dickinson, Germany). The animals are placed into the cage, and successful implantation of the E-Mitter is controlled by Vital View software. Their health status is controlled for seven days before infection.

8.5.3.2.3 Histology and Immunohistology

Mice are treated with the nebulized extract (10 mg/ml) three times at 9 am, 12 am, and 3 pm for 10 min or with H_2O. Immediately after the treatment, the mice are killed and the collected lungs are fixed in buffered 4%

paraformaldehyde, and stained with hematoxylin–eosin. Lectin staining of lung sections is performed with *Sambucus nigra* agglutinin (SNA; Vector Laboratories) for sialic acid 2, 6 linked to galactose and *Maackia amurensis* agglutinin (MAL II; Vector Laboratories) for sialic acid 2, 3 linked to galactose. Secondary staining is done with the ABC-kit for 30 min at room temperature, and substrate reaction with the Diaminiobenzidine (DAB) kit.

8.5.3.3 Dengue Virus

The dengue virus (DENV) is a mosquito-borne *Flavivirus* (*Flaviviridae*) virus with a positive sense single-stranded RNA [115,116] of about 11,000 bases, which codes for capsid protein C; membrane protein M; envelop protein E; seven nonstructural NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5; and short noncoding regions on 5′ and 3′ ends [115,117]. Further classification of each serotype (1–5) into genotypes often relates to the region where particular strains are found.

8.5.3.3.1 Virus Inhibition Assay

The inhibitory potential of a test drug can be evaluated in suckling mice [118]. Briefly, the serial twofold dilutions of test agent are mixed with 100 LD_{50} of virus in equal proportions and incubated for 1 h at room temperature. After incubation, 0.02 ml of the mixture (virus + drug) per mouse pup should be inoculated intracerebrally along with the virus and mice control, and observed daily for signs of Dengue, that is, weight loss, slow gait, inability to suck mother's milk, and flaccid paralysis followed by death. On postinoculation days 5–6, the mouse pups are collected, killed (chloroform inhalation), and stored at −20 °C for RNA extraction. In addition to the preincubation inhibition assay, pretreatment and posttreatment of the test drug are attempted as per the same protocol, except that the time of drug addition is varied.

RT-PCR: In order to demonstrate the presence or absence of viral RNA in infected C6/36 cells and mouse brain, RT-PCR can be performed [118], employing group specific primers. Briefly, RNA is extracted and eluted and then subjected to RT-PCR after initial reverse transcription with Moloney murine leukemia virus reverse-transcriptase (MMLV-RT) to synthesize the cDNA. The cDNA synthesis is usually carried out in a 10-µl volume with RT-mix (5 × -RT buffer, 10 mM dNTPs, 5U RNasin, 5U MMLV-RT) at 37 °C for 1 h using a downstream consensus primer (MP4). The amplification of cDNA is carried out by PCR (50 µl volume) congaing PCR mix (10 × -buffer, 1.5 mM MgCl2, 10 mM dNTPs, 2.5 U Taq-DNA Polymerase) using an upstream consensus primer (MP3) in a DNA thermal cycler. The thermal profile of the PCR reaction includes initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 94 °C 30 s, annealing at 54 °C, 2 min, and extension at 72 °C, 2 min, and final extension at 72 °C, 10 min. The amplified product can be analyzed on a 2% agarose gel for the presence of 511 bp virus-specific amplicon.

8.6 FUTURE PROSPECTS AND DIRECTIONS

Not only are the existing viral diseases fatal but also there is an upsurge of new viral infections worldwide. The currently available antivirals, though effective, are costly and beyond the reach of a vast majority of people. Thus, the development of safe, effective, and low cost antiviral drugs such as RT inhibitors is among the top priorities, as many viruses are not yet curable and have high mortality rates. Recently, considerable work has been done on medicinal plants focusing on anti-HIV activity [119–122], and there has been a considerable rise in the use of over-the-counter plant products containing orthodox drugs. The rationale is to reduce the side effects and to produce synergistic effects. But since in most cases pharmacological mechanisms of the combinations are not studied, adverse effects or therapeutic failures have been observed [123]. The most important consideration involving medicinal plants is to identify and standardize the method of preparation of extract, appropriate season of collecting plant material, and details of its administration [20,124]. Since a significant number of plant extracts has yielded positive results, it seems reasonable to conclude that there are probably many potential antiviral agents, and further characterization of active ingredients of those potential plants will reveal useful compounds.

References

[1] Chattopadhyay D, Sarkar MC, Chatterjee T, Sharma DR, Bag P, Chakraborti S, et al. Recent advancements for the evaluation of anti-viral activities of natural products. N Biotechnol 2009;25:347–68.
[2] Chattopadhyay D, Chakraborty MS, Saha GC. Viruses, the acellular parasites of cellular hosts: biology and pathology with special reference to HIV. Ind J STD AIDS 1999;20:54–60.
[3] Wagner EK, Hewlett MJ. Basic virology. 1st ed. Blackwell Science, Inc; 1999. ISBN 1-4051-0346-9.
[4] Chattopadhyay D, Bhattacharya SK. ethnopharmacology: a new search engine for the development of antivirals from naturaceuticals. In: Eddouks M, editor. Handbook of ethnopharmacology. Trivandrum: Research Signpost Publication; 2008. p. 129–97.
[5] Chattopadhyay D, Naik TN. Antivirals of ethnomedicinal origin: structure–activity relationship and scope. Mini Rev Med Chem 2007;7:275–301.
[6] Tombacz K, Patterson R, Grierson SS, Werling D. Lack of genetic diversity in newly sequenced porcine circovirus type 1 strains isolated 20 years Apart. Genome Announc 2014;2:e00156–14.
[7] Khan MT, Ather A, Thompson KD, Gambari R. Extracts and molecules from medicinal plants against herpes simplex viruses. Antiviral Research 2005;67:107–19.

[8] Chattopadhyay D. Ethnomedicinal antivirals: scope and opportunity. In: Ahmad I, Aquil F, Owais M, editors. Modern phytomedicine: turning medicinal plants into drugs. Wiley-VCH; 2006. p. 313–38.
[9] Naithani R, Huma LC, Holland LE, Shukla D, McCormick DL, Mehta RG, et al. Antiviral activity of phytochemicals: a comprehensive review. Mini Rev Med Chem 2008;8:1106–33.
[10] Bag P, Ojha D, Mukherjee H, Halder UC, Mondal S, Chandra NS, et al. An indole alkaloid from a tribal folklore inhibits immediate early event in HSV-2 infected cells with therapeutic efficacy in vaginally infected mice. PLoS One 2013;8:e77937.
[11] Bag P, Ojha D, Mukherjee H, Halder UC, Mondal S, Biswas A, et al. A dihydro-pyrido-indole potently inhibits HSV-1 infection by interfering the viral immediate early transcriptional events. Antiviral Res 2014;105:126–34.
[12] Mukherjee H, Ojha D, Bag P, Chandel HS, Bhattacharyya S, Chatterjee TK, et al. Anti-herpes virus activities of *Achyranthes aspera*: an Indian ethnomedicine, and its triterpene acid. Microbiol Res 2013;168:238–44.
[13] Christopher ME, Wong JP. Recent developments in delivery of nucleic acid-based antiviral agents. Curr Pharm Des 2006;12: 1995–2006.
[14] Kleymann G. New antiviral drugs that target herpesvirus helicase primase enzymes. Herpes 2003;10:46–52.
[15] Miserocchi E, Modorati G, Galli L, Rama P. Efficacy of valacyclovir vs acyclovir for the prevention of recurrent herpes simplex virus eye disease: a pilot study. Am J Ophthalmol 2007;144:547–51.
[16] Sweetman S. The complete drug reference. 34th ed. London: Pharmaceutical Press; 2004.
[17] Narayana K. A purine nucleoside analogue-acyclovir [9-(2-hydroxyethoxymethyl)-9h-guanine] reversibly impairs testicular functions in mouse. J Toxicol Sci 2008;33:61–70.
[18] Sawyer MH, Webb DE, Balow JE, Straus SE. Acyclovir-induced renal failure, clinical course and histology. Am J Med 1988;84: 1067–71.
[19] Greco M, Silva AP, Merchán-Hamann E, Jeronymo ML, Andrade JC, Greco DB. Differences in HIV-risk behaviour of bisexual men in their relationships with men and women. Revista de Saúde Pública 2007;41:109–17.
[20] Chattopadhyay D, Khan MTH. Ethnomedicines and ethnomedicinal phytophores against herpes viruses. Biotechnology Annual Review 2008;14:297–348.
[21] Jadhav P, Kapoor N, Thomas B, Lal H, Kshirsagar N. Antiviral potential of selected Indian medicinal (Ayurvedic) plants against herpes simplex virus 1 and 2. N Am J Med Sci 2012; 4:641–7.
[22] McCutcheon AR, Roberts TE, Gibbons E, Ellis SM, Babiuk LA, Hancock RE, et al. Antiviral screening of British Columbian medicinal plants. J Ethnopharmacol 1995;49:101–10.
[23] Chamorro C, de Latorre FJ, Montero A, Sánchez-Izquierdo JA, Jareño A, Moreno JA, et al. Comparative study of propofol versus midazolam in the sedation of critically ill patients: results of a prospective, randomized, multicenter trial. Crit Care Med 1996;24:932–9.
[24] Chamorro G, Salazar M, Favila L, Bourges H. Pharmacology and toxicology of *Spirulina* alga. Rev invest clin 1996;48:389–99.
[25] Clercq De E. Novel compounds in preclinical/early clinical development for the treatment of HIV infections. Rev Med Virol 2000;10:255–77.
[26] Schaeffer DJ, Krylov VS. Anti-HIV activity of extracts and compounds from algae and cyanobacteria. Ecotoxicol Environ Saf 2000;45:208–27.
[27] Alvarez AL, Habtemariam S, Juan-Badaturuge M, Jackson C, Parra F. In vitro anti HSV-1 and HSV-2 activity of *Tanacetum vulgare* extracts and isolated compounds: an approach to their mechanisms of action. Phytother Res 2011;25:296–301.
[28] Kurokawa M, Basnet P, Ohsugi M, Hozumi T, Kadota S, Namba T, et al. Anti-herpes simplex virus activity of moronic acid purified from *Rhus javanica* in vitro and in vivo. J Pharmacol Exp Ther 1999;289:72–8.
[29] Ojha D, Mukherjee H, Ghosh S, Bag P, Mondal S, Chandra NS, et al. Evaluation of anti-infective potential of a tribal folklore *Odina wodier* Roxb against some selected microbes and herpes simplex virus associated with skin infection. J Appl Microbiol 2013;115:1317–28.
[30] Bag P, Chattopadhyay D, Mukherjee H, Ojha D, Mandal N, Sarkar MC, et al. Anti-herpes virus activities of bioactive fraction and isolated pure constituent of *Mallotus peltatus*: an ethnomedicine from andaman islands. Virol J 2012;9:98.
[31] Lin LT, Chen TY, Chung CY, Noyce RS, Grindley TB, McCormick C, et al. Hydrolyzable tannins (chebulagic acid and punicalagin) target viral glycoprotein–glycosaminoglycan interactions to inhibit herpes simplex virus 1 entry and cell-to-cell spread. J Virol 2011;85:4386–98.
[32] Zhou M, Xu M, Ma XX, Zheng K, Yang K, Yang CR, et al. Antiviral triterpenoid saponins from the roots of *Ilex asprella*. Planta Med 2012;78:1702–5.
[33] Bharitkar YP, Bathini S, Ojha D, Ghosh S, Mukherjee H, Kuotsu K, et al. Antibacterial and antiviral evaluation of sulfonoquinovosyldiacylglyceride: a glycolipid isolated from *Azadirachta indica* leaves. Lett Appl Microbiol 2014;58:184–9.
[34] Yarmolinsky L, Huleihel M, Zaccai M, Ben-Shabat S. Potent antiviral flavone glycosides from Ficus benjamina leaves. Fitoterapia 2012;83:362–7.
[35] Chuanasa T, Phromjai J, Lipipun V, Likhitwitayawuid K, Suzuki M, Pramyothin P, et al. Anti-herpes simplex virus (HSV-1) activity of oxyresveratrol derived from Thai medicinal plant: mechanism of action and therapeutic efficacy on cutaneous HSV-1 infection in mice. Antiviral Res 2008;80:62–70.
[36] Suedee A, Tewtrakul S, Panichayupakaranant P. Anti-HIV-1 integrase activity of *Mimusops elengi* leaf extracts. Pharm Biol 2014; 52:58–61.
[37] Liang CQ, Luo RH, Yan JM, Li Y, Li XN, Shi YM, et al. Structure and bioactivity of triterpenoids from the stems of *Schisandra sphenanthera*. Arch Pharmacal Res 2014;37:168–74.
[38] Qian Y, Guan T, Tang X, Huang L, Huang M, Li Y, et al. Maslinic acid, a natural triterpenoid compound from *Olea europaea*, protects cortical neurons against oxygen–glucose deprivation-induced injury. Eur J Pharmacol 2011;670:148–53.
[39] Asada Y, Sukemori A, Watanabe T, Malla KJ, Yoshikawa T, Li W, et al. Stelleralides A-C, novel potent anti-HIV daphnane-type diterpenoids from *Stellera chamaejasme* L. Organic Letter 2011; 13:2904–7.
[40] Lubbe A, Seibert I, Klimkait T, Van der Kooy F. Ethnopharmacology in overdrive: the remarkable anti-HIV activity of *Artemisia annua*. J Ethnopharmacol 2012;141:854–9.
[41] Paskaleva EE, Arra M, Liu Y, Guo H, Swartz G, Kennedy JS, et al. Evaluation of potential genotoxicity of HIV entry inhibitors derived from natural sources. PLoS One 2014;9:e93108.
[42] Jadulco RC, Pond CD, Van Wagoner RM, Koch M, Gideon OG, Matainaho TK, et al. 4-Quinolone alkaloids from *Melochia odorata*. J Nat Prod 2014;77:183–7.
[43] Talwar GP, Dar SA, Rai MK, Reddy KV, Mitra D, Kulkarni SV, et al. A novel polyherbal microbicide with inhibitory effect on bacterial, fungal and viral genital pathogens. Int J Antimicrob Agents 2008;32:180–5.
[44] Helfer M, Koppensteiner H, Schneider M, Rebensburg S, Forcisi S, Müller C, et al. The root extract of the medicinal plant *Pelargonium sidoides* is a potent HIV-1 attachment inhibitor. PLoS One 2014;9:e87487.
[45] Dai JP, Zhao XF, Zeng J, Wan QY, Yang JC, Li WZ, et al. Drug screening for autophagy inhibitors based on the dissociation of

Beclin1-Bcl2 complex using BiFC technique and mechanism of eugenol on anti-influenza A virus activity. PLoS One 2013a;8: e61026.
[46] Dai JP, Wu LQ, Li R, Zhao XF, Wan QY, Chen XX, et al. Identification of 23-(s)-2-amino-3-phenylpropanoyl-silybin as an antiviral agent for influenza A virus infection in vitro and in vivo. Antimicrob Agents Chemother 2013b;57:4433–43.
[47] Ehrhardt C, Dudek SE, Holzberg M, Urban S, Hrincius ER, Haasbach E, et al. A plant extract of *Ribes nigrum folium* possesses anti-influenza virus activity *in vitro* and *in vivo* by preventing virus entry to host cells. PLoS One 2013;8:e63657.
[48] He W, Han H, Wang W, Gao B. Anti-influenza virus effect of aqueous extracts from dandelion. Virol J 2011;8:538.
[49] Yang F, Zhou WL, Liu AL, Qin HL, Lee SM, Wang YT, et al. The protective effect of 3-deoxysappanchalcone on *in vitro* influenza virus-induced apoptosis and inflammation. Planta Med 2012;78: 968–73.
[50] Dai J, Wang G, Li W, Zhang L, Yang J, Zhao X, et al. High-throughput screening for anti-influenza A virus drugs and study of the mechanism of procyanidin on influenza A virus-induced autophagy. J Biomol Screen 2012;17:605–17.
[51] Kiyohara H, Ichino C, Kawamura Y, Nagai T, Sato N, Yamada H, et al. *In vitro* anti-influenza virus activity of a cardiotonic glycoside from *Adenium obesum* (Forssk). Phytomedicine 2012;19: 111–4.
[52] Park JY, Jeong HJ, Kim YM, Park SJ, Rho MC, Park KH, et al. Characteristic of alkylated chalcones from Angelica keiskei on influenza virus neuraminidase inhibition. Bioorg Med Chem Lett 2011;21:5602–4.
[53] Garozzo A, Timpanaro R, Stivala A, Bisignano G, Castro A. Activity of *Melaleuca alternifolia* (tea tree) oil on Influenza virus A/PR/8: study on the mechanism of action. Antiviral Res 2011; 89:83–8.
[54] Shin HB, Choi MS, Ryu B, Lee NR, Kim HI, Choi HE, et al. Antiviral activity of carnosic acid against respiratory syncytial virus. Virol J 2013;10:303.
[55] Glatthaar-Saalmüller B, Rauchhaus U, Rode S, Haunschild J, Saalmüller A. Antiviral activity in vitro of two preparations of the herbal medicinal product Sinupret against viruses causing respiratory infections. Phytomedicine 2011;19:1–7.
[56] Wang KC, Chang JS, Lin LT, Chiang LC, Lin CC. Antiviral effect of cimicifugin from *Cimicifuga foetida* against human respiratory syncytial virus. Am J Chin Med 2012;40:1033–45.
[57] Zhao Y, Geng CA, Sun CL, Ma YB, Huang XY, Cao TW. Polyacetylenes and anti-hepatitis B virus active constituents from *Artemisia capillaris*. Fitoterapia 2014;95:187–93.
[58] Zhou WB, Zeng GZ, Xu HM, He WJ, Tan NH. Astataricusones A-D and astataricusol A, five new anti-HBV shionane-type triterpenes from *Aster tataricus* L. f. Molecules 2013;18:14585–96.
[59] Wang HL, Geng CA, Ma YB, Zhang XM, Chen JJ. Three new secoiridoids, swermacrolactones A-C and anti-hepatitis B virus activity from Swertia macrosperma. Fitoterapia 2013;89:183–7.
[60] Jiang ZY, Liu WF, Zhang XM, Luo J, Ma YB, Chen JJ. Anti-HBV active constituents from *Piper longum*. Bioorg Med Chem Lett 2013;23:2123–7.
[61] Wei W, Li X, Wang K, Zheng Z, Zhou M. Lignans with anti-hepatitis B virus activities from *Phyllanthus niruri* L. Phytother Res 2012;26:964–8.
[62] Ratnoglik SL, Aoki C, Sudarmono P, Komoto M, Deng L, Shoji I, et al. Antiviral activity of extracts from *Morinda citrifolia* leaves and chlorophyll catabolites, pheophorbide and pyropheophorbide a, against hepatitis C virus. Microbiol Immunol 2014;58: 188–94.
[63] Fu SB, Yang JS, Cui JL, Sun DA. Biotransformation of ursolic acid by *Syncephalastrum racemosum* CGMCC 3.2500 and anti-HCV activity. Fitoterapia 2013;86:123–8.
[64] Haid S, Novodomská A, Gentzsch J, Grethe C, Geuenich S, Bankwitz D, et al. A plant-derived flavonoid inhibits entry of HCV genotypes into human hepatocytes. Gastroenterology 2012;143:213–22.
[65] Rehman S, Ashfaq UA, Riaz S, Javed T, Riazuddin S. Antiviral activity of *Acacia nilotica* against Hepatitis C Virus in liver infected cells. Virol J 2011;8:220.
[66] Takeshita M, Ishida Y, Akamatsu E, Ohmori Y, Sudoh M, Uto H, et al. Proanthocyanidin from blueberry leaves suppresses expression of subgenomic hepatitis C virus RNA. J Biol Chem 2009;284: 21165–76.
[67] Tsujimoto K, Sakuma C, Uozaki M, Yamasaki H, Utsunomiya H, Oka K, et al. Antiviral effect of pyridinium formate, a novel component of coffee extracts. Int J Mol Med 2010;25:459–63.
[68] Visintini Jaime MF, Redko F, Muschietti LV, Campos RH, Martino VS, Cavallaro LV. In vitro antiviral activity of plant extracts from Asteraceae medicinal plants. Virol J 2013;10:245.
[69] Melo FL, Benati FJ, Roman Jr WA, de Mello JC, Nozawa C, Linhares RE. The in vitro antiviral activity of an aliphatic nitro compound from *Heteropteris aphrodisiaca*. Microbiol Res 2008; 163:36–9.
[70] Semple SJ, Pyke SM, Reynolds GD, Flower RL. *In vitro* antiviral activity of the anthraquinone chrysophanic acid against poliovirus. Antiviral Res 2001;49:169–78.
[71] Semple SJ, Nobbs SF, Pyke SM, Reynolds GD, Flower RL. Antiviral flavonoid from *Pterocaulon sphacelatum*, an Australian aboriginal medicine. J Ethnopharmacol 1999;68:283–8.
[72] Kang SY, Kang JY, Oh MJ. Antiviral activities of flavonoids isolated from the bark of *Rhus verniciflua* stokes against fish pathogenic viruses in Vitro. J Microbiol 2012;50:293–300.
[73] Micol V, Caturla N, Pérez-Fons L, Más V, Pérez L, Estepa A. The olive leaf extract exhibits antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV). Antiviral Res 2005;66: 129–36.
[74] Li SY, Chen C, Zhang HQ, Guo HY, Wang H, Wang L, et al. Identification of natural compounds with antiviral activities against SARS-associated coronavirus. Antiviral Res 2005;67:18–23.
[75] Fiore C, Eisenhut M, Krausse R, Ragazzi E, Pellati D, Armanini D, et al. Antiviral effects of *Glycyrrhiza* species. Phytother Res 2008;22:141–8.
[76] De Tommasi N, Conti C, Stein ML, Pizza C. Structure and in vitro antiviral activity of triterpenoid saponins from *Calendula arvensis*. Planta Med 1991;57:250–3.
[77] Cella M, Riva DA, Coulombié FC, Mersich SE. Virucidal activity presence in *Trichilia glabra* leaves. Rev Argent Microbiol 2004;36: 136–8.
[78] Yamai M, Tsumura K, Kimura M, Fukuda S, Murakami T, Kimura Y. Antiviral activity of a hot water extract of black soybean against a human respiratory illness virus. Biosci Biotechnol Biochem 2003;67:1071–9.
[79] Shang L, Qu Z, Sun L, Wang Y, Liu F, Wang S, et al. Astragaloside IV inhibits adenovirus replication and apoptosis in A549 cells in vitro. J Pharm Pharmacol 2011;63:688–94.
[80] Lazreg Aref H, Gaaliche B, Fekih A, Mars M, Aouni M, Pierre Chaumon J, et al. In vitro cytotoxic and antiviral activities of *Ficus carica* latex extracts. Nat Prod Res 2011;25:310–9.
[81] Oliveira GL, Cardoso SK, Lara CR, Vieira Jr TM, Guimarães EF, Figueiredo LS, et al. Chemical study and larvicidal activity against *Aedes aegypti* of essential oil of *Piper aduncum* L. (Piperaceae). An Acad Bras Ciênc 2013;85:1227–34.
[82] Zandi K, Teoh BT, Sam SS, Wong PF, Mustafa MR, Abubakar S. Novel antiviral activity of baicalein against dengue virus. BMC Complement Alternat Med 2012;12:214.
[83] Parida MM, Upadhyay C, Pandya G, Jana AM. Inhibitory potential of neem (*Azadirachta indica* Juss.) leaves on dengue virus type-2 *replication*. J Ethnopharmacol 2002;79:273–8.

[84] Sudo K, Konno K, Yokota T, Shigeta S. A sensitive assay system screening antiviral compounds against herpes simplex virus type 1 and type 2. J Virol Methods 1994;49:169–78.
[85] Wan Z, Lu Y, Liao Q, Wu Y, Chen X. Fangchinoline inhibits human immunodeficiency virus type 1 replication by interfering with gp160 proteolytic processing. PLoS One 2012;7: e39225.
[86] Berghe Vanden DA, Haemers A, Vlietinck AJ. Antiviral agents from higher plants and an example of structure–activity relationship of 3-methoxyflavones. In: Colegate SM, Milyneux RJ, editors. Bioactive natural products: detection, isolation, and structural determination. Florida: CRC Press; 1993. p. 405–40.
[87] Vlietinck AJ, De Bruyne T, Vanden Berghe D. Plant substances as antiviral agents. Curr Org Chem 1997;1:307–44.
[88] Apers S, Cimanga K, Vanden Berghe D, Van Meenen E, Longanga AO, Foriers A, et al. Antiviral activity of simalikalactone D, a quassinoid from *Quassia Africana*. Planta Med 2002; 68:20–4.
[89] Kim JW, Park SJ, Lim JH, Yang JW, Shin JC, Lee SW, et al. Triterpenoid saponins isolated from *Platycodon grandiflorum* inhibit hepatitis C virus replication. Evid-Based Complement Alternat Med 2013;2013:560417.
[90] Takebe Y, Saucedo CJ, Lund G, Uenishi R, Hase S, Tsuchiura T, et al. Antiviral lectins from red and blue–green algae show potent in vitro and in vivo activity against hepatitis C virus. PLoS One 2013;8:e64449.
[91] Kimura H, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, et al. Quantitative analysis of Epstein–Barr virus load by using a real-time PCR assay. J Clin Microbiol 1999;37:132–6.
[92] Laue T, Emmerich P, Schmitz H. Detection of dengue virus RNA inpatients after primary or secondary dengue infection by using the TaqMan automated amplification system. J Clin Microbiol 1999;37:2543–7.
[93] Tanaka N, Kimura H, Iida K, Saito Y, Tsuge I, Yoshimi A, et al. Quantitative analysis of cytomegalovirus load using a real-time PCR assay. J Med Virol 2000;60:455–62.
[94] Josefsson A, Livak K, Gyllensten U. Detection and quantification of human papillomavirus by using the fluorescent 59 exonuclease assay. J Clin Microbiol 1999;37:490–6.
[95] Locatelli G, Santoro F, Veglia F, Gobbi A, Lusso P, Malnati MS, et al. Real-time quantitative PCR for human herpesvirus 6 DNA. J Clin Microbiol 2000;38:4042–8.
[96] Loeb KR, Jerome KR, Goddard J, Huang M, Cent A, Corey L. High-throughput quantitative analysis of hepatitis B virus DNA in serum using the TaqMan fluorogenic detection system. Hepatology 2000;32:626–9.
[97] Schutten M, van den Hoogen B, van der Ende ME, Gruters RA, Osterhaus AD, Niesters HG. Development of a real-time quantitative RT-PCR for the detection of HIV-2 RNA in plasma. J Virol Method 2000;88:81–7.
[98] Smith JS, Robinson NJ. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. J Infect Dis 2002;186:S3–28.
[99] Hardin TC. Sexually transmitted diseases. In: Herfindal ET, Gourley DR, editors. Textbook of therapeutics-drug and disease management. Baltimore: Williams & Wilkins; 1996. p. 1389–404.
[100] Tyring SK. Advances in the treatment of herpesvirus infection: the role of famciclovir. Clin Ther 1998;20:661–70.
[101] Cowan FM, French RS, Mayaud P, Gopal R, Robinson NJ, de Oliveira SA, et al. Seroepidemiological study of herpes simplex virus types 1 and 2 in Brazil, Estonia, India, Morocco, and Sri Lanka. Sex Transm Infect 2003;79:286–90.
[102] White MK, Gorrill TS, Khalili K. Reciprocal transactivation between HIV-1 and other human viruses. Virology 2006;352: 1–13.
[103] Nagot N, Ouédraogo A, Foulongne V, Konaté I, Weiss HA, Vergne L, et al. Reduction of HIV-1 RNA levels with therapy to suppress herpes simplex virus. N Engl J Med 2007;356:790–9.
[104] Stanberry LR. Clinical trials of prophylactic and therapeutic herpes simplex virus vaccines. Herpes 2004;11:161A–9A.
[105] Koelle DM, Huang J, Hensel MT, McClurkan CL. Innate immune responses to herpes simplex virus type 2 influence skin homing molecule expression by memory CD4+ lymphocytes. J Virol 2006;80:2863.
[106] Zhang Y, But PP, Ooi VE, Xu HX, Delaney GD, Lee SH, et al. Chemical properties, mode of action, and *in vivo* anti-herpes activities of a lignin–carbohydrate complex from *Prunella vulgaris*. Antiviral Res 2007;75:242–9.
[107] Yim KC, Carroll CJ, Tuyama A, Cheshenko N, Carlucci MJ, Porter DD, et al. The cotton rat provides a novel model to study genital herpes infection and to evaluate preventive strategies. J Virol 2005;79:14632–9.
[108] Gordon YJ, Romanowski E, Araullo-Cruz T. A fast, simple reactivation method for the study of HSV-1 latency in the rabbit ocular model. Invest Ophthalmol Vis Sci 1990;31:921–4.
[109] Gordon YJ, Araullo-Cruz TP, Romanowski E, Ruziczka L, Balouris C, Oren J, et al. The development of an improved reactivation model for the study of HSV-1 latency. Invest Ophthalmol Vis Sci 1986;27:1230.
[110] Gordon YJ, Armstrong JA, Brown SI, Becker Y. The role of herpesvirus type 1 thymidine kinase in experimental ocular infections. Am J Ophthalmol 1983;95:175.
[111] McKinlay MA, and Steinberg BA. Oral efficacy of WIN 51711 in mice infected with human poliovirus. Antimicrobial Agents and Chemotherapy 29:30–2.
[112] Lodmell DL, Esposito JJ, Ewalt LC. Rabies virus antinucleoprotein antibody protects against rabies virus challenge in vivo and inhibits rabies virus replication in vitro. J Virol 1993;67(10):6080–6.
[113] Finke S, Conzelmann KK. Replication strategies of rabies virus. Virus Res 2005;111:120–31.
[114] Admasu P, Deressa A, Mengistu A, Gebrewold G, Feyera T. In vivo antirabies activity evaluation of hydroethanolic extract of roots and leaves of *Phytolacca dodecandra*. Global Veterinaria 2014;12:12–8.
[115] Rodenhuis-Zybert IA, Wilschut J, Smit JM. Dengue virus life cycle: viral and host factors modulating infectivity. Cell Mol Life Sci 2010;67:2773–86.
[116] WHO. Dengue guidelines for diagnosis, treatment, prevention and control. World Health Organization; 2009. ISBN 92-4-154787-1.
[117] Hanley KA, Weaver SC. Frontiers in dengue virus research. Caister Academic Press; 2010. ISBN 978-1-904455-50-9.
[118] Parida MM, Upadhyay C, Pandya G, Jana AM. Inhibitory potential of neem (*Azadirachta indica* Juss) leaves on Dengue virus type-2 replication. J Ethnopharmacol 2002;79:273–8.
[119] Hussein G, Miyashiro H, Nakamura N, Hattori M, Kawahata T, Otake T, et al. Inhibitory effects of Sudanese plant extracts on HIV-1 replication and HIV-1 protease. Phytother Res 1999;13: 31–6.
[120] Premanathan M, Kathiresan K, Yamamoto N, Nakashima H. *In vitro* anti-human immunodeficiency virus activity of polysaccharide from *Rhizophora mucronata* Poir. Biosci Biotechnol Biochem 1999;63:1187–91.
[121] Calabrese C, Berman SH, Babish JG, Ma X, Shinto L, Dorr M, et al. A phase I trial of andrographolide in HIV positive patients and normal volunteers. Phytother Res 2000;14:333–8.
[122] Asres K, Bucar F, Kartnig T, Witvrouw M, Pannecouque C, De Clercq E. Antiviral activity against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) of ethnobotanically selected Ethiopian medicinal plants. Phytother Res 2001;15:62–9.

[123] Chan PK, Ng HK, Cheung JL, Cheng AF. Survey for the presence and distribution of human herpesvirus 8 in healthy brain. J Clin Microbiol 2000;38:2772–3.
[124] Chattopadhyay D, Arunachalam G, Mandal AB, Bhattacharya SK. Dose dependent therapeutic antiinfectives from ethnomedicines of Bay Islands. Chemotherapy 2006;52:151–7.
[125] Bufalo MC, Figueiredo AS, de Sousa JP, Candeias JM, Bastos JK, Sforcin JM. Anti-poliovirus activity of *Baccharis dracunculifolia* and propolis by cell viability determination and real-time PCR. J Appl Microbiol 2009;107:1669–80.
[126] Li J, Huang H, Feng M, Zhou W, Shi X, Zhou P. In vitro and in vivo anti-hepatitis B virus activities of a plant extract from *Geranium carolinianum* L. Antiviral Res 2008;79:114–20.
[127] Hana Y-Q, Huang Z-M, Yang X-B, Liud H-Z, Wua G-X. *In vivo* and *in vitro* anti-hepatitis B virus activity of total phenolics from *Oenanthe javanica*. J Ethnopharmacol 2008;118:148–53.
[128] Chavez JH, Leal PC, Yunes RA, Nunes RJ, Barardi CR, Pinto AR, et al. Evaluation of antiviral activity of phenolic compounds and derivatives against rabies virus. Vet Microbiol 2006;116: 53–9.
[129] Lee JH, Park DY, Lee KJ, Kim YK, So YK, Ryu JS, et al. Intracellular reprogramming of expression, glycosylation, and function of a plant-derived antiviral therapeutic monoclonal antibody. PLoS One 2013;8:e68772.
[130] Zandi K, Teoh BT, Sam SS, Wong PF, Mustafa MR, Abubakar S. Antiviral activity of four types of bioflavonoid against dengue virus type-2. Virol J 2011;8:560.
[131] Zandi K, Lim TH, Rahim NA, Shu MH, Teoh BT, Sam SS, et al. Extract of *Scutellaria baicalensis* inhibits dengue virus replication. BMC Complement Alternat Med 2013;13:91.

LIST OF ABBREVIATIONS

AHA Azidohomoalanine
AIDS Acquired immunodeficiency syndrome
AZT Azidothymidine
BSA Bovine serum albumin
CC_{50} 50% cytotoxic concentration
CCR5 C–C chemokine receptor type 5
CD4 Cluster of differentiation 4
CNS Central nervous system
CO_2 Carbon dioxide
CPE Cytopathic effects
DAB kit Diaminiobenzidine (DAB) as substrate used by Vectastain ABC kit (Vector Labs, USA)
DEAE Diethylaminoethyl
DHBV Duck hepatitis B virus
DMEM Dulbecco's modified Eagle's medium
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid
dNTP Deoxynucleotide triphosphates
DOC Deoxycholate
EC_{50} Concentration of compound producing 50% inhibition of virus-induced cytopathic effect
ECL Enhanced chemiluminescence
EDTA Ethylenediaminetetraacetic acid
FACS Fluorescence-activated cell sorting
FDA Food and Drug Administration
FITC Fluorescein isothiocyanate
GAPDH Glyceraldehyde-3-phosphate dehydrogenase gene
GFP Green fluorescent protein
H_2SO_4 Sulfuric acid
HA Hemagglutination activation
HBsAg HBV antigens
HBV Hepatitis B virus
HCC Hepatocellular carcinoma
HCF Host cell factor
HCl Hydrochloric acid
HCV Hepatitis C virus
HIV Human immunodeficiency virus
HSV Herpes simplex virus
IC_{50} half minimal (50%) inhibitory concentration.
ICP0 Infected cell protein 0
IgG Immunoglobulin G
LAT Latency associated transcript
LD_{50} Median lethal dose
LTR Long terminal repeat
MAb Monoclonal antibodies
MEM Minimum Essential Medium
$MgCl_2$ Magnesium chloride
MMLV-RT Moloney Murine Leukemia Virus Reverse Transcriptase
MNTD Maximum Nontoxic Dose
MOI Multiplicity of infection
mRNA messenger RNA
MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl Sodium chloride
NP-40 Nonyl phenoxypolyethoxylethanol-40
NRTIs Nucleoside analog reverse-transcriptase inhibitors
OD Optical density
OptiMEM Reduced Serum Media is a modification of MEM
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
PBST PBS solution with 0.1% Tween 20
PCR Polymerase chain reaction
PFA Paraformaldehyde
PFU Plaque-forming unit
PI Propidium iodide
PVDF Polyvinylidene difluoride
qRT-PCR quantitative real-time polymerase chain reaction
RBCs Red blood cells
RF Reduction factors
RNA Ribonucleic acid
RSV Respiratory syncytial virus
RT Reverse transcriptase
RTQ-PCR Quantitative reverse transcription PCR
SARS Severe acute respiratory syndrome
SDS Sodium dodecyl sulfate
SHIV Simian-HIV
SI selectivity index
siRNA Small interfering RNA
SIV Simian immunodeficiency virus
TBE Tris-borate-EDTA
$TCID_{50}$ Titration expressed in 50% tissue culture infectious doses
TCM Traditional Chinese Medicine
TI Therapeutic index
TMB 3,3′,5,5′-tetramethyl-benzidine
UV Ultraviolet
VGE Viral genome equivalent
VHSV Viral hemorrhagic septicemia.
VSV Vesicular stomatitis virus

CHAPTER

9

Harmonization of Regulatory Requirements in Europe to Ensure Quality, Safety and Efficacy of Herbal Medicinal Products

Werner Knöss

Federal Institute for Drugs and Medical Devices, Bonn, Germany

OUTLINE

9.1 INTRODUCTION

Medicinal plants have been used in Europe since ancient times. The earliest documents about medicinal plants and their usage were written in Greece more than 2000 years ago. The systematic knowledge was extended in the medieval age and famous textbooks were also created in central Europe. During the past centuries of the second millennium the natural constituents of medicinal plants became more and more the subject of research and education, especially in pharmacy and medicine.

Legal regulation of herbal medicinal products in Europe has been developed since the second half of the twentieth century. Basically legal obligations intended to ensure quality, efficacy, and safety of herbal medicinal products. However, in the Member States of the European Union there are diverse traditions with

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00009-X

respect to usage of medicinal plants and different approaches were followed at the national level. In order to harmonize assessment of herbal medicinal products and to facilitate access to the market in different Member States of the European Union, a common law was enforced. The legal framework provided a set of basic definitions, laid down the options to grant access to the market, and set the basic framework to have common standards and requirements for herbal medicinal products in the European Union. A Committee on Herbal Medicinal Products (HMPC) was established at the European Medicines Agency (EMA) in 2004 [1]. This scientific expert committee is following a set of legal tasks. The most important one is the development of so-called Community Monographs harmonized standard for safety and efficacy of herbal substances and preparations thereof, which is the basic recommendation for decision by the national competent authorities of the Member States of the European Union. The Monographs are based on current assessment of the available literature. Following the legal requirements the existing level of evidence could result in a well-established use Monograph, which is the precondition for a marketing authorization as herbal medicinal product. If the scientific evidence is not sufficient the European legal framework offers the option for a Monograph addressing the traditional use. This may be the base for registration as a traditional herbal medicinal product. The HMPC is an excellent model of how scientific evaluation of herbal medicines can be harmonized and set accepted and science-based standards to ensure public health.

9.2 HMPC: ESTABLISHMENT AND WORKING STRUCTURE

The HMPC was established as one of seven scientific expert committees of the EMA in 2004 (Table 9.1). Details were laid down in directives and regulations of the European Union [1–3]. The EMA is located in London and was built to coordinate the network of the national competent authorities for medicinal products of the Member States of the European Union.

Ten years before the HMPC started its work there had been a working party on herbal medicinal products that was elaborating first steps toward harmonized evaluation of herbal medicinal products. However, although the working party played a substantial role in initiating exchange between the Member States in the field of herbal medicines, the impact of the HMPC was much stronger because the tasks were legally defined. Moreover, the decisions and scientific opinions of the HMPC were intended to create a common standard, which due to the specific procedure could be legally binding or should be considered as a strong recommendation.

TABLE 9.1 Scientific Committees at the EMA

CHMP	Committee on Medicinal Products for Human Use
COMP	Committee on Orphan Medicinal Products
PDCO	Paediatric Committee
HMPC	Committee on Herbal Medicinal Products
CAT	Committee on Advanced Therapies
CVMP	Committee on Medicinal Products for Veterinary Use
PRAC	Pharmacovigilance Risk Assessment Committee

The legal tasks of the HMPC are defined as follows. The HMPC shall perform the following:

- Prepare Community Herbal Monographs on herbal substances or herbal preparations that may be used for full marketing authorizations of well-established herbal medicinal products or simplified registrations
- Prepare a list of traditional herbal substances/preparations/combinations
- At the request of a Member State draw up an opinion on the adequacy of the evidence of the long-standing use
- After referral of a Member State draw up a Community Herbal Monograph on traditional herbal products used less than 15 years within the Community
- Be responsible for arbitration/referral procedures originating from different views among Member States on registered traditional herbal medicinal products
- Give an opinion on other medicinal products containing herbal substances for human use referred to the EMA

Each Member State of the European Union is nominating one delegate and one alternate member of the HMPC. The competence is complemented by up to five so-called co-opted members who are elected by the HMPC and who are representing specific fields of expertise. Currently, the following subjects are covered by the co-opted members: pediatrics, toxicology, pharmacology, clinical pharmacology, and general

medicine. Decisions of the HMPC should strive for a consensus, but can be also based on a majority. Delegates and co-opted members are allowed to vote (i.e., currently 28 delegates and five co-opted members; June 2014). The regular plenary meetings of the HMPC also include delegates from Norway and Iceland, which are members of the European Economic Association and observers from European Union candidate countries. A secretariat is established at the EMA, which is supporting the work administratively as well as scientifically. In the same way the EMA provides legal or regulatory advice to the HMPC, if necessary, and ensures an adequate coordination with other committees established at the EMA. Plenary meetings of HMPC are held six times a year every other month in London. The meetings have been scheduled so far for one or one and a half day.

There are three subgroups that have been established to support the work of the HMPC:

- Organizational Matters Drafting Group (ORGAM DG)
- Quality Drafting Group (Q DG)
- Working Party on Monographs and List Entries (MLWP)

The ORGAM DG is composed of about 10 experts who are elected by the HMPC. The task of this drafting group is to develop suitable procedures to organize the work, to provide appropriate templates to facilitate the work, and to give advice to the HMPC and its subgroups whenever questions address procedural or organizational topics. Meetings are usually held four times per year. Initially all meetings were face-to-face meetings, whereas at present the work is performed during virtual meetings. The Q DG is bringing together the knowledge of 10 experts in the field of quality of herbal medicines. For all issues identified by the HMPC related to the quality of herbal medicines, the Q DG is preparing draft decisions or comments for further consideration and discussion at the HMPC. Meetings are also organized routinely four times per year; the Q DG meetings are performed either as face-to-face meetings or as virtual meetings. The third subgroup, MLWP, has been established as a permanent subgroup. The MLWP is composed of 20–25 members, including the five co-opted members of the HMPC. The MLWP is meeting six times per year in London for two and a half day. Its contribution is of high importance for the core task of the HMPC because the MLWP is drafting Monographs and related documents for final discussion and decision by the HMPC. Without any doubt a major part of scientific discussion and evaluation of herbal substances and preparations derived thereof is taking place at the MLWP.

9.3 BASIC LEGAL DEFINITIONS AND ACCESS TO THE MARKET

Worldwide there is no unique definition for "herbal medicines." Regulation in Canada is using the term "natural health products," and in the United States the term "botanicals" is applied. In the European Union the basic definitions are laid down in Directive 2001/83/EC [2]. The European regulatory framework is providing in article 1 definitions for herbal medicinal products, traditional herbal medicinal products, herbal substances, and herbal preparations:

9.3.1 Herbal Medicinal Products

It is any medicinal product exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations.

9.3.2 Traditional Herbal Medicinal Products

It is a herbal medicinal product that fulfills the conditions laid down in Article 16a(1) of Directive 2001/83/EC.*Vitamins and minerals may be added if their action is ancillary to the herbal constituent*(*s*). (As this is the original basic definition no further explanation is given here, but the criteria are explained in more detail in Chapter 10.4).

9.3.3 Herbal Substances (Synonym "Herbal Drug" According to the European Pharmacopeia [4])

Herbal substances are mainly whole, fragmented, or cut plants, plant parts, algae, fungi, or lichen in an unprocessed, usually dried, form, but sometimes fresh. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal substances. Herbal substances are precisely defined by the plant part used and the botanical name according to the binomial system (genus, species, variety, and author).

9.3.4 Herbal Preparations (Synonym "Herbal Drug Preparation" According to the European Pharmacopeia [4])

These are preparations obtained by subjecting herbal substances to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. These include comminuted or powdered herbal substances, tinctures, extracts, essential oils, expressed juices, and processed exudates.

The basic approach in the European Union is to assess quality, efficacy, and safety of herbal medicinal products before they have access to the market. An application for marketing authorization or registration has to be submitted via a distinct procedure. The following procedures are legally established [2,5]:

- *Centralized procedure*: This procedure for marketing authorization is directed to the EMA and is linked to an assessment coordinated by the EMA. If the marketing authorization is granted a medicinal product can be marketed in all Member States of the European Union. This procedure is foreseen for a defined set of indications (e.g., oncological or neurological indications) or medicinal products of special importance for public health. Currently, this procedure is very rarely used for herbal medicinal products.
- *Decentralized procedure* (DCP): This procedure for marketing authorization or registration is directed to a subset of Member States. A Reference Member State is taking the lead for the assessment and the other Member States involved (Concerned Member States) are mainly checking the assessment of the Reference Member State. At the end of a successful procedure a marketing authorization or registration is granted in the Member States participating.
- *Mutual Recognition Procedure* (MRP): If a medicinal product is already authorized or registered in one Member State, a procedure may be started that is built up on the existing assessment. At the end of a successful procedure a marketing authorization or registration is granted in the Member States participating.
- *National procedure*: An application can be directed to a single national competent authority and finally only a marketing authorization or registration for one Member State is granted.

During the past years there has been growing experience with DCP, but national procedures still play an important role. The application has to be made in a distinct format with respect to the data provided in the dossier (Figure 9.1).

9.3.5 Marketing Authorization

- Full application: for new herbal medicinal products
- Bibliographic application: for known herbal medicinal products with well-established use
- Hybrid forms may be used

9.3.6 Registration

- Bibliographic application with additional data on safety if necessary: for traditional herbal medicinal products.

FIGURE 9.1 Overview of requirements for traditional/herbal medicinal products with respect to type of application.

9.4 MONOGRAPHS AND LIST ENTRIES: WELL-ESTABLISHED USE AND TRADITIONAL USE

The Community Monographs are intended to facilitate marketing authorization of herbal medicinal products and registration of traditional herbal medicinal products in the Member States of the European Union. They basically follow the structure of a summary of product characteristics in order to give the national competent authorities the backbone for a products-specific assessment.

9.4.1 Sections of Community Monographs and List Entries

- Qualitative and quantitative composition
- Pharmaceutical form
- Clinical particulars
  - Therapeutic indications
  - Posology, method of administration
  - Contraindications
  - Special warnings and precautions for use
  - Interactions
  - Pregnancy and lactation, fertility
  - Effects on the ability to drive and use machines
  - Undesirable effects
  - Overdose
- Pharmacological properties
  - Pharmacodynamic properties
  - Pharmacokinetic properties
  - Preclinical safety data
- Pharmaceutical particulars

The Monographs for herbal substances and herbal preparations are reflecting the harmonized European

view and should be interpreted as a strong recommendation to applicants and national competent authorities if there is no new scientific knowledge or if no product-specific data are made available. The Monographs on well-established use and/or traditional use are published by the EMA. In contrast, List Entries are developed by the same process like Monographs but are published by the European Commission and their content is binding to all Member States. List Entries are only developed for traditional use.

9.4.2 Well-Established Use

The concept of well-established use was implemented in the European legislation not only for herbal medicinal products but also for other "old" medicinal products that were already on the market when the legislation was developed. The facilitation was based on the strategy that the applicant shall **not** be required to provide the results of toxicological and pharmacological tests or the results of clinical trials if he or she can demonstrate that the constituent or constituents of the medicinal product have a well-established medicinal use with recognized efficacy and an acceptable level of safety, by means of a detailed scientific bibliography. This option should help to avoid unnecessary tests and trials. Nonclinical and clinical characteristics shall be addressed in a detailed scientific bibliography of published scientific literature, which is discussed in the dossier for application by an expert.

The time of accepted medicinal use in the European Union must be at least 10 years. The HMPC agreed that it is also important to consider quantitative aspects of the use of the active substance, the degree of scientific interest in the use of the substance, and the coherence of scientific assessments and published scientific literature. Basic reflections were laid down in the guideline on the assessment of clinical safety and efficacy (EMEA/HMPC/104613/05). In the development of Monographs for well-established use a systematic review of all clinical data is performed, taking into account the quality of the clinical trials (e.g., sufficient number of patients, good clinical practice). According to the guideline at least one controlled clinical study (clinical trial, postmarketing study, epidemiological study) of good quality is required to substantiate efficacy for a well-established use Monograph.

9.4.3 Traditional Use

When experiences with evaluation of well-established use of herbal medicinal products were reflected in Europe, it became obvious that the requirements could probably not be fulfilled for many herbal medicines with a tradition in the European market. Accordingly, a new legislation was established—Directive 2004/24/EC [3], which was amending the overall Directive 2001/83/EC[2]—in order to create an option for a simplified registration for those products that had a long tradition of usage, which could be accepted as a substitute for the data especially on safety and efficacy. The time to establish a tradition was set to 30 years, at least for 15 years of which a product should have been in medicinal use in the European Union. The second part of 15 years of medicinal use could be not only be in the European Union but also in any other part of the world. In article 16 (1) of Directive 2001/83/EC [2] as amended the following inclusion criteria were defined as a precondition for traditional use:

- indication(s) appropriate to traditional herbal medicinal products;
- use without the supervision of a medical practitioner for diagnosis, prescription, or monitoring of treatment;
- specified strength/posology;
- only oral use, external use, and inhalation;
- sufficient data on traditional use of the product (safety);
- pharmacological effects/efficacy plausible on the basis of long-standing use and experience.

The objective of these inclusion criteria was to assure that only safe traditional herbal medicinal products are subject to a registration. If necessary, a national competent authority could ask for additional data on safety. The quality of traditional herbal medicinal products must meet the same criteria as any other herbal medicinal product. The legislation demands a specific labeling for traditional herbal medicinal products. The package leaflet must include a statement that the product is a traditional herbal medicinal product for use in specified indications exclusively based on long-standing use and the user should consult a doctor or qualified health care practitioner if the symptoms persist during the use of the product or if adverse effects not mentioned in the package leaflet occur.

9.5 PROCEDURE TO ESTABLISH MONOGRAPHS

The HMPC established a priority list of herbal substances, for which a Monograph and or a List Entry should be established. Important parameters for prioritization were interests from the Member States of the European Union, suggestions from interested parties, and also inclusion in other sets of Monographs. The process of development of a Monograph is started by

approval of a rapporteur, which is suggested by the MLWP to the HMPC. At the same time ideally a peer reviewer is nominated who is responsible for cross-checking the documents at specific steps of the process in order to achieve an appropriate quality and consistency. The EMA is publishing a general call for scientific data, which are, e.g., provided by interested parties, and the rapporteur is requesting data on existing medicinal products from all Member States. Moreover, the rapporteur is carefully collecting all scientific data that are available in the public domain. Subsequently, the rapporteur elaborates a draft assessment report and a draft reference list, from which a Monograph is derived. The draft documents are discussed at the MLWP, and the documents are improved and rediscussed until the MLWP decides to forward the draft Monograph and the accompanying documents to the HMPC. Before discussion at the HMPC the peer reviewer is controlling quality of the documents and consistency with decisions made so far. If the HMPC agrees with the draft suggested by the MLWP it is adopted for public consultation. Monograph, assessment report, and reference list are published at the web site of EMA for a period of 3 months to enable interested parties and the public in general to submit comments. At the end of the consultation period the rapporteur compiles an overview of comments and based on this document the MLWP discusses whether a modification of the Monograph is justified. When the whole package of Monograph, assessment report, list of references, and overview of comments is finalized by the MLWP, the documents are peer reviewed again and then forwarded to the HMPC for final adoption. After final adoption of a Monograph all the documents for a herbal substance are published at the Web site of EMA (www.ema.europa.eu) [6] and are available to the public by just three mouse clicks.

When assessing the data for development of a Monograph it is checked in parallel whether a List Entry could be drafted. However, in praxis there are always some data missing (e.g., data on genotoxicity). Therefore, only a limited number of List Entries have been released so far. In case that the HMPC is adopting a List Entry the final decision and publication is up to the European Commission.

The HMPC also experienced that it may not be possible to develop a Monograph. A simple reason may be the overall lack of sufficient data and the fact that the projects are routinely put on hold in order to avoid investment of additional resources. In some cases there may be legal reasons, which do not allow establishment of a Monograph, or there may be some concerns on the safety of a herbal substance. In this situation the process is finalized by releasing a public statement that explains the reasons that hindered establishment of a Monograph. Until March 2014 the HMPC released 125 Monographs, 12 List Entries, and 12 Public Statements. The majority of Monographs resulted in an assignment of traditional use, 12 Monographs concluded well-established and traditional use for different herbal preparations of a herbal substance and 12 Monographs concluded for well-established use only. An overview of the results of the projects finished, under evaluation, and started so far is given in Tables 9.2–9.4.

After the HMPC initiated the process in the first years after its establishment the committee could meanwhile finish about 20 Monographs per year. An important step to guarantee sustainability of the system was the start of a revision process. Each Monograph will be regularly updated and modified according to the needs of current scientific knowledge every 5 years. Following this approach the set of Community Monograph will be a valuable and official standard in the Member States of the European Union for the next decades.

In comparison with other sets of Monographs, e.g., by European Scientific Cooperative on Phytotherapy (ESCOP) [7] and the World Health Organization [8], the process is more advanced with respect to transparency and public availability. At the end of the twentieth century in Germany and other countries the Monographs of the Commission E [9] were regarded as a gold standard, but this historic set is outdated because the Monographs had not been updated to current knowledge.

9.6 USAGE AND ACCEPTANCE OF MONOGRAPHS

Until May 2014 about 1500 registrations for traditional herbal medicinal products have been granted by the Member States of the European Union. About 1000 applications are under assessment [6]. Many of these applications are based on Community Monographs. In most of the Member States of the European Union the standards are accepted and applied when decisions are made at the national level. The pharmaceutical companies are increasingly exploring the benefits of European procedures addressing a selection of Member States, DCP, and MRP. If a harmonized decision is predictable, a company needs only to create one application file and the national competent authorities share their resources used for assessment. More and more examples are demonstrating that the system is working appropriately.

The indications that are granted for registered traditional herbal medicinal products are rather

TABLE 9.2 Herbal Substances Evaluated by the HMPC and Decisions Adopted

T	Absinthii herba	*Artemisia absinthium* L.	Wormwood
PS	Adhatodae vasicae folium	*Adhatoda vasica* Nees	Malabar nut leaf
T, W	Agni casti fructus	*Vitex agnus-castus* L.	Agnus castus fruit
T	Agropyri repentis rhizoma	*Agropyron repens* (L.) P. Beauv.	Couch grass rhizome
PS	Allii cepae bulbus	*Allium cepa* L.	Onion
W	Aloe bardadensis/Aloe capensis	*Aloe barbadensis* Miller/*Aloe ferox* Miller	Aloes
T	Althaeae radix	*Althaea officinalis* L.	Marshmallow root
PS	Angelicae sinensis radix	*Angelica sinensis (Oliv.)* Diels	Winter cherry root
T	Anisi aetheroleum	*Pimpinella anisum* L.	Anise oil
LE, T	Anisi fructus	*P. anisum* L.	Aniseed
T	Arctii radix	*Arctium lappa* L.	Burdock root
T	Avenae fructus	*Avena sativa* L.	Oat fruit
T	Avenae herba	*A. sativa* L.	Oat herb
T	Betulae folium	*Betula pendula* Roth/*Betula pubescens* Ehrh.	Birch leaf
T	Boldi folium	*Peumus boldus* Molina	Boldo leaf
T	Bursae pastoris herba	*Capsella bursa-pastoris* (L.) Medikus	Shepherds purse
LE, T	Calendulae flos	*Calendula officinalis* L.	Calendula flower
T	Camelliae non fermentatum folium	*Camellia sinensis* (L.) Kuntze, non *fermentatum folium*	Green tea
T	Caryophylli floris aetheroleum	*Syzygium aromaticum* (L.) Merill et L. M. Perry	Clove oil
PS	Caryophylli flos	*S. aromaticum* (L.) Merill et L. M. Perry	Clove
T	Centaurii herba	*Centaurium erythraea* Rafn.	Centaury
PS	Centellae asiaticae herba	*Centella asiatica* L. Urban	Centella
T	Chamomillae romanae flos	*Chamaemelum nobile* (L.) All. (*Anthemis nobilis* L.)	Roman chamomile flower
PS	Chelidonii herba	*Chelidonium majus* L.	Greater celandine
T	Cichorii intybi radix	*Cichorium intybus* L.	Chicory root
W	Cimicifugae rhizoma	*Cimicifuga racemosa* (L.) Nutt.	Black Cohosh
T	Cinnamomi cortex	*Cinnamomum veri* J. S. Presl (*Cinnamomum zeylancium* Nees)	Cinnamon
T	Cinnamomi corticis aetheroleum	*C. verum* J. S. Presl (*C. zeylanicum* Nees)	Cinnamon Bark oil
PS	Citri bergamia aetheroleum	*Citrus bergamia* Risso & Poiteau	Bergamot oil
T	Colae semen	*Cola nitida* (Vent.) Schott et Endl. And its varieties and *Cola acuminata* (*P. Beauv.*) Schott et Endl.	Cola
T	Cucurbitae semen	*Cucurbita pepo* L.	Pumpkin seed

Continued

TABLE 9.2 Herbal Substances Evaluated by the HMPC and Decisions Adopted—cont'd

T	Curcumae longae rhizoma	*Curcuma longa* L.	Turmeric
T	Curcumae xanthorrhizae rhizoma	*Curcuma xanthorrhiza* Roxb. (*C. xanthorrhiza* D. Dietrich).	Javanese turmeric
T	Cynarae folium	*Cynara scolymus* L.	Artichoke leaf
T	Echinaceae angustifoliae radix	*Echinacea angustifolia* DC.	Narrow-leaved coneflower root
T	Echinaceae pallidae radix	*Echinacea pallida* (Nutt.) Nutt.	Pale coneflower root
LE, T, W	Echinaceae purpureae herba	*Echinacea purpurea* (L.) Moench	Purple coneflower herb
T	Echinaceae purpureae radix	*E. purpurea* (L.) Moench.	Purple coneflower Root
LE, T	Eleutherococci radix	*Eleutherococcus senticosus (Rupr. et Maxim.) Maxim.*	Eleutherococcus
T	Equiseti herba	*Equisetum arvense* L.	Equisetum stem
T	Eucalypti aetheroleum	*Eucalyptus globulus* Labill.; *Eucalyptus polybractea* R.T. Baker; *Eucalyptus smithii* R.T. Baker.	Eucalyptus oil
T	Eucalypti folium	*E. globulus* Labill.	Eucalyptus leaf
PS	Euphrasiae herba	*Euphrasia officinalis* L. (mainly subsp. *E. rostkoviana* Hayne)	Eyebright
T	Filipendulae ulmariae flos	*Filipendula ulmaria* (L.) Maxim. (= *Spiraea ulmaria* L.).	Meadowsweet flower
T	Filipendulae ulmariae herba	*F. ulmaria* (L.) Maxim. (= *S. ulmaria* L.).	Meadowsweet
LE, T	Foeniculi amari fructus	*Foeniculum vulgare* Miller subsp. *vulgare var. vulgare*	Bitter fennel
T	Foeniculi amari fructus aetheroleum	*F. vulgare* Miller subsp. *vulgare var. vulgare*	Bitter fennel fruit oil
LE, T	Foeniculi dulcis fructus	*F. vulgare* Miller subsp. *vulgare var. dulce* (Miller) Thellung.	Sweet fennel
W	Frangulae cortex	*Rhamnus frangula* L.	Frangula bark
T	Fraxini folium	*Fraxinus* excelsior L. and *F. angustifolia* Vahl, folium	Ash leaf
T	Fumariae herba	*Fumaria officinalis* L.,	Fumitory
T	Gentianae radix	*Gentiana lutea* L.	Gentian root
T	Ginseng radix	*Panax ginseng* C. A. Meyer	Ginseng
T	Grindeliae herba	*Grindelia robusta* Nutt., *Grindelia squarrosa* (Pursh) Dunal, *Grindelia humilis* Hook. Et Arn., Grindel	Gumweed herb
T	Hamamelidis cortex	*Hamamelis virginiana* L.	Hamamelis bark
T	Hamamelidis folium	*H. virginiana* L.	Hamamelis leaf
LE, T	Hamamelidis folium et cortex aut ramunculus destillatum	*H. virginiana* L.	Hamamelis distillate
T	Harpagophyti radix	*Harpagophytum procumbens* DC; *Harpagophytum zeyheri* Decne	Devil's claw root
T, W	Hederae helicis folium	*Hedera helix* L.	Ivy leaf
T	Hippocastani cortex	*Aesculus hippocastanum* L.	Horse chestnut bark
W, T	Hippocastani semen	*A. hippocastanum* L.	Horse chestnut seed

TABLE 9.2 Herbal Substances Evaluated by the HMPC and Decisions Adopted—cont'd

W, T	Hyperici herba	*Hypericum perforatum* L.	St. John's wort
T	Juglandis folium	*Juglans regia* L.	Walnut leaf
T	Juniperi aetheroleum	*Juniperus communis* L.	Juniper oil
T	Juniperi pseudo-fructus	*J. communis* L.	Juniper berry
T	Lavandulae aetheroleum	*Lavandula angustifolia* Mill. (*L. officinalis* Chaix)	Lavender oil
T	Lavandulae flos	*L. angustifolia* Mill. (*L. officinalis* Chaix)	Lavender
T	Leonuri cardiacae herba	*Leonurus cardiaca* L.	Motherwort
T	Levistici radix	*Levisticum officinale* Koch	Lovage root
LE, W, T	Lini semen	*Linum usitatissimum* L.	Linseed
T	Liquiritiae radix	*Glycyrrhiza glabra* L. and/or *Glycyrrhiza inflata* Bat. and/or *Glycyrrhiza uralensis* Fisch.	Liquorice root
T	Lupuli flos	*Humulus lupulus* L.	Hop strobile
T	Marrubii herba	*Marrubium vulgare* L.	White horehound
T	Mate folium	*Ilex paraguariensis* St. Hil.	Maté leaf
T	Meliloti herba	*Melilotus officinalis* (L.) Lam.	Melilot
T	Melissae folium	*Melissa officinalis* L.	Melissa leaf
LE, W, T	Menthae piperitae aetheroleum	*Mentha x piperita* L.	Peppermint oil
T	Menthae piperitae folium	*M. x piperita L.*	Peppermint leaf
T	Millefolii flos	*Achillea millefolium* L.	Yarrow flower
T	Millefolii herba	*A. millefolium* L.	Yarrow
T	Myrrha, gummi-resina	*Commiphora molmol* Engler	Myrrh
T	Oenotherae biennis oleum	*Oenothera biennis* L.; *Oenothera lamarckiana* L.	Evening primrose oil
T	Oleae folium	*Olea europaea* L.	Olive leaf
T	Ononidis radix	*Ononis spinosa* L. and *Ononis arvensis* L.	Restharrow root
T	Origani dictamni herba	*Origanum dictamnus* L.	Dittany of crete herb
T	Orthosiphonis folium	*Orthosiphon stamineus* Benth.	Java tea
T	Passiflorae herba	*Passiflora incarnata* L.	Passion flower
T	Paulliniae semen	*Paullinia cupana* Kunth, semen	Guarana
T	Pelargonii radix	*Pelargonium sidoides* DC; *Pelargonium reniforme* Curt.	Pelargonium root
T	Phaseoli fructus (sine semine)	*Phaseolus vulgaris* L.	Green bean pod
T	Plantaginis lanceolatae folium	*Plantago lanceolata* L.	Ribwort plantain
W	Plantaginis ovatae semen	*Plantago ovata* Forsk.	Ispaghula seed
W	Plantaginis ovatae seminis tegumentum	*P. ovata* Forsk.	Ispaghula husk
T	Polypodii rhizoma	*Polypodium vulgare* L.	Polypody rhizome
T	Primulae flos	*Primula veris* L.; *Primula elatior* (L.) Hill	Primula flower

Continued

TABLE 9.2 Herbal Substances Evaluated by the HMPC and Decisions Adopted—cont'd

T	Primulae radix	*P. veris* L.; *P. elatior* (L.) Hill	Primula root
W	Psyllii semen	*Plantago afra* L.; *Plantago indica* L.	Psyllium seed
T	Quercus cortex	*Quercus robur* L.; *Quercus petraea* (Matt.) Liebl.; *Quercus pubescens* Willd.	Oak bark
W	Rhamni purshianae cortex	*Rhamnus purshianus* D.C.	Cascara
W	Rhei radix	*Rheum palmatum* L.; *Rheum officinale* Baillon	Rhubarb
T	Rhodiolae roseae rhizoma et radix	*Rhodiola rosea* L.	Arctic root
T	Ribis nigri folium	*Ribes nigrum* L.	Blackcurrant leaf
T	Rosmarini aetheroleum	*Rosmarinus officinalis* L.	Rosemary oil
T	Rosmarini folium	*Rosmarinus officinalis* L.	Rosemary leaf
T	Rubi idaei folium	*Rubus idaeus* L.	Raspberry leaf
T	Rusci rhizoma	*Ruscus aculeatus* L.	Butcher's broom
W, T	Salicis cortex	*Salix* [various species including *S. purpurea* L.; *S. daphnoides* Vill.; *S. fragilis* L.]	Willow bark
PS	Salviae officinalis aetheroleum	*Salvia officinalis* L.	Sage oil
T	Salviae officinalis folium	*S. officinalis* L.	Sage leaf
T	Sambuci flos	*Sambucus nigra* L.	Elder flower
	Sambuci fructus	*S. nigra* L.	Elderberry
W	Sennae folium	*Cassia senna* L.; *Cassia angustifolia* Vahl	Senna leaf
W	Sennae fructus	*C. senna* L.; *C. angustifolia* Vahl	Senna pods
T	Solani dulcamarae stipites	*Solanum dulcamara* L.	Woody nightshade stem
T	Solidaginis virgaureae herba	*Solidago virgaurea* L.	European goldenrod
T	Tanaceti parthenii herba	*Tanacetum parthenium* (L.) Schultz Bip.	Feverfew
T	Taraxaci folium	*Taraxacum officinale* Weber ex Wigg.	Dandelion leaf
T	Taraxaci radix cum herba	*T. officinale* Weber ex Wigg.	Dandelion root with herb
LE, T	Thymi aetheroleum	*Thymus vulgaris* L.; *Thymus zygis* Loefl. ex L.	Thyme oil
T	Thymi herba	*T. vulgaris* L.; *T. zygis* Loefl. ex L.	Thyme
T	Thymi herba/Primulae radix	*T. vulgaris* L.; *T. zygis* Loefl. ex L./ *Primula veris* L.; *Primula elatior* (L.) Hill	Thyme/primula root
T	Tiliae flos	*Tilia cordata* Miller, *Tila platyphyllos* Scop., *Tilia x vulgaris* Heyne or their mixtures	Lime flower
PS	Tiliae tomentosae flos	*Tilia tomentosa* Moench	Silver lime flower
Z	Tormentillae rhizoma	*Potentilla erecta (L.)* Raeusch.	Tormentil
Z	Trigonellae foenugraeci semen	*Trigonella foenum-graecum* L.	Fenugreek
Z	Urticae folium	*Urtica dioica* L.; *Urtica urens* L.	Nettle leaf
Z	Urticae herba	*U. dioica* L.; *U. urens* L.	Nettle herb

TABLE 9.2 Herbal Substances Evaluated by the HMPC and Decisions Adopted—cont'd

Z	Urticae radix	*U. dioica* L.; *U. urens* L.	Nettle root
Z	Uvae ursi folium	*Arctostaphylos uva-ursi* (L.) Spreng.	Bearberry leaf
LE, T	Valerianae radix	*Valeriana officinalis* L.	Valerian root
W, T	Valerianae radix/Lupuli flos	*V. officinalis* L./*Humulus lupulus* L.	Valerian root/hop strobile
T	Verbasci flos	*Verbascum thapsus* L.; *V. densiflorum* Bertol. (*V. thapsiforme* Schrad); *V. phlomoides* L.	Mullein flower
T	Violae tricoloris herba cum flore	*Viola tricolor* L.	Wild pansy
PS	Visci albi herba	*Viscum album* L.	Mistletoe
LE, W, T	Vitis viniferae folium	*Vitis vinifera* L.	Grapevine leaf
PS	Withania somnifera radix	*Withania somnifera* (L.) Dunal	Winter cherry root
W, T	Zingiberis rhizoma	*Zingiber officinale* Roscoe	Ginger

T, Traditional Use Monograph; W, Well-Established Use Monograph; LE, List Entry; PS, Public Statement.

representative for therapeutic use of medicinal plants in European phytotherapy (see listing below). Nevertheless, some indications are still under debate, e.g., the majority of Member States does not regard cardiovascular indication acceptable for traditional herbal medicinal products because of safety considerations linked to an obligate diagnosis. In Austria and Germany this type of indication is still granted for traditional herbal medicinal products due to a long-standing tradition.

The following herbal substances were most frequently approved as single active substance traditional herbal medicinal products [6]:

- Harpagophyti radix
- Hyperici herba
- Pelargonii radix
- Valerianae radix
- Passiflorae herba
- Ginseng radix

TABLE 9.3 Herbal Substances Under Evaluation by the HMPC, Draft has Already Been Published for Consultation

Agrimoniae herba	*Agrimonia eupatoria* L.	Agrimony
Allii sativi bulbus	*Allium sativum* L.	Garlic
Andrographidis paniculatae folium	*Andrographis paniculata* Nees, folium	Kalmegh
Arnicae flos	*Arnica montana* L.	Arnica flower
Fucus vesiculosus, thallus	*Fucus vesiculosus* L.	Bladderwrack
Ginkgo folium	*Ginkgo biloba* L.	Ginkgo leaf
Lichen islandicus	*Cetraria islandica* (L.) *Acharius s.l.*	Iceland moss
Matricariae aetheroleum	*Matricaria recutita* L.	Matricaria oil
Matricariae flos	*M. recutita* L.	Matricaria flower
Melaleucae alternifoliae aetheroleum	*Melaleuca alternifolia* (Maiden and Betche) Cheel	Tea tree oil
Rosae flos	*Rosa centifolia* L.; *Rosa gallica* L.; *Rosa damascena* Mill.	Rose flower
Sisymbrii officinalis herba	*Sisymbrium officinale* (L.) Scop., herba	Hedge mustard
Symphyti radix	*Symphytum officinale* L.	Comfrey root

TABLE 9.4 Herbal Substances, the Evaluation of Which by the HMPC Has Been Started

Calendulae herba		Marigold
Capsici fructus	*Capsicum annuum* L. var. minimum (Miller) Heiser	Capsicum
Carvi aetheroleum	*Carum carvi* L.	Caraway oil
Carvi fructus	*C. carvi* L.	Caraway fruit
Cisti cretici folium/resinum	*Cisti creticus* L.	Pink rock-rose
Crataegi folium cum flore	*Crataegus* spp, folium cum flore	Hawthorn leaf and flower
Crataegi fructus	*Crataegus monogyna* Jacq. (Lindm.); *Crataegus laevigata* (Poir.) D.C.	Hawthorn berries
Cyani flos		Cornflower
Epilobii herba		Willow herb
Eschscholtziae herba cum flore	*Eschscholtzia california* Cham.	California poppy
Fragariae folium	*Fragaria vesca* L.	Wild strawberry leaf
Glycine max, lecithin	*Glycine max* (L.) Merr.	Soybean
Helichrysi flos		Sandy everlasting
Myrtilli folium	*Vaccinium myrtillus* L.	Billberry leaf
Myrtilli fructus siccus	*V. myrtillus* L.	Billberry fruit
Origani majoranae herba	*Origanum majorana* L.	Marjoram
Paeoniae radix	*Paeonia* spec. (to be defined)	Peony root
Picrorhizae kurroae rhizoma et radix	*Picrorhiza kurroa* Royle ex. Benth.	Katula
Pilosellae herba cum flore	*Hieracium pilosella* L.	Mouse-ear hawkweed
Pistacia lentiscus, resinum	*Pistacia lentiscus* L.	Mastic tree resin
Polygoni avicularis herba	*Polygonum aviculare* L.	Knotweed herb, common
Prunus africanae cortex	*Prunus africana* (Hook f.) Kalkm.	*Pygeum africanum* bark
Ricini oleum	*Ricinus communis* L.	Castor oil
Sabalis serrulatae fructus	*Serenoa repens* (Bartram) Small (*Sabal serrulata* (Michaux) Nichols	Saw palmetto fruit
Saccharomyces cerevisiae/*Saccharomyces boulardii*		Yeast
Salviae trilobae folium	*Salvia triloba* L.	Sage leaf
Sideritis herba	*Sideritis* spec. (to be defined)	Ironwort
Silybi mariani fructus	*Silybum marianum* L. Gaertner	Milkthistle fruit
Uncariae tomentosae cortex	*Uncariae tomentosae* (Willd.) DC.	Cat's claw

The following are the therapeutic areas of major importance for traditional herbal medicinal products approved [6]:

- Cough and cold
- Mental stress and mood disorders
- Gastrointestinal disorders
- Urinary tract and gynecological disorders
- Sleep disorders and temporary insomnia
- Pain and inflammation
- Skin disorders and minor wounds
- Fatigue and weakness
- Mouth and throat disorders
- Venous circulatory disorders (Germany and Austria)
- Loss of appetite

9.7 GUIDANCE ON QUALITY, EFFICACY, AND SAFETY: COORDINATION

Traditional and herbal medicinal products are defined by the manufacturing procedure and a set of specifications. The reproducible quality is a precondition to assure safe and effective therapeutic use of these products. The quality must be demonstrated at all steps of the manufacturing process:

- harvest or collection of the plant material
- herbal substance
- herbal preparation
- finished herbal medicinal product or finished traditional herbal medicinal product

The European Pharmacopeia provides standards on methodology in general Monographs and basic quality requirements for herbal substances and selected herbal preparations in specific Monographs, whereas the quality guidance of the HMPC is addressing quality issues that have to be regarded when providing a dossier for application. The HMPC installed a Q DG that is supporting the HMPC in establishing harmonized positions and guidance with respect to requirements and assessments linked to applications for marketing authorizations or registrations. For example, guidelines are addressing quality requirements, specifications, stability testing, and labeling. Meanwhile, the harmonization is also targeting very specific and tricky issues, for instance, by publishing guidance about the level of purification of herbal preparations or the application of marker concepts. In future the HMPC will include a statement in the assessment report of a Monograph to clarify the classification of herbal preparations (standardized, quantified, or other extract). The HMPC and European Directorate for Quality of Medicines & Health Care have established a very valuable coordination of their activities in order to complement the requirements and to offer a complete set of guidance to applicants and national competent authorities(Table 9.5)

The guidance on nonclinical efficacy and safety is addressing on one hand the evaluation criteria for establishingMonographs. On the other hand, topics of specific and multidisciplinary interest are addressed. These may refer to safety concerns (e.g., recommendations for thresholds for the levels of thujone) or cover a special field like genotoxicity. Because traditional knowledge is not suitable to substitute data on cancerogenicity and genotoxicity there was an approach to have a basic investigation of genotoxicity for traditional herbal medicinal products by means of an AMES test. If there is no concern from literature and the AMES test is negative, then no further data are required. In case of concerns or a positive AMES test the investigations have to follow a decision tree (Tables 9.6–9.8).

An intrinsic part of the European regulatory framework is to include all medicinal products in a phamacovigilance system, which is surveying the market after marketing authorization or registration in order to detect signals from recording adverse events. Herbal and traditional herbal medicinal products are embedded in this system, but the legal provisions are following an approach to take into account the particular characteristics of these products. For example periodic safety update reports are only requested if there is a distinct safety concern.

Manufacturing site inspections are principally possible. Responsibilities are attributed following different concepts (centralized or federal), but basic requirements are also valid for traditional and herbal medicinal products.

9.8 OUTLOOK

The legal framework for herbal medicinal products in the European Union has been established to set up a harmonized approach to offer European citizens herbal medicinal products with appropriate quality, safety, and efficacy. Pharmaceutical companies can follow defined requirements to develop successful applications. Despite the long tradition of medicinal use, data on many herbal medicinal products are still limited. The current framework was set up to avoid disappearance of all these products due to regulation. Such products, provided they are very safe, can be marketed as traditional herbal medicinal products. However, their use is restricted and the usage is mostly not recommended for special groups of patients like pregnant or lactating women or younger children. These patients are under special protection of the European legislation. There were even incentives in legislation to improve the availability of medicinal products to the pediatric population [10].

The European set of Community Monographs on herbal substances and preparations derived thereof is defining a unique standard. It is an excellent model of harmonization of scientific assessment among a large set of countries with different traditional backgrounds in application of herbal medicines. As set of Community Monographs is developed in close communication with the scientific community and interested parties from pharmaceutical industry the standards provided are quite robust. There are still some issues for which Member States of the European Union have a divergent opinion, but these are made public together with the

TABLE 9.5 Selected Examples of Guidance Documents on Quality of Traditional and Herbal Medicinal Products Released by the HMPC

Quality	
Title	**Reference number**
Reflection paper on microbiological aspects of herbal medicinal products and traditional herbal medicinal products	EMA/HMPC/95714/2013
Questions & answers (Q&A) on quality of herbal medicinal products/traditional herbal medicinal products	EMEA/HMPC/41500/2010 Rev. 2
Quality of essential oils as active substances in herbal medicinal products/traditional herbal medicinal products	EMEA/HMPC/84789/2013
Reflection paper on the use of recovered/recycled solvents in the manufacture of herbal preparations for use in herbal medicinal products/traditional herbal medicinal products	EMEA/HMPC/453258/2013
Use of recovered/recycled solvents in the manufacture of herbal preparations for use in herbal medicinal products/traditional herbal medicinal products	EMEA/HMPC/13658/2013
Reflection paper on stability testing of herbal medicinal products and traditional herbal medicinal products	EMEA/HMPC/3626/09
Reflection paper on level of purification of extracts to be considered as herbal preparations	EMEA/HMPC/186645/08
Development of a guideline on preparation on herbal teas	EMEA/HMCP/451978/08
Markers used for quantitative and qualitative analysis of herbal medicinal products and traditional herbal medicinal products	EMEA/HMPC/253629/07
Declaration of herbal substances and herbal preparations in herbal medicinal products/traditional herbal medicinal products in the Summary of Product Characteristics (SPC)	EMEA/HMPC/CHMP/CVMP/287539/05 Rev. 1
Quality of combination herbal medicinal products/traditional herbal medicinal products	EMEA/HMPC/CHMP/CVMP/214869/06
Nonpharmacopoeial reference standards for herbal substances, herbal preparations, and herbal medicinal products/traditional herbal medicinal products	EMA/HMPC/312890/2012
Quality of herbal medicinal products/traditional herbal medicinal products	CPMP/QWP/2819/00 Rev. 2
Test procedures and acceptance criteria for herbal substances, herbal preparations, and herbal medicinal products/traditional herbal medicinal products	CPMP/QWP/2820/00 Rev. 2
The use of fumigants	EMEA/HMPC/125562/06
Good agricultural and collection practice for starting materials of herbal origin	EMEA/HMPC/246816/05

Monographs and accompanying documents. Together with the basic quality requirements defined by the European Pharmacopeia the Community Monographs provide a complete system for regulation of herbal medicinal products in the market.

As plants are not only used as herbal medicines but also for other purposes, there is still a problem of classification of products. There is an existing borderline area where products derived from plants may also be marketed as food (especially food supplements), medical devices, or cosmetics. These different categories of products are covered by different legal frameworks and until now the final classification is not harmonized but is still left to the responsibility of the individual Member States. This situation is not satisfying and there is a need for better definitions to minimize problems and confusion of citizens with borderline products.

Beside the approach of a harmonized European legislation the market of herbal products has also been challenged by globalization during the past decades. European herbal medicinal products have been exported worldwide, and vice versa, herbal and traditional medicines from other parts of the world were brought to the European Union. The HMPC initiated a project within its work program that is exploring options and limitations of the European regulatory framework to handle herbal traditional medicines of non-European origin. Reflections about this issue and an overview of relevant parts of legislation were published in a Question & Answer document. Globally, there is no unique definition of herbal medicines or related products. Without any doubt there is a similar approach by regulators to assess quality, efficacy, and safety but criteria for evaluation and requirements

TABLE 9.6 Selected Examples of Guidance Documents on Nonclinical Issues of Traditional and Herbal Medicinal Products Released by the HMPC

Nonclinical	
Title	**Reference number**
Ethanol content in herbal medicinal products and traditional herbal medicinal products used in children	EMA/HMPC/85114/08
Selection of test materials for genotoxicity testing for traditional herbal medicinal products/herbal medicinal products	EMEA/HMPC/67644/09
Assessment of genotoxicity of herbal substances/preparations	EMEA/HMPC/107079/07
Nonclinical documentation for herbal medicinal products in applications for marketing authorization (bibliographical and mixed applications) and in applications for simplified registration	EMEA/HMPC/32116/05

are divergent. Obviously there is a need to discuss regulation of herbal and traditional medicines at a global level to improve exchange and availability of safe products of reasonable quality all over the world. Moreover, the scientific community should increase current knowledge with research initiatives including

TABLE 9.7 Selected Examples of Guidance Documents on Efficacy and Safety of Traditional and Herbal Medicinal Products Released by the HMPC

Clinical efficacy and safety	
Title	**Reference number**
Reflection paper on the necessity of initiatives to stimulate the conduct of clinical studies with herbal medicinal products in the pediatric population	EMA/HMPC/833398/2009
Reflection paper on the adaptogenic concept	EMEA/HMPC/102655/07
Clinical assessment of fixed combinations of herbal substances/herbal preparations	EMEA/HMPC/166326/05
Assessment of clinical safety and efficacy in the preparation of Community Herbal Monographs for well-established and of Community Herbal Monographs/entries to the community list for traditional herbal medicinal products/substances/preparations	EMEA/HMPC/104613/05

TABLE 9.8 Selected Examples of Multidisciplinary Guidance Documents on Traditional and Herbal Medicinal Products Released by the HMPC

Multidisciplinary	
Title	**Reference number**
Use of herbal medicinal products containing toxic, unsaturated pyrrolizidine alkaloids	EMA/HMPC/893108/2011
Use of herbal medicinal products containing thujone	EMA/HMPC/732886/2010 Rev.1
Reflection paper on the risks associated with furocoumarins contained in preparations of *Angelica archangelica* L.	EMEA/HMPC/317913/06
Herbal medicinal products containing *Cimicifugae racemosae* rhizoma—serious hepatic reactions	EMEA/269259/2006
Assessment of case reports connected to herbal medicinal products containing *C. racemosae* rhizoma (black cohosh, root)	EMEA/HMPC/269258/2006 Rev. 1
Chamomilla containing herbal medicinal products	EMEA/HMPC/138309/2005
Allergenic potency of herbal medicinal products containing soya or peanut protein	EMEA/HMPC/138139/2005
Use of herbal medicinal products containing asarone	EMEA/HMPC/139215/2005
Risks associated with the use of herbal products containing *Aristolochia* sp.	EMEA/HMPC/138381/2005
Use of herbal medicinal products containing pulegone and menthofuran	EMEA/HMPC/138386/2005
Capsicum/capsaicin-containing herbal medicinal products	EMEA/HMPC/138379/2005
Use of herbal medicinal products containing estragole	EMEA/HMPC/137212/2005
Use of herbal medicinal products containing methyleugenol	EMEA/HMPC/138363/2005

new methodology and technology. Better knowledge about multitarget mode of actions, synergies and interactions and availability in the human body will be a precondition for future use of traditional and herbal medicinal products.

CONFLICT OF INTEREST

The views expressed in this article are the views of the authors and may not be understood or quoted as

being made on behalf of or reflecting the position of the European Medicines Agency or one of its Committees or Working Parties. There is no conflict of interest.

The data and figures provided are based on data available in May 2014.

References

[1] Regulation (EC) No 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down Community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency.
[2] Consolidated Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use as amended by Directive 2002/98/EC, Directive 2003/63/EC, Directive 2004/24/EC, Directive 2004/27/EC and Directive 2008/29/EC.
[3] Directive 2004/24/EC of the European Parliament and of the Council of 31 March 2004 amending, as regards traditional herbal medicinal products, Directive 2001/83/EC on the Community code relating to medicinal products for human use.
[4] European pharmacopeia. 7th ed. Strasbourg: EDQM; 2011.
[5] Marketing Authorisation, The Rules governing Medicinal Products in the European community, Notice to applicants, Volume 2A, Chapter 1; EU Pharmaceutical legislation - EudraLex Volume 1.
[6] European Medicines Agency: http://www.ema.europa.eu – Regulatory – Human Medicines.
[7] ESCOP Monographs.1st and 2nd Edition and supplements. In: European scientific cooperative on phytotherapy. Stuttgart: Georg ThiemeVerlag; 2009.
[8] WHO Monographs on selected medicinal plants, Vols. 1–4. Geneva: World Health Organisation; 2009.
[9] Blumenthal M, Busse WR, Goldberg A, Gruenwald J, et al., editors. The complete German commission E monographs. Austin Texas: American Botanical Council; 1998.
[10] Regulation (EC) Nr. 1901/2006 of the European Parliament and of the Council of 12 December 2006 on medicinal products for paediatric use.

LIST OF ABBREVIATIONS

DCP Decentralized Procedure
EC European Community
EDQM European Directorate for Quality of Medicines & Health Care
EMA European Medicines Agency
GCP Good Clinical Practice
HMPC Committee on Herbal Medicinal Products
MLWP Working Party on Community Monographs and List Entries
MRP Mutual Recognition Procedure
ORGAM DG Organizational Matters Drafting Group
Q DG Quality Drafting Group

CHAPTER

10

Bioavailability of Herbal Products: Approach Toward Improved Pharmacokinetics

Pulok K. Mukherjee, Ranjit K. Harwansh, Sauvik Bhattacharyya

School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

OUTLINE

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00010-6

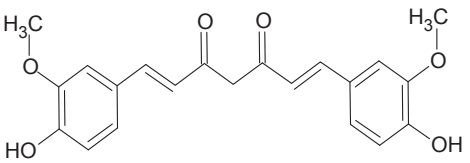
10.1 INTRODUCTION

The therapeutic and phytochemical importance of herbal medicine has been built for the improvement of human health, but its broader application is restricted due to the low bioavailability. The nature of the molecule plays an essential role in enhancing the rate and extent of absorption of molecules when administered through any path. Generally, the problems come with poor lipid-soluble compounds due to limited membrane permeability [1]. Many herbal products demonstrated low therapeutic action due to their solubility problems which finally resulted in low bioavailability despite their extraordinary potential [2]. The strength of any herbal product depends on the delivery of effective levels of the therapeutically active compound. To overcome these limitations of absorption, developing novel herbal drug delivery system with better absorption profile is of premier importance. The therapeutic indices of the associated drugs are improved by increasing the drug concentration at the site of action. On the other hand, their biodistribution is altered in favor of the diseased tissue [3]. In the past century, attention has been focused on the approaches toward improved bioavailability and pharmacokinetics of herbal drugs through development of a novel drug delivery system (NDDS). The application of NDDS is an important approach toward solving bioavailability-related problems associated with phytomolecules. Several novel herbal drug delivery systems specifically liposomes, transfersomes, ethosomes, niosomes, phytosomes, dendrimers, micro/nanoparticles, micro/nanoemulsions (NEs), micelles, etc., have been successfully employed for the delivery of phytopharmaceuticals. The novel formulations have notable advantages compared to conventional formulations like enhancement of solubility and stability, membrane permeability and bioavailability, improved pharmacological activity through sustained-release profile, and reduced toxicity. The novel systems of herbal medicine have the capability to deliver the drug at a rate directed by the needs of the body for an extended period of time, and it should channel the bioactive molecule of herbal products to the site of action [4]. Therefore, the NDDS has a great future for enhancing the therapeutic activity and overcoming problems attributed to herbal medicine [4]. Most bioactive agents (e.g., nutraceuticals and pharmaceuticals) intended for oral administration are found as highly hydrophobic compounds with low water solubility and poor bioavailability. Furthermore, poor solubility also leads to lower absorption in the gastrointestinal (GI) tract (GIT) and therefore limited therapeutic activity [5]. The application of nanotechnology/nanoencapsulation to food, medical, and pharmaceutical industries has received great attention from the scientific community [6]. Forced back by the increasing consumer demand for quality and safer medicinal food products for the promotion of better health, researchers are currently concentrating their efforts in nanotechnology to address topics relevant to food as medication. The NDDS offers various advantages over conventional systems: (1) stabilization in aqueous systems (foodstuffs) of lipophilic bioactive compounds with scarce solubility in water, (2) protection of herbal drugs against degradation reactions with food constituents and minimization of the revision of the food matrix, (3) controlled release by engineering the delivery systems, and (4) enhancement of cell uptake and bioavailability. Especially, the pharmacokinetic profile of nanoencapsulated herbal drugs are enhanced due to their nanoparticle size distribution range.

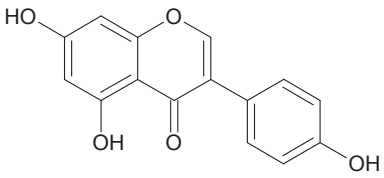
Cutting down the particle size to values below cell size ($\sim$500 nm) produces higher absorption of the active ingredient and higher particle uptake, by enhancing the mechanisms of passive transport through the intestinal membranes [7]. NDDS has been set up for effective and efficacious herbal drug delivery. The present discussion highlights the current status of pharmacokinetics and bioavailability of herbal drugs/products and implication of novel herbal drug delivery technology with particular emphasis on phospholipid-based complex system.

10.2 FACTORS AFFECTING BIOAVAILABILITY AND PHARMACOKINETICS OF HERBAL PRODUCTS

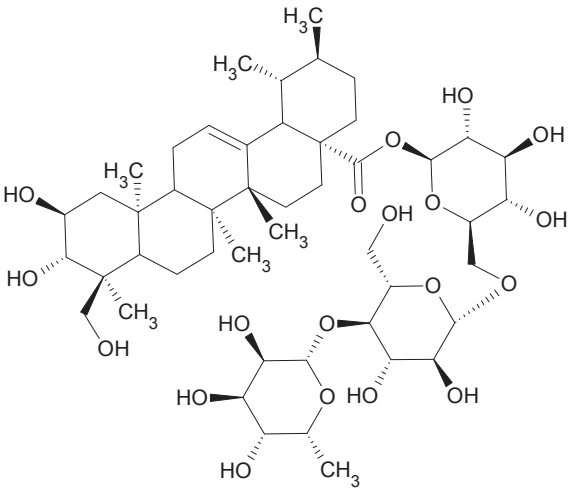
Biological properties of phytochemicals depend on their physical and chemical properties. The chemical structure of phytochemicals determines their rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma. The bioavailability studies in humans show that the quantities of phytochemicals found intact in urine vary from one compound to another. Bioavailability is low for quercetin and rutin (0.3–1.4%), but it is relatively higher for catechin in green tea, genistein and daidzein in soy, and anthocyanidins in red wine (3–26%). A major part of the polyphenols ingested (75–99%) is not found in urine. This means that they have either not been absorbed through the gut barrier, or may be absorbed, but excreted in the bile or metabolized by our own tissues or colonic microflora [8].



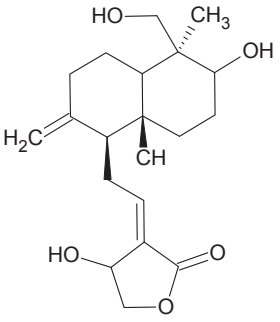
Curcumin (**1**)



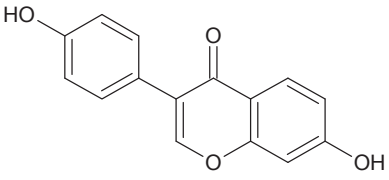
Genistein (**2**)



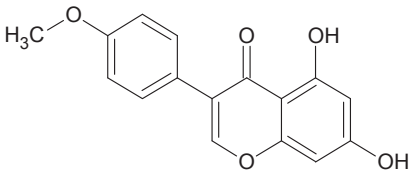
Asiaticoside (**3**)



Andrographolide (**4**)



Daidzein (**5**)



Biochanin A (**6**)

Bioavailability of a compound cannot be accurately predicted; however, analysis by Lipinski's "rule of five" provides some insight. In general, a compound will have better bioavailability when it contains not more than five hydrogen bond donors, not more than 10 hydrogen bond acceptors, molecular mass not greater than 500 Da, log $p \leq 5$, and ≤ 10 rotatable bonds [9]. Most of the phytopharmaceuticals, including curcumin (**1**), asiaticoside (**3**), andrographolide (**4**) and catechin, do not come within these specifications and exhibited low bioavailability. However, compounds such as genistein (**2**), daidzein (**5**), biochanin A (**6**) have good absorptive properties and are excreted by an efflux mechanism into the gut at a high rate that limits their bioavailability. Another rate-limiting factor is solubility and first-pass metabolism [8]. These factors affect the absorption, distribution, metabolism, and elimination (ADME) process of the phytochemicals. Figure 10.1 demonstrated the factors affecting the ADME, which in turn affects their bioavailability and pharmacokinetic profile.

10.2.1 Absorption of the Phytochemicals from GI Lumen

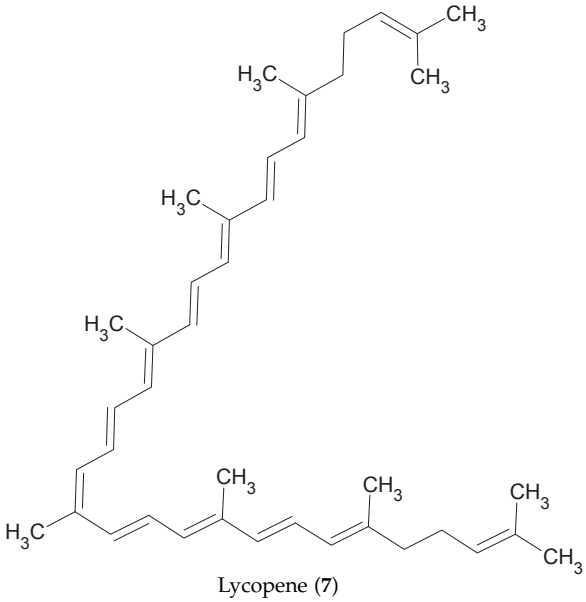
Absorption is the transfer of a drug from its site of administration into the bloodstream. The oral absorption of the drug molecules from GIT basically depends on the dissolution and membrane permeation processes.

10.2.1.1 The Solubility of the Herbal Products

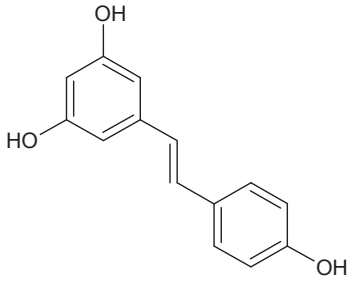
The dissolution of a phytochemical depends on various factors like the log p value, pKa of the compound as well as the pH of GI and intestinal fluids, particle size, and surface area. For example, ellagic acid (EA) (**10**), curcumin (**1**), and resveratrol (**8**) have poor bioavailability due to their poor solubility in aqueous media [8]. The majority of other phytochemicals, such as polyphenols and carotenoids, are either poorly soluble or lipophilic like silybin (**9**), lycopene (**7**), phytosterols, and ω-3 fatty acids [10].

10.2.1.2 The Absorption of Natural Products via Passive Diffusion

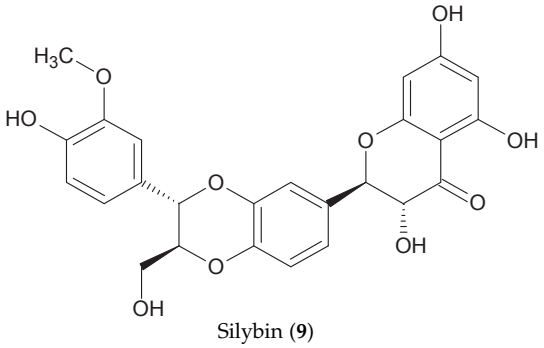
Various physicochemical factors are involved in the absorption of phytochemicals through passive diffusion mechanism. This is because the unionized molecules (acidic compounds) prefer to pass through the lipid barriers of the GI wall, whereas the basic compounds from the intestine. The process of absorption also depends on GIT length, surface area, motility, and blood flow. For example, the log p value is a major parameter for diffusion across a biological membrane and how they might partition in the lipid membrane. The quercetin (11) (log p 1.2 ± 0.1), whereas quercetin-3-O-rhamnoglucoside (rutin), (log p 0.37 ± 0.06), showing greater hydrophilicity comparatively [11].



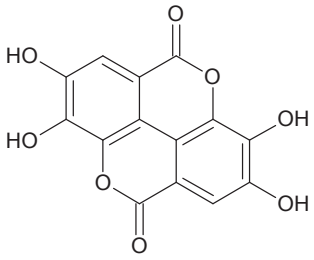
Lycopene (**7**)



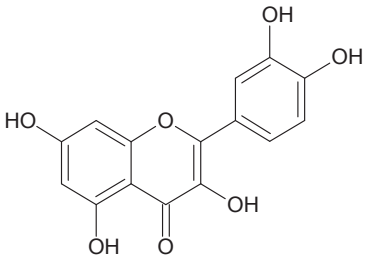
Resveratrol (**8**)



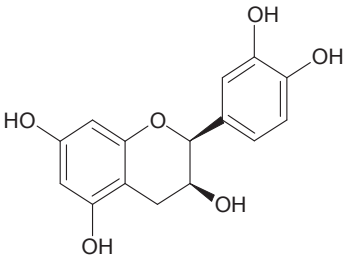
Silybin (**9**)



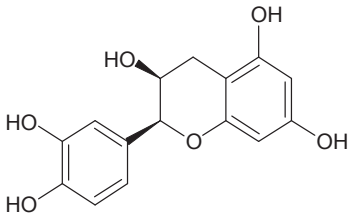
Ellagic acid (**10**)



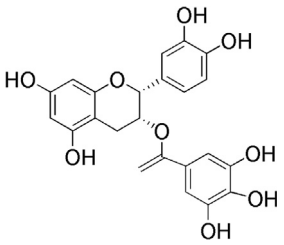
Quercetin (**11**)



(+) Catechin (**12**)



(−) Epicatechin (**13**)



(−) Epicatechin-3-gallate (**14**)

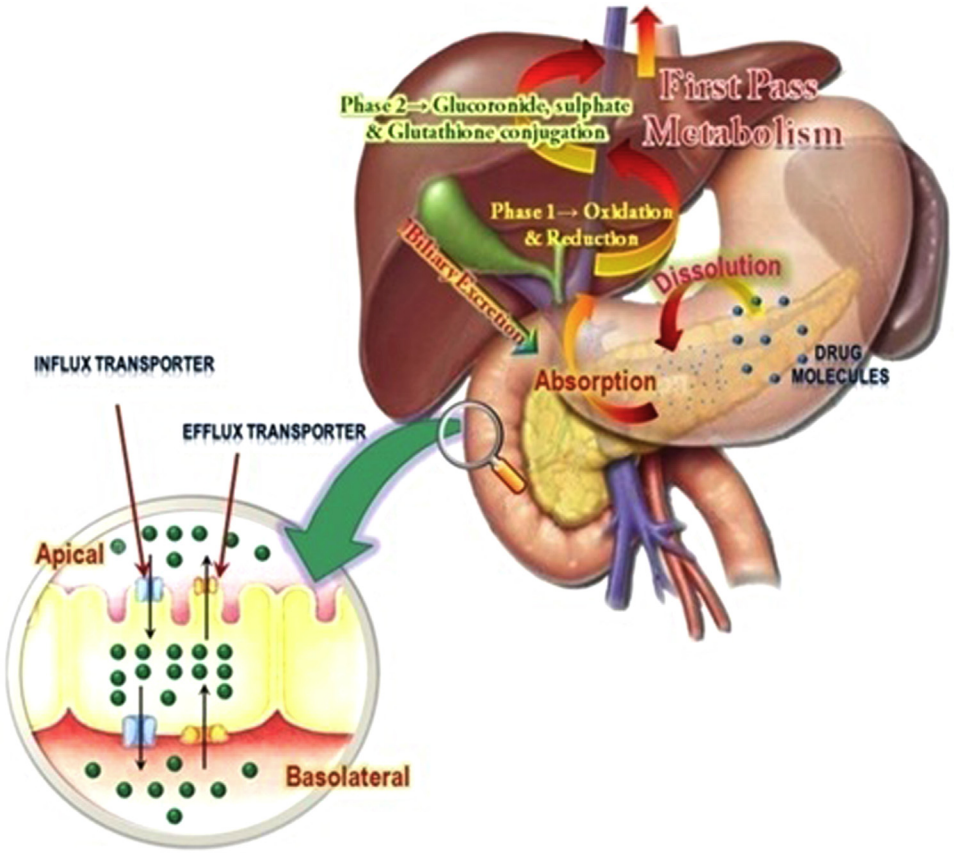


FIGURE 10.1 Different factors affecting the absorption, distribution, metabolism, and elimination (ADME) process of phytochemicals.

Unlike the other flavonoids anthocyanin gets absorbed in the body, although to a limited extent. Absorption is rapid and anthocyanins can be detected within less than 1.5 h after intake, indicating that absorption probably occurs from the stomach or small intestine and is ≤0.1% of the ingested amount. (+)-Catechin (**12**) showed quite high absorption when compared with (−)-epicatechin (**13**), (−)-epicatechin-3-gallate (**14**), and (−)-epigallocatechin-3-gallate [12]. There are only a few studies on the bioavailability of flavanones in humans. Like other flavonoids, the efficiency of absorption for flavanones is poor. The C_{max} of these compounds were in the nanomolar range. But the aglycones appear to be absorbed more rapidly (T_{max} 2−4 h) than the glycosides (T_{max} 6−7 h). This low absorption of flavonoids from the diet is due to the fact that the majority of food flavonoids are bound in glycoside form, which are less absorbed because of their high molecular weight. For example, ellagitannins from *Punica gratum* are very low. They are hydrolyzed to EA in the gut with low absorption profile due to rate-limiting factors [13].

10.2.1.3 *Active Transport Mechanism Involved in the Influx or Efflux of Phytomolecules*

Phytochemicals having large molecular structure cannot be absorbed by passive diffusion, but might be absorbed through carriers by an active transport process. The transport proteins present in the plasma membrane act as channels, pumps, or carriers. The channels operate as specific pathways for the rapid permeation of ions down an electrochemical gradient at the expense of adenosine triphosphate (ATP). Their protein structure is composed of an ATP-binding cassette (ABC) with a conserved consensus sequence, where ATP binding and hydrolysis occurs. Other transporters are solute carriers that promote the transmembrane movement of molecules without hydrolyzing ATP. If only a single type of molecule is transported down an electrochemical gradient, it is classified as a uniporter, while if it moves two molecules at the same time in either the same or opposite directions, it is named a symporter or an antiporter, respectively. Influx and efflux transporters move solutes either into or out of the cells. In absorptive epithelial cells lining the intestine, nutrient influx transporters may be uniporters located in the apical (luminal) domain of the cell, facilitating the diffusion of solutes until an electrochemical equilibrium is established across the membrane. One such influx transporter is glucose transporter 2 [14]. The quercetin glycosides are absorbed intact across the intestine via glucose-specific transporters. Hollman et al. (1999) proposed that flavonoid glycosides actually could be absorbed intact in the small intestine, using the sodium-dependent glucose transporter-1 (SGLT1). Bilitranslocase, a membrane protein originally isolated from rat livers, is an important carrier of flavonoids and anthocyanins [15]. In enterocytes, ABC pumps occur in both the luminal and the basolateral plasma membrane domains, and promote efflux of substrates at both sides. The cellular efflux of flavonoids and other phytochemicals occurs via these transporters and this might be the reason for their poor bioavailability [14].

10.2.2 Metabolism of Phytochemicals

Metabolism is a process of biochemical modification through a set of enzymatic pathways by living organisms, which modifies the chemical structure of compounds that are foreign to the organism's natural biology. These processes are often intended to detoxify phytochemicals, which are converted from lipophilic chemical compounds to more hydrophilic products. The metabolism may occur in the GIT before absorption of the phytochemical or may occur in the hepatic microsomal system after getting adsorbed.

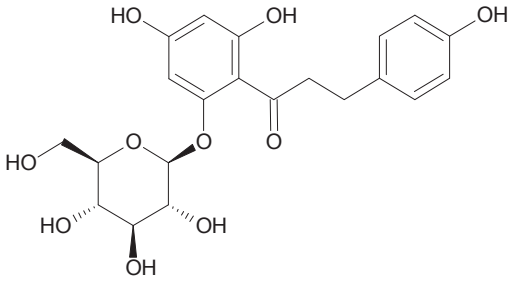
10.2.2.1 *Metabolism in GIT*

The metabolism at GIT is an important aspect for the bioavailability of the compounds. These involve the enzymes of the digestive system (protease, glycosidase, etc.) or the gut microflora at the colon level. The flavonoids such as flavonols, isoflavones, flavones, and anthocyanins are usually glycosylated by 1−3 sugar moieties like glucose, rhamnose, galactose, arabinose, xylose, or glucuronic acid [16]. Flavonoids could be hydrolyzed in the small intestine by enzymes (glycosidase). When quercetin derivatives were administered to volunteers, quercetin-3-O-rhamnoglucoside was absorbed more slowly than quercetin-4-O-glucoside (maximum concentration at 6 and 0.5 h, respectively) into the small intestine (gut microflora) after hydrolysis [15]. Chlorogenic acid (CA) is a caffeic acid ester linked to quinic acid, and no esterase in human tissues is able to release caffeic acid from CA; it is metabolized by the colonic microflora for absorption. Similarly, ferulic acid and its homologs are released by colonic microflora (xylanases and esterases). Ellagitannins are also hydrolyzed by colonic gut microflora to EA and further converted to hydroxy-6H-dibenzo[b,d]-pyran-6-one derivatives known as urolithins A and B [17]. Catechin is excreted in the bile as glucuronide conjugates (33−44%), but other metabolites [*m*- and *p*-hydroxyphenylpropionic acid, d-(3-hydroxyphenyl)-g-valerolactone, and d-(3,4-dihydroxyphenyl)-g-valerolactone] are excreted from the gut microflora [17]. Naringenin (**21**) is metabolized to microbial derived metabolites such as 3-(4-hydroxyphenyl) propionic acid and resveratrol is metabolized to dihydroresveratrol by the colon microflora [18].

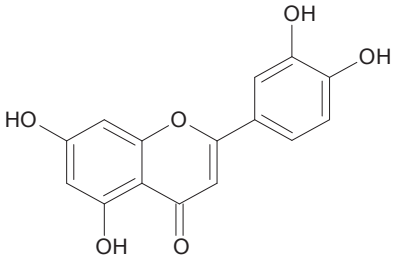
10.2.2.2 *Metabolism in the Hepatic Microsomal System*

After the drug gets absorbed in the blood it undergoes first-pass metabolism in the hepatic microsomal system. This process is biphasic and consists of stepwise

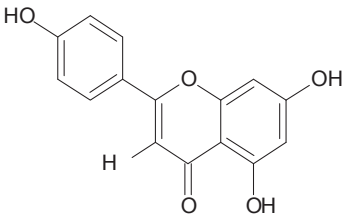
biotransformation and synthesis reactions. Phase I (biotransformation) consists of the oxidation (hydroxylation), hydrolysis, or reduction of a lipid-soluble or nonpolar drug. Phase II (synthesis) consists of the conjugation of a drug or its metabolite with an endogenous compound (predominantly glycine, sulfate, or glucuronic acid). The result of either of these phases of metabolism is the production of metabolites that are more prone to polar groups than the parent ring and readily excreted in the bile or urine. One of the most common oxidation mechanisms is catalyzed by cytochrome-P450-(CYP450)-dependent mixed function oxidase system. The majority of the phytochemicals is metabolized by CYP450. These enzyme complexes act to incorporate an atom of oxygen into nonactivated hydrocarbons, which can result in either the introduction of hydroxyl groups or N-, O-, and S-dealkylation of substrates. In this regard, galangin (by ring hydroxylation) and kaempferide (by O-demethylation) were metabolized to kaempferol. CYP2C9 was the most efficient isoform for the oxidation of galangin, followed by CYP1A2 and CYP1A1. For the oxidation of kaempferide, CYP1A2 clearly predominated, followed by CYP2C9 and CYP1A1. Surprisingly, chrysin was not oxidized by any of these human enzyme preparations, but was oxidized by the liver microsomes [18].



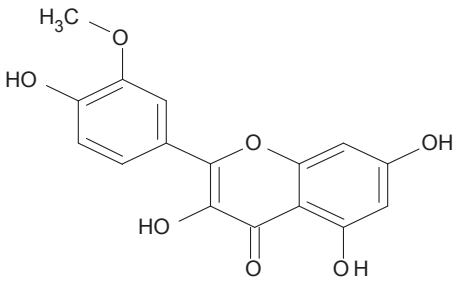
Phloridzin (**15**)



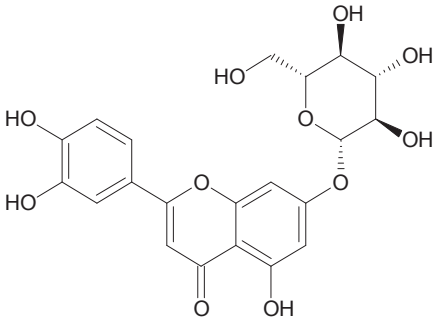
Luteolin (**16**)



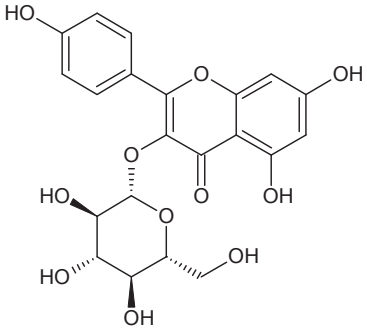
Kaempferol (**17**)



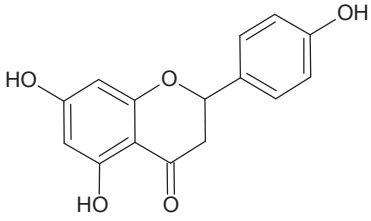
Isorhamnetin (**18**)



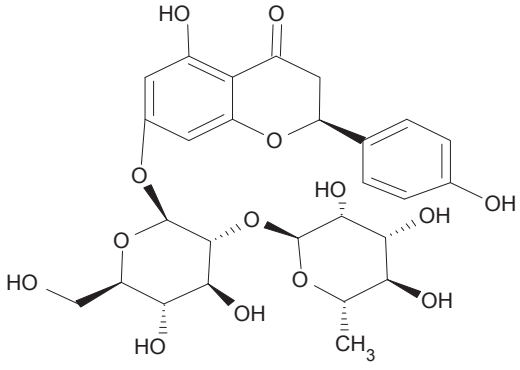
Luteolin-7-O-glucoside (**19**)



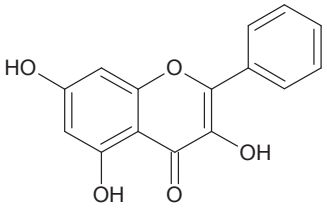
Kaempferol-3-O-glucoside (**20**)



Naringenin (**21**)



Naringin (Naringenin-7-rhamnoglucoside) (**22**)



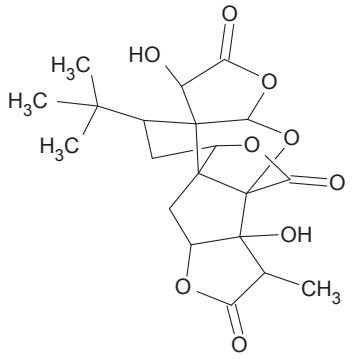
Galangin (**23**)

Most of the flavonoid glycosides are first deglycosylated and then converted to glucuronides or sulfates with or without methylation, as shown for phloridzin (**15**), luteolin (**16**), luteolin-7-O-glucoside (**19**), quercetin glycosides, kaempferol-3-O-glucoside (**20**), genistein, daidzein, quercetin, catechin, kaempferol, and naringin (naringenin-7-rhamnoglucoside) (**21**) [17]. Similarly, isorhamnetin (**18**), or 3′-O-methylquercetin, has also been reported in human studies [19]. The main metabolites of galangin (**23**) were found to be kaempferol by oxidation, glucuronidation, and sulfation conjugations [18]. Resveratrol was administered at high dose; high levels of resveratrol metabolites were detected in plasma, whereas the aglycone did not reach concentrations higher than 7 μM and exhibited a relatively short life of about 8–14 min [13]. These results suggested an intense phase II metabolism (due to the action of detoxifying enzymes) [18] and support the fact that at higher administered doses, higher levels of metabolites can be detected in plasma. EA methyl ether glucuronides have been detected in human plasma and urine showing that free EA is absorbed and extensively metabolized by phase II enzymes [13].

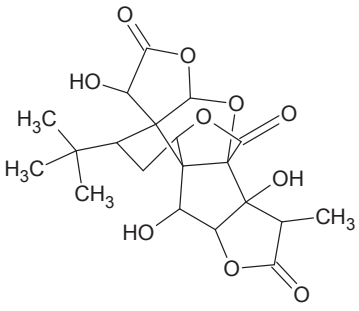
bioavailability. The solubility of some of these phytochemicals in GI fluid is low, which leads to poor absorption and hence poor bioavailability. Also, majority of the compounds are present in the food as glycosides, which are known to have poor absorption. The pharmacokinetic profiles of some phytomolecules are illustrated in Table 10.1.

10.3.1 *Gingko biloba* (Family: Ginkgoaceae)

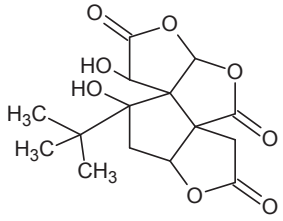
The pharmacokinetic profile of bilobalide and ginkgolides A and B were studied after oral administration of 80 mg of *Gingko biloba* (GB) extract (~24% flavonoids and 6% terpenoids) [20]. The absolute bioavailability of ginkgolides A and B were ≤80%, while ginkgolide C showed very low bioavailability. Bioavailability of bilobalide was 70% after administration of 120 mg extract. In another pharmacokinetic study, the mean bioavailabilities of ginkgolide A (**24**), ginkgolide B (**25**), and bilobalide (**26**) were found to be 80%, 88%, and 79%, respectively [21].



Ginkgolide A (**24**)



Ginkgolide B (**25**)



Bilobalide (**26**)

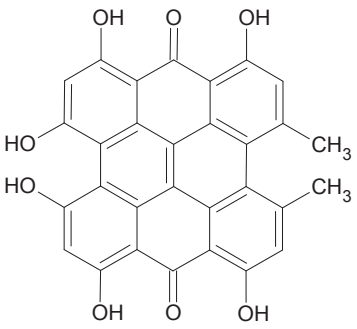
10.3 THE BIOAVAILABILITY AND PHARMACOKINETICS OF SOME HERBS AND PHYTOCONSTITUENTS

Medicinal herbal products are composed of different chemical constituents, including flavonoids, alkaloids, glycosides, tannins, xanthonoids, and cinnamates, and possess diverse therapeutic activity. But unfortunately the beneficial roles of potent plant secondary metabolites are greatly limited due to their poor

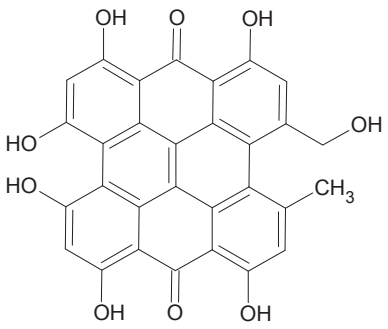
The $t_{1/2}$ of ginkgolides A and B and bilobalide were 4.5, 10.6 and 3.2 h, respectively [22]. The bioavailability of ginkgolides A and B and bilobalide was studied in rats after single oral administration of the dose at 30, 55, and 100 mg/kg GB extracts [23].

10.3.2 *Hypericum perforatum* (Family: Hypericaceae)

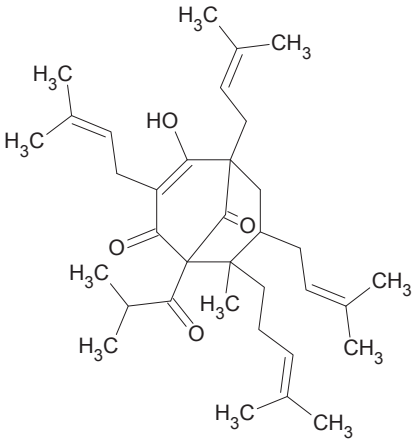
Hypericum perforatum (HP) consists of hypericin (**27**), pseudohypericin (**28**), hyperforin (**29**), and adhyperforin (**30**) as the chief constituents [20].



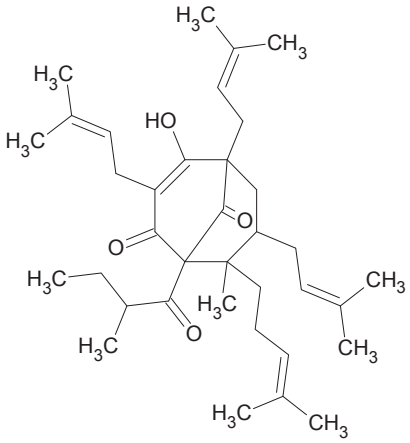
Hypericin (**27**)



Pseudohypericin (**28**)



Hyperforin (**29**)



Adhyperforin (**30**)

TABLE 10.1 The Pharmacokinetics of Different Phytochemicals after Oral Administration (Mean ± SD)

Phytochemicals	Subject	C_{max}(ng/mL)	T_{max}(h)	$T_{1/2el}$(h)	C_L	Bioavailability	Reference
Ginkgolide A	Human	15 41.8 ± 14	1–2 h 2	4–6 2.63 ± 0.45	130–200 mL/min –	80–98% –	[21] [22] [19]
Ginkgolide B	Human	4 5.6 ± 2.2	1–2 h 2	5–11 2.34 ± 0.38	140–250 mL/min –	80–90% –	
Ginkgolide C	Human	12 37.6 ± 14.2	1–2 h 2	~3 2.30 ± 0.24	600 mL/min	70–80%	
Hypericin	Human	30.6 ± 12.6 14–22 0.9–3.3	4.4 ± 2.7 4.0–10 4.0–8.0	36.1 ± 22.6 25–31 14.7–57.8	5.8 ± 2.3 L/h 2.3–3.1 L/h 34.7–238 L/h	–	[117] [118] [24]
Pseudohypericin	Human	1.1–7.1 7–12	2.0–5.0 3.0–3.6	13.9–27.9 16–24	89.2–511 L/h 10.8–17.4 L/h	–	[118] [119] [24]
Hyperforin	Human	153.2 ± 22	3.6 ± 0.6	9.5 ± 1.1	11.9 ± 1.7 L/h	–	
	Rat	370	3	6	70 mL/min	–	

(Continued)

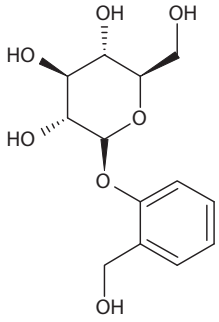
TABLE 10.1 The Pharmacokinetics of Different Phytochemicals after Oral Administration (Mean ± SD)—cont'd

Phytochemicals	Subject	C_{max}(ng/mL)	T_{max}(h)	$T_{1/2el}$(h)	C_L	Bioavailability	Reference
Escin	Human	–	–	10–19	–	–	[28]
	Rat	–	–	–	–	12.5%	[29]
Salicin	Human	1200 (As salicylic acid)	1	2.45	15.8	–	[32]
Silybin	Human	523.7 ± 292	0.6–4.6	6	–	–	[33]
Quercetin	Human	86	4.9	15.1	–	–	[34]
Rutin	Human	320	7	11.8	–	–	[35]
Andrographolide	Rats	1620 230	1 0.495	1.7 2.37	7.17 mL/min –	– 2.67%	[37] [38]
Resveratrol	Human	1942 470 nM	2.80 0.5	1.06 0.82	– –	– –	[120] [121]
	Dogs	1700–2600	1–2	2–4	41–58 L/h	–	[122]
	Rats	5900 3750	0.17 0.25	1.16 1.97	74.51 mL/min 0.310 L/min	– –	[107] [123]
Curcumin	Rats	500 86.55 ± 9.55	0.75 0.60 ± 0.13	1.45 1.21 ± 0.23	92.26 L/h –	– –	[106] [124]
	Rabbits	230	2	–	27.54 ± 3.09 L/h	–	[125]
Mangiferin	Human	19.94 ± 3.47	2.42 ± 0.71	4.47 ± 0.25	0.83 ± 0.22 L/h	–	[54]
	Rats	2730 ± 236 874.9 190	4.8 ± 1.0 4.22 0.25	0.39 ± 0.04 2.80 5.1	– 10.1L/h –	– – –	[126] [127] [128]
Genistein	Human	39 ± 3.7 261.84	6.7 ± 1.1 7.00	10.2 ± 0.9 7.96	37.6 ± 10.9 L/h –	–-	[129] [58]
	Rats	4876.19	2	–	–	30.75%	[57]
Diadzein	Human	46 ± 4.4 96.02	7.4 ± 0.9 6.25	11.4 ± 1.2 6.67	7.8 ± 1.0 L/h –	– –	[129] [58]
Ellagic acid	Human	3.65 ± 1.71 0.06 ± 0.01 μmol/L	1.98 ± 2.87 0.98 ± 0.06	8.41 0.71 ± 0.09	– –	– –	[60] [61]
	Rats	1750.7 ± 769	0.264 ± 0.034	5.811 ± 0.93	–	–	[130]
Gallic acid	Human	1.83 ± 0.16 μmol/L	1.27 ± 0.20	1.19 ± 0.07	8.4 ± 2.4 L/h	–	[131]
	Rats	175.13 ± 45.2	0.2	0.57	–	–	[63]
Chlorogenic acid	Rats	1013.91 242	0.36 0.5	2.54 8.55	– –	– –	[58] [132]
	Rabbit	839	0.58	–	–	–	[68]
Chrysin	Human	13	1	4.6	–	–	[71]
	Rats	19.47 ± 13.1 90.22 ± 19.5	0.3 ± 0.1 0.40 ± 0.15	6.6 ± 0.8 9.72 ± 3.16	17.0 ± 11.0 L/h 2.72 ± 0.67 L/h	– –	[73] [72]
Naringenin	Mice	2910.6	0.2	4.69	–	–	[133]
	Beagle dogs	146.56 ± 167.98	5.3 ± 2.1	2.75 ± 1.33	–	–	[134]
	Rats	1034 ± 402 1088 ± 198.7	13.6 ± 0.89 0.42 ± 0.21	5.00 ± 1.46 4.43 ± 0.60	– 0.012 L/h	– –	[135] [136]
Hesperetin	Mice	776.06	0.2	5.26	–	–	[133]
	Rats	0.58 ± 0.31 791 ± 165	8.0 ± 2.8 0.42 ± 0.21	4.3 ± 1.5 4.85 ± 0.51	– 0.003 L/h	– –	[137] [136]

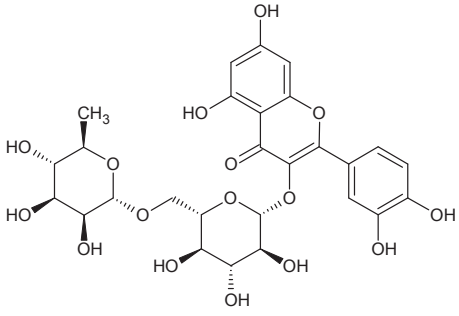
Pharmacokinetic studies of HP extract have been reported by several researchers. Pharmacokinetic profile of hypericin has been reported by Biber et al. (1998) with the oral administration of HP extract (equivalent to 0.3% hypericin) as coated tablets [24]. The terminal elimination $t_{1/2}$ of hypericin increased significantly from 25 to 48 h at higher doses. The bioavailability of hypericin and pseudohypericin from the extract was estimated to be 14% and 21%, respectively [25]. Pharmacokinetic parameter study of hypericin was performed in monkeys after administration of an intravenous (IV) dose of 2 mg/kg. Elimination $t_{1/2}$ and mean plasma C_L were found to be 26 ± 14 h and 6 ± 2 mL/kg/h, respectively. Another pharmacokinetic study demonstrated that the C_{max} of hyperforin was found to be 370 ng/mL at 3 h after oral administration of HP extract at the dose of 300 mg/kg in rats (~5% hyperforin). [24]. The $t_{1/2}$ and C_L were observed as 6 h and 70 mL/min/kg, respectively [26].

10.3.3 *Aesculus hippocastanum* (Family: Sapindaceae)

It contains β-escin and α-escin as the active ingredients [20,27]. The bioavailability has been studied after administration of a single dose and multiple doses (2 tablets ~50 mg escin) every 12 h for 4 days [28]. The $t_{1/2}$ of escin was determined to be 10–19 h. The pharmacokinetics and bioavailability of escin has been reported after oral and IV administration in rats; about 66% and 33% of the dose was excreted in the bile and urine, respectively, after IV administration. The oral bioavailability of escin was found to be 12.5% [29].



Salicin (**31**)



Rutin (**32**)

10.3.4 *Salix alba* (Family: Salicaceae)

Salicin (**31**) is the active component of *Salix alba*. It is converted to salicylic acid through metabolism [20]. The bioavailability studies revealed that when 4000 mg pure salicin was administered, the plasma concentration of the main metabolite salicylic acid was found to be 110 mg/L and 86% was eliminated through urine within 24 h of administration [30]. In contrast, lower doses (single dose of extract equivalent to 54.9 mg of salicin) resulted in average peak plasma concentrations of only 0.13 mg/L of salicylic acid [31]. In another study, the extract (equivalent to 240 mg of salicin) was given in two equal doses (at 0 and 3 h) to 10 volunteers [32]. Peak plasma levels of salicylic acid were recorded at 1.2 mg/L. Maximum concentrations were found at 1 h after administration, while the $t_{1/2}$ was 2.45 h.

10.3.5 *Silybum marianum* (Family: Asteraceae)

Silymarin mainly consists of silybin (**9**) (50–70%) with small amounts of other constituents including isosilybin, silydianin, and silychristin [20]. A pharmacokinetic study of silybin has been done by Weyhenmeyer et al. (1992) after a single dosage of 102, 153, 203, and 254 mg. The area under the curve (AUC) correlated linearly with the dose of about 10% of total silybin in plasma, which was present as conjugate. Elimination $t_{1/2}$ of silybin was found to be ~6 h. Only 5% of the dose was excreted in urine as total silybin. Differences in the bioavailability were attributed to the in vitro dissolution characteristics of silybin in various marketed products. It has been found that at least 70% of silymarin has to be released in vitro within 15 min or 80% within 30 min to achieve desirable plasma concentrations [33].

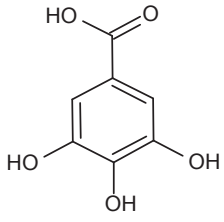
10.3.6 Rutin

In the pharmacokinetic study of rutin it was found that profound lag time of several hours has been achieved with C_{max} within ~7 h [34]. C_{max} and

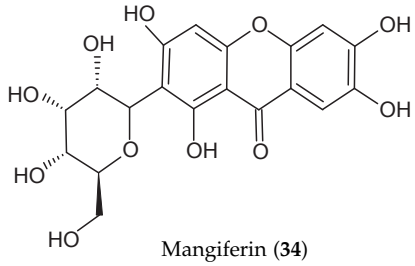
$AUC_{(0-24)}$ were found to vary linearly in a dose-dependent manner. After intake of 400 mg rutin (**32**) [~200 mg quercetin aglycone], mean maximum plasma concentrations were 0.32 μg/mL at 7 h, while the mean $AUC_{(0-24)}$ was 2.5 μgh/mL. Absorption of the quercetin aglycone was faster than rutin but slower compared to other glucosides. T_{max} was in the range 1.9–4.9 h [34]. Mean $AUC_{(0-32)}$ and C_{max} values were similar after intake of comparable doses of free quercetin and rutin [34,35]. After oral administration only traces of free quercetin were found in the plasma, which means that rutin was converted to quercetin metabolites. The $t_{1/2}$ of quercetin was found to be 3.8 h [36].

10.3.7 Andrographolide

Pharmacokinetic profile of andrographolide (**4**) (AP) has been observed after oral administration of tablets (equivalent to 10 mg/kg) in rats. The different parameters were observed by a one-compartment open model with $T_{max} = 59.69 \pm 3.61$ min, $C_{max} = 1.62 \pm 0.11$ μg/mL, $V_d = 1056.90 \pm 83.42$ mL, and $AUC_{0-\infty} = 348.75 \pm 24.41$ μg min/mL [37]. The absolute bioavailability of AP was found to be 2.67%. AP has poor oral bioavailability because of its rapid biotransformation and efflux by P-glycoprotein (P-gp) [38].



Gallic acid (**33**)



Mangiferin (**34**)

10.3.8 Resveratrol

Resveratrol (**8**), [3,4,5-trihydroxystilbene] exhibited low bioavailability in various pharmacokinetic studies [39]. Several parameters have been studied after oral administration of resveratrol (~25 mg/70 kg) to healthy male subjects as white wine, white grape juice, or vegetable juice. The C_{max} was achieved after 30 min and concentrations declined rapidly after reaching baseline levels within 4 h due to rapid and extensive metabolism. However, low plasma levels of free resveratrol were observed (<40 nmol/L) [40]. Resveratrol-3-O-sulfate, resveratrol-4′-O-glucuronide, and resveratrol-3-O-glucuronide were found to be the major metabolites of resveratrol [41,42].

10.3.9 Curcumin

Curcumin, a hydrophobic polyphenol, exhibited low bioavailability [43] due to extensive metabolism. In pharmacokinetic studies, Holder et al. (1978) has reported that the major biliary metabolites of curcumin were glucuronides of tetrahydrocurcumin and hexahydrocurcumin in rats. Negligible amount of curcumin was observed in blood plasma after oral administration of 1 g/kg; it revealed that curcumin was poorly absorbed from the gut [44,45]. After oral administration of 400 mg of curcumin to rats, no curcumin was found in blood/plasma, whereas a trace amount (≤5 μg/mL) was found in the portal blood [46]. In another study, curcumin was given orally at a dose of 2 g/kg in rats; a maximum serum concentration of 1.35 ± 0.23 μg/mL was observed at 0.83 h. In humans, the same dose of curcumin resulted in either undetectable or extremely low (0.006 ± 0.005 μg/mL at 1 h) serum levels [47,48]. Similarly, in a human clinical trial, curcumin (3.6 g) produced plasma levels of 11.1 nmol/L after an hour of dosing [49,50]. The absorption and elimination $t_{1/2}$ of orally administered curcumin (2 g/kg) in rats were reported to be 0.31 ± 0.07 and 1.7 ± 0.5 h, respectively [51].

10.3.10 Mangiferin

The bioavailability of mangiferin (**34**) [1,3,6,7-tetrahydroxyxanthone-C-2-β-d-glucoside] has been reported to be very low [52] at 1.2% [53]. Pharmacokinetic study of mangiferin was performed by Liu et al. (2010) after administration of single oral dose of mangiferin of 17.5, 35, and 70 mg/kg to rats. The $t_{1/2}$ seemed to be dose dependent and ranged from 2.34 to 5.10 h. The times to reach C_{max} and T_{max} were prolonged to 3.0 and 4.0 h, respectively. Another study has been investigated after oral administration of 0.1, 0.3, and

0.9 g mangiferin to 21 healthy volunteers. The pharmacokinetic profile of mangiferin was determined using noncompartmental model. Mangiferin reached plasma concentration 38.64 ± 6.75 ng/mL about 1 h after oral administration of 0.9 g, and the apparent elimination $t_{1/2}$ was 7.85 ± 1.72 h [54].

10.3.11 Genistein

The pharmacokinetics of genistein (**2**) has been studied in rats and humans [55], and low bioavailability has been demonstrated due first-pass effect [56]. After oral administration of different doses of genistein (4, 20, 40 mg/kg), its bioavailabilities were found to be 38.58, 24.34, and 30.75%, respectively. The T_{max}, C_{max}, and $AUC_{0-\infty}$ of genistein after oral administration (40 mg/kg) were 2 h, 4876.19 ng/mL, and 31,269.66 ng h/mL, respectively [57].

Further investigation demonstrated the pharmacokinetics of daidzein (**5**) and genistein, after ingestion of soy beverage compared with soy extract capsules in postmenopausal Thai women [58]. They received either two soy extract capsules equivalent to genistein content 3.90 ± 0.04 mg/capsule and daidzein content 11.29 ± 0.17 mg/capsule or 15 g of soy beverage (genistein and daidzein content 0.62 ± 0.005 and 0.70 ± 0.01 mg/g). The first peak of plasma daidzein concentration was reached ~1 h after ingestion of both preparations, whereas the second peak was attained at higher plasma concentrations after 5.92 ± 2.43 h for soy beverage, and 6.25 ± 2.26 h for soy extract capsules. The C_{max} values were 96.31 ± 36.18 ng/mL and 96.02 ± 27.71 ng/mL for soy beverage and soy extract capsules, respectively. It also revealed that the elimination $t_{1/2}$ was 7.68 ± 4.14 h for soy beverage and 6.67 ± 1.65 h for soy extract capsules [58].

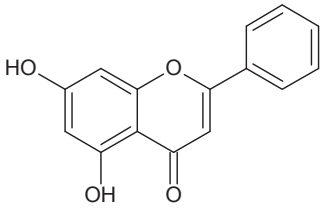
10.3.12 Ellagic Acid

The pharmacokinetics of EA (**10**) has been determined in healthy volunteers fed with freeze-dried black raspberries 45 g (equivalent to 13.5 mg EA) for 7 days [59]. After 1 day, C_{max}, T_{max}, AUC, and mean $t_{1/2}$ were found to be 3.65 ± 1.71 ng/mL, 1.98 ± 2.87 h, 16.56 ± 7.66 ng h/mL, and 8.41 h respectively. Similarly, 3.25 ± 1.52 ng/mL, 2.00 ± 1.97 h, 17.61 ± 9.15 ng h/mL and 8.57 h, respectively, were observed after 7 days [60].

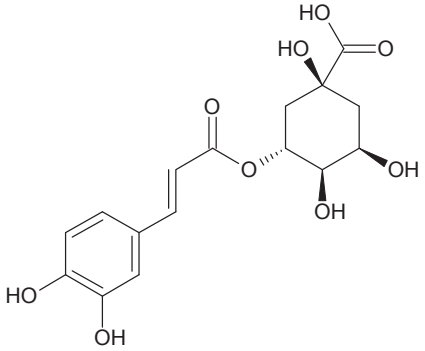
In another study, EA was given to 18 human volunteers through a single dose of pomegranate juice 180 mL (~12 mg EA). The C_{max} was 0.06 ± 0.01 μmol/L, and the corresponding AUC [0.17 ± 0.02 (μmol.h)·L^{-1}] was attained while T_{max} was 0.98 ± 0.06 h. The elimination $t_{1/2}$ was found to be 0.71 ± 0.08 h [61].

10.3.13 Gallic Acid

Pharmacokinetics of gallic acid (GA) (**33**) [3,4,5-trihydroxybenzoic acid] has been studied through different trials and low bioavailability was found [62]. In an experiment, healthy human volunteers were given two *Acidum gallicum* tablets (25 mg GA each tablet) or 125 mL Assam black tea brew (~50 mg GA). The oral absorption of GA from both sources was fast (T_{max}: 1.27 ± 0.20 h for *A. gallicum* tablets and 1.39 ± 0.21 h for the tea). But the highest GA concentrations observed in plasma were 1.83 ± 0.16 μmol/L (tablets) and 2.09 ± 0.22 μmol/L (tea). The elimination $t_{1/2}$, elimination rate constant, and C_L were found to be 1.19 ± 0.07 h, 0.58 ± 0.03 h^{-1}, and 8.4 ± 2.4 L h^{-1}, respectively, for the tablets and 1.06 ± 0.06 h, 0.65 ± 0.04 h^{-1}, and 8.4 ± 2.0 L h^{-1} respectively, for the tea brew [63]. In another experiment, the pharmacokinetic profile has been evaluated after oral administration of 50, 100, and 150 mg/kg grape seed extract (equivalent to 91 mg GA/gm) in male Sprague Dawley rats. Absorption of GA was observed to be significantly higher from a single oral dose of 150 mg/kg. AUC and C_{max} were significantly higher. For GA, AUC was found to increase by 198% upon dose escalation [64].



Chrysin (**35**)



Chrorogenic acid (**36**)

10.3.14 Chlorogenic Acid

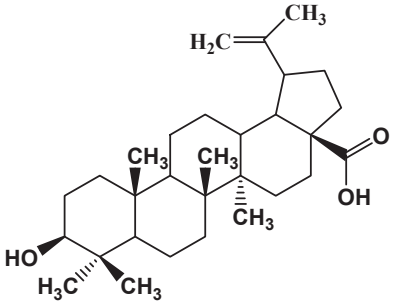
CA (**36**) is an ester of caffeic acid and quinic acid [65]. CA was hydrolyzed by intestinal microflora into caffeic acid and quinic acid metabolites [66]. The pharmacokinetic profile of CA has been determined in rat models after oral administration of *Flos lonicerae*. A two-compartment model was selected for calculation of the different parameters. At the administered doses of 200, 400, and 600 mg/kg, the absorption half-lives ($t_{1/2\ Ka}$) were 10.23, 18.66, and 28.13 min, respectively, while elimination $t_{1/2}$ were 231.64, 337.23, and 420.81 min, respectively. The V_d at the three doses were 55.26, 35.56, 32.22 L/kg, respectively. The $AUC_{0-\infty}$ was not proportional to the administered dose and found have nonlinear kinetics [67].

In another experiment, the plasma levels and pharmacokinetics of CA have been determined in rabbit after single administration of *F. lonicerae* extract at a dose of 10 g/kg (~220 mg CA) body weight [68]. The plasma CA level reached a C_{max} of 0.839 ± 0.35 μg/mL at 34.7 ± 1.1 min. A second peak of CA appeared at 273.4 ± 39.6 min in the plasma with a concentration 0.367 μg/mL. The various pharmacokinetic parameters were: AUC, 140 ± 65.9 μg min/mL and K_{el}, $0.0130 \pm 0.0023\ min^{-1}$ [69].

10.3.15 Chrysin

Pharmacokinetic studies as well as extensive systemic metabolism suggested strongly that the oral bioavailability of chrysin (**35**) [5,7-dihydroxyflavone] in humans may be low [70]. In a study, 200-mg capsules of chrysin were orally administered to seven healthy human volunteers for pharmacokinetic investigation. C_{max} and T_{max} was found to be 3–16 ng mL^1 and 1 h, respectively, with correspondingly large interindividual variability in AUC values (5–193 ng mL^{-1}h). The apparent $t_{1/2}$ was 4.6 h. The mean plasma concentrations of chrysin sulfate in the seven subjects exceeded those of chrysin by approximately 30-fold, with AUC values of 450–4220 ng mL^{-1}h [71]. In a different study, the pharmacokinetic profile of chrysin was performed with oral administration of the extract of *Scutellaria baicalensis* to male Wistar rats at a dose of 8 mL/kg (~0.23 mg/mL of chrysin). The maximum concentration appeared at 0.40 ± 0.15 h with a C_{max} 90.22 ± 19.55 ng/mL. The other pharmacokinetic properties such as $t_{1/2}$, $AUC_{0-\infty}$, and C_L were calculated as 9.72 ± 3.16 h, 746.9 ± 239.8 ng h/mL, and 2.72 ± 0.67 L/h/kg, respectively [72].

Another pharmacokinetic study of chrysin has been performed after oral administration of 16.0 g/kg Tang-min-Ling pill (equivalent to 1.12 mg/kg chrysin). Chrysin was rapidly absorbed with a C_{max} 19.47 ± 13.14 ng/mL at 0.3 h. The $t_{1/2}$, $AUC_{0-\infty}$, and C_L were calculated as 6.6 ± 0.8 h, 107.5 ± 77.9 ng h/mL, and 17.0 ± 11.0 L/h kg, respectively [73].



Betulinic acid (**37**)

10.3.16 Betulinic Acid

Betulinic acid (BA) (**37**) exhibited low bioavailability due to limited aqueous solubility [74]. The pharmacokinetic study of BA has been screened in the CD-1 mice, to which BA was administered at a dose of 250 and 500 mg/kg i.p. The serum concentrations reached peak level at 0.15 and 0.23 h, respectively. It has been observed that BA has an elimination $t_{1/2}$ of 11.5 and 11.8 h and total clearances of 13.6 and 13.5 L/kg/h at a dose of 250 and 500 mg/kg, respectively [75].

10.4 CHALLENGES IN DEVELOPING A HERBAL FORMULATION

Herbal medicine is still a challenge for developing into suitable dosage forms for promotion and development of human health. There are several challenges for developing an appropriate delivery system, due to their limited solubility and permeability through biological membranes, so much so their low therapeutic efficacy and bioavailability. Research is now being concurrently conducted on basic as well as applied fields of herbal medicines, and this has led to the need for research in the delivery system of herbal drugs for maximum concentration as well as bioavailability [3].

10.4.1 Modification for Half-Life of Herbal Drugs

The biological half-life ($t_{1/2}$) has an immense role in the therapeutic efficacy and potency of drug molecules

at the site where it is administered. If drugs have shorter $t_{1/2}$, then they possess low bioavailability when compared to those with higher $t_{1/2}$. The biological membrane permeability of such molecules offers more activity for lipophilic action, hence its chances of availability in the blood/plasma are more in comparison to hydrophilic compounds. However, 40% of the active pharmaceutical ingredients obtained from high-throughput screening are poorly soluble molecules. Lower bioavailability results from poor solubility and incomplete dissolution in vivo. This often holds back continuous development and coming into the market of some promising new chemical entities (NCEs), or elicits insufficient therapeutic effects from certain drugs. The increasing numbers of poorly soluble drugs require innovative formulation approaches to acquire a sufficient bioavailability level after oral administration. In other words, the hepatic biotransformation of the drug molecules is related to more renal elimination, which lowers the bioavailability of drugs [76]. A lot of research is going on to overcome the problem associated with herbal medicine by applying NDDS.

10.4.2 Drug Delivery Systems to Enhance Bioavailability of Herbal Medicine

A great deal of herbal products for therapeutic application are being acquired through the use of herbs, and information is increasing quickly with greater understanding of molecular mechanisms of diseases. Nevertheless, favorable drug action alone against the disease is insufficient to satisfy the medical community. In addition, avoiding unwanted side effects at the site of action is equally important. The pharmacological activity of any administered drug relies not only on its therapeutic efficacy but also on the bioavailability at the administered site. Several phytopharmaceuticals possess low aqueous solubility, which means poor membrane permeability and therefore low oral bioavailability. Conception and evolution into a suitable pharmaceutical formulation for delivery of certain phytoconstituents is of premier importance. The promotion of novel technologies is providing a large platform for novel delivery systems of herbal medicine to improve the therapeutic activity along with bioavailability of drugs that have poor aqueous solubility. Promising novel approaches are being developed including phytosome/herbosome, liposome, nanostructured lipid carriers (NLCs), NE, polymeric nanoparticles (NPs), dendrimers, micelles, and so on [77]. These novel technologies can modulate the pharmacokinetics of existing drugs, and it may be helpful to enhance delivery of herbal medicine to target sites. In this regard, the discussion on some of the delivery systems has made an impact either by enhancing the delivery of the herbal drugs to their target tissues or by increasing their bioavailability by manyfold [8].

10.5 NOVEL DRUG DELIVERY TECHNOLOGY FOR HERBAL FORMULATION

In the development of novel therapeutics, the ability to devise a suitable pharmaceutical formulation for delivery is of utmost importance. Therefore delivery of the phytomolecules is critical for effective prevention and treatment of diseases. The emergence of new technologies has engendered great interest in developing NDDS to advance both the pharmacological and therapeutic properties of herbal drugs. There is continuous quest for information and technology to overcome the shortcomings associated with herbal medicine for therapeutic effect as well bioavailability enhancement. In this context, the NDDS is pioneering to curb the problems related to herbal drugs, which is systematically represented in Figure 10.2.

The nanosized NDDSs of herbal drugs have a potential future for enhancing the activity and overcoming problems associated with plant medicines. The applications of NDDS in herbal medicine are explained through Figure 10.3.

In this section, we would discuss some of the delivery methods that have already made an impact either by enhancing the delivery of different herbal products to their target tissues or by increasing their bioavailability by manyfold [78].

10.5.1 Liposome

Liposome is a bilayer lipidic vesicular carrier system of phospholipids/cholesterol that varies in size from 25 to 2.5 nm. The distinct advantages are their ability to encapsulate various materials and their structural versatility. Liposome can encapsulate drugs with widely varying solubility or lipophilicity, either entrapped in the aqueous core of the phospholipid bilayer or at the bilayer interface. Liposome composed of natural lipids is biodegradable, biologically inactive, nonimmunogenic, and possesses limited intrinsic toxicity. The structure of liposome is described in Figure 10.4.

Therefore, drugs encapsulated in liposomes are expected to be transported without rapid degradation, and this results in minimum side effects. Liposome is increasingly used with herbal products to deliver certain drugs for the prevention or treatment of a variety of diseases. Several formulations have been developed and studied with regard to relative stability, pharmacokinetic

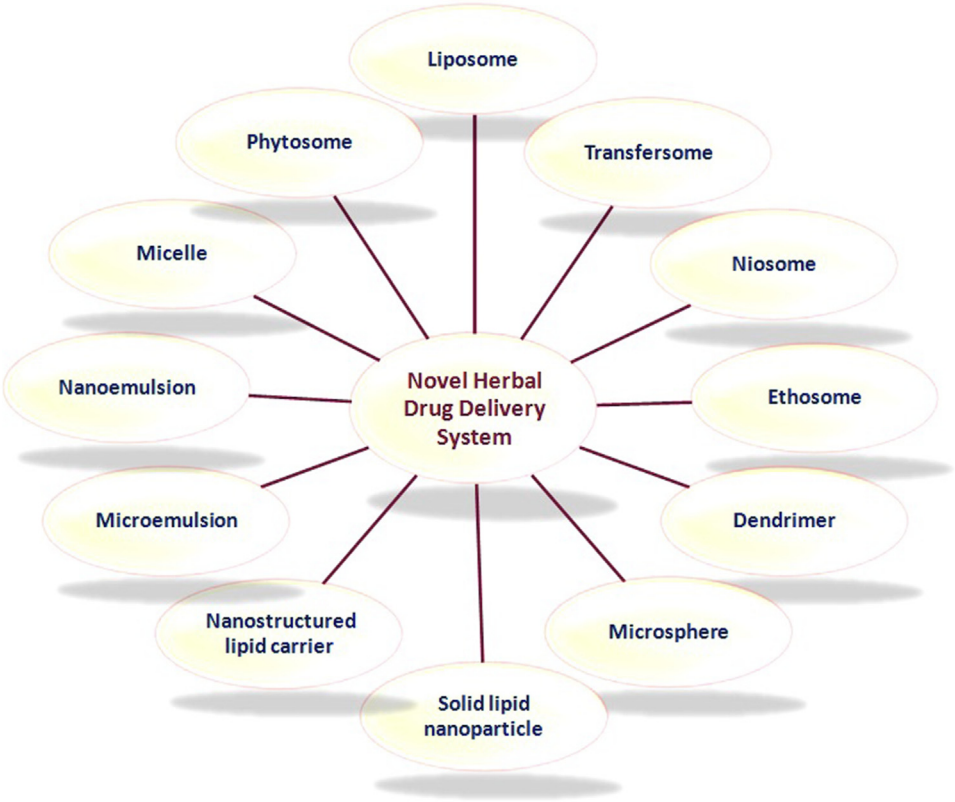


FIGURE 10.2 Different novel delivery systems for herbal drugs.

properties, biodistribution, and toxicity. Moreover, liposomes are able to deliver drugs into target site by fusion or endocytosis. Despite the many advantages of liposomes, including safety and biocompatibility, their main drawback is instability in plasma [8]. El-Samaligy et al. (2006) prepared silymarin-encapsulated hybrid liposomes, which show successful preparation with efficient encapsulation of silymarin. Liposome Herbasec®, one of the liposomal powders marketed with standardized botanical extracts using white and green tea, white hibiscus, guarana, and aloe, successfully improved the activities of their herbal ingredients. This is a novel means of delivering drugs in a controlled manner to enhance bioavailability and get the therapeutic effect over a longer period of time [79].

10.5.2 Transfersome

Transfersomes are vesicular system consisting of phospholipids as the main ingredient with 10–25% surfactant (such as sodium cholate) and 3–10% ethanol. The surfactants work as "edge activators," conferring ultradeformability on the structure of transfersome, which helps them to squeeze through pores in the stratum corneum (SC). Transfersomes can squeeze through SC layers spontaneously at ~500 nm pore size, while liposomes are too large to pass through pore size ≤50 nm [80]. Transfersomes are also known as elastic vesicles due to their deformability , and they can pass through intact skin under the influence of hydration gradient, transporting therapeutic agents only when applied under nonocclusive conditions [81]. The method of preparation of transfersome is similar to that of liposome. The hypothesized mechanism of action of transfersome is described as followings: (1) vesicles act as drug carriers and intact vesicles enter the SC carrying vesicle-bound drug molecules into the skin and (2) vesicles act as penetration enhancers and enter the SC and then modify the intercellular lipid lamellae and consequently facilitate the penetration of unbound drug molecules into and across the SC [81]. It can be used as novel delivery systems for phytopharmaceuticals to enhance their permeability as well as bioavailability. Transfersome can penetrate the SC and supply the nutrients locally to access its functions resulting in maintenance of skin; in this context, the transfersome of capsaicin has been prepared, which exhibited better topical absorption in comparison to pure capsaicin [4].

10.5.3 Niosome

Niosomes are hydrated vesicular systems of nonionic surfactants with phospholipid or cholesterol and deliver drugs to target sites. The lamellar structures of these

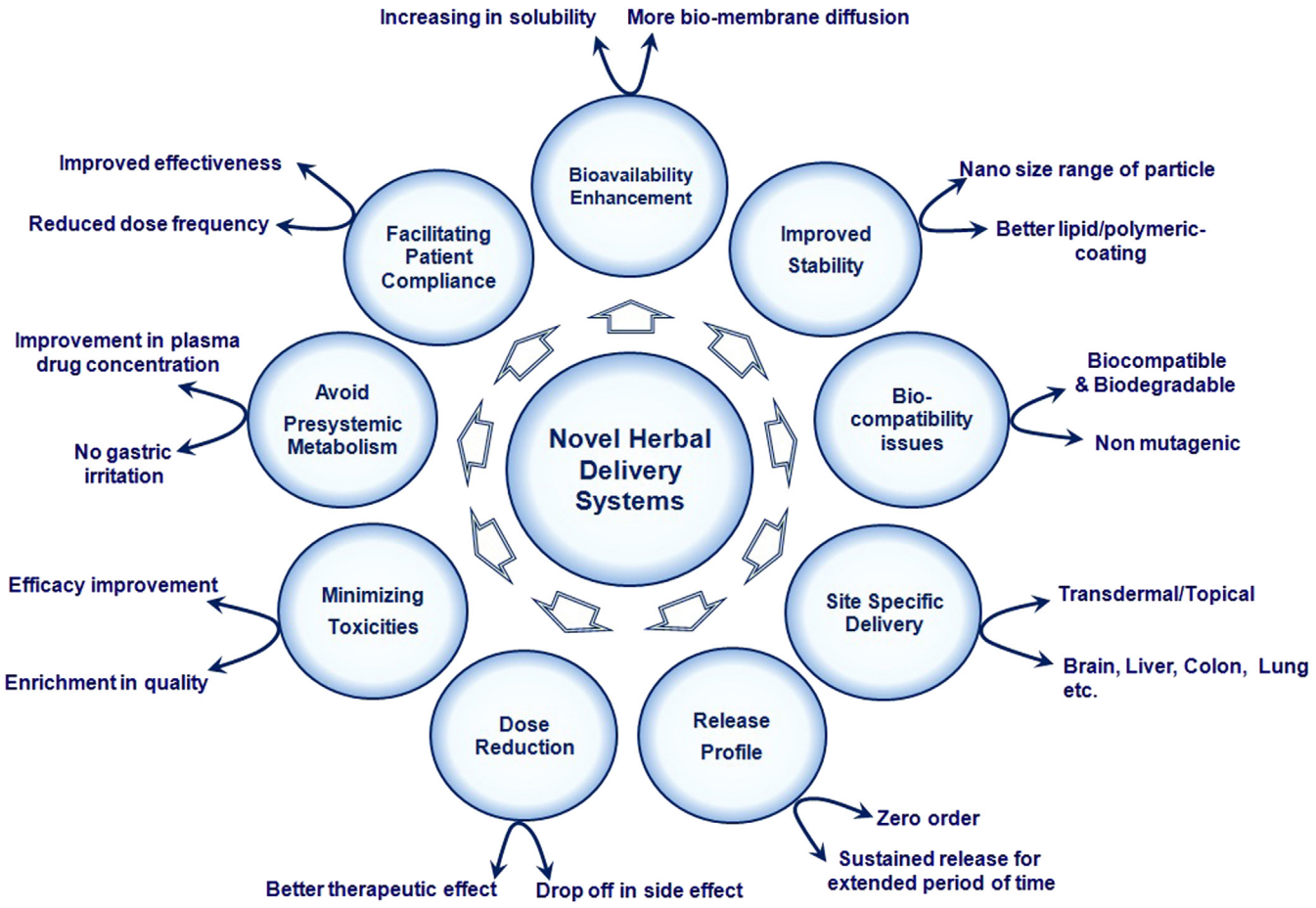


FIGURE 10.3 Salient features of novel herbal delivery system.

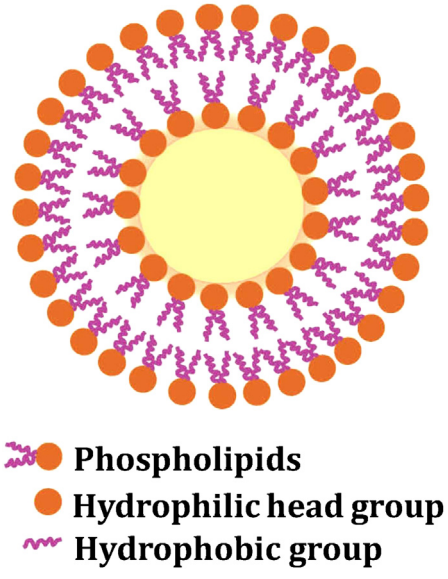


FIGURE 10.4 Structure of liposome.

vesicular systems are fabricated of amphiphilic molecules and surrounded by an aqueous compartment. Vesicular systems are applicable for both hydrophilic and hydrophobic drug delivery, wherein the drug is encapsulated in the interior hydrophilic compartment and the outer lipid layer, respectively [82]. It is biodegradable, biocompatible, nontoxic, and stable over a longer period of time in different conditions and is capable of encapsulating large quantities of material in a relatively small volume of vesicles. Furthermore, these are versatile carrier systems that can be administered through various routes including intramuscularly, IV injection, peroral, ocular, pulmonary, and transdermal [83]. The structure of niosome is represented in Figure 10.5.

Niosomes are bilayer lipidic systems consisting of nonionic surfactants. Nonionic surfactants are used due to their ability to increase solubility of poorly water-soluble drugs and therefore enhanced bioavailability. Compared to liposome, they have longer shelf

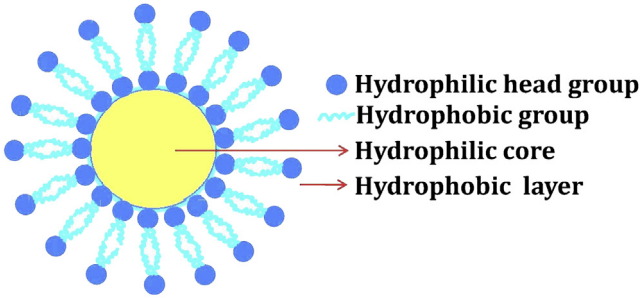


FIGURE 10.5 Structure of niosome.

life, stability, and ability to deliver drugs at the target site in a controlled or sustained manner for extended periods. Niosomes increased the permeability and fluidity of biological membranes of drug molecules like podophyllotoxin, etoposide, and methotrexate and show enhanced bioavailability upon transdermal application [82]. Junyaprasert et al. (2012) studied the EA-loaded niosome where the in vitro skin permeation revealed that permeation of EA depends on vesicle size, amount of EA entrapped, and the added solubilizers, which may act as permeation enhancer. From skin distribution study, the EA-loaded niosome showed more efficiency in the delivery of the EA through human epidermis and dermis than EA solution [83].

10.5.4 Ethosome

Ethosomes are lipid vesicular systems with alcohol (ethanol) capable of enhancing penetration to the deep tissues of skin and then systemic circulation. It is assumed that the alcohol interacts with ethosomal lipids and SC bilayer lipids, thus allowing the soft, malleable ethosomes to penetrate [80]. Recently, ethosomes have been shown to be promising and novel vesicular systems that have appeared in the fields of herbal product and drug delivery. This system has interesting characteristics correlated with its ability to permeate intact through the SC due to its high deformability. Indeed, ethosomes are soft, malleable vesicles tailored for enhanced delivery of phytomolecules. It has been reported that the physicochemical features of ethosomes allow this vesicular carrier to transport drug molecules more efficaciously through the SC into the deeper layers of the skin than conventional liposomes. This is an important aspect in the design of the carriers to be applied topically for delivery of herbal drugs [84]. Moreover, it facilitates percutaneous absorption of matrine as an antiinflammatory agent [4]. In addition, ethosomes elicited an increase in the percutaneous permeation of ammonium glycyrrhizinate thereby enhancing the anti-inflammatory activity of this drug in an in vivo model. These results are very encouraging and confirm that ethosomes are a very promising career in topical administration due to the enhanced delivery of drugs through the skin [84].

10.5.5 Dendrimer

Dendrimers are three-dimensional hyperbranched, treelike polymers having massive potential in drug delivery, targeting, and diagnosis and as carriers for deoxyribonucleic acid/gene delivery. Dendrimers have hydrophilic exteriors and hydrophilic interiors, which is responsible for its unimolecular micellar nature [85]. Dendrimers have some interesting features because of their globular form and interior cavities. Another feature is the possibility to encapsulate drug molecules in the macromolecule interior. The interactions between drug molecules and poly(amidoamine) (PAMAM) dendrimers by covalent conjugation are complexed by van der Waals interactions, or they are incorporated in the empty spaces within branches. This complexation is important in terms of stability, controlled release, and high drug loading, and reduced toxicity leads to the higher bioavailability of the drugs. In addition, dendrimers can be surface engineered to release the drug at the site specific for targeted drug delivery. This property along with the solubilization behavior could increase the therapeutic efficacy of drugs [85]. Abderrezak et al. (2012) studied the interaction of several dendrimers of different compositions such as mPEG-PAMAM (G3), mPEG-PAMAM (G4), and PAMAM (G4) with hydrophilic and hydrophobic drugs cisplatin, resveratrol, genistein, and curcumin at physiological conditions. Structural investigation showed that cisplatin binds dendrimers in hydrophilic mode via "Pt" cation and polymer terminal NH_2 groups, while curcumin, genistein, and resveratrol were found in the cavities binding through both hydrophobic and hydrophilic channels [86].

10.5.6 Microsphere

Microsphere refers to spherical microparticles with a diameter of 1–1000 μm. Biodegradable polymers are frequently used for the development of microsphere matrixes such as polylactic acid and copolymer of lactic acid and glycolic acid. Apart from them, there is an extensive range of microspheres prepared from albumin, albumin dextran sulfate, and fibrinogen. Administration of medication via microparticulate systems is advantageous because microspheres can be ingested or injected; they can be tailored for desired release profiles and used for site-specific delivery of drugs and in some cases can even provide organ-targeted release. So far, a series of phytomedicines such as rutin, camptothecin,

zedoary oil, tetrandrine, quercetin and *Cynara scolymus* extract have been successfully exploited through this delivery system. In addition, reports on immune microsphere and magnetic microsphere are also common in recent years. Immune microsphere possesses immune competence because the antibody and antigen were coated or adsorbed on the polymer microspheres [4].

10.5.7 Nanoparticle

Nanoparticles range in size from 10 to 1000 nm and can be synthesized from lipids, proteins, and carbohydrates, as well as several natural and synthetic polymers. For delivery, a drug is dissolved, entrapped, encapsulated, or attached to an NP matrix. NPs improve the therapeutic index of encapsulated drugs by protecting them from enzymatic degradation, by altering pharmacokinetics, by reducing toxicity, or by providing controlled release over extended periods of time. NPs may enhance the oral bioavailability of poorly soluble drugs and the tissue uptake after parenteral administration, through adherence to the capillary wall. The nanoparticulate systems of herbal medicines have attracted much attention, for example, nanonized curcuminoids [87], paclitaxel [88], and praziquantel, which have a mean particle size of 450, 147.7, and even higher than 200 nm, respectively. Furthermore, glycyrrhizic acid, quercetin, berberine, and artemisinin have been incorporated in the NPs, which was found to improve their bioavailability and bioefficacy [89].

10.5.8 Solid Lipid Nanoparticles and NLCs

Solid lipid nanoparticles (SLNs) are prepared from lipids that are solid at room temperature as well as at body temperature. Different solid lipids are exploited to produce SLNs, such as, tripalmitin, cetyl alcohol, cetyl palmitate, glyceryl monostearate, trimyristin, tristearin, stearic acid, etc. There are several advantages of SLN formulations, such as: (1) protection from degradation in the external environment (during storage) and in the gut, (2) improved bioavailability, (3) biocompatibility, (4) ease of scaling up at industrial production level [90]. The schematic diagram of SLN is depicted in Figure 10.6.

In case of NLCs, spatially very different lipid molecules are mixed to create a lipid particle matrix as imperfect as possible. Generally, solid and liquid lipids are mixed to produce NLCs that are still solid at room temperature as well as at body temperature. Due to many imperfections in NLCs, drug-loading capacity is enhanced and drug expulsion during storage is minimized. NLCs have several advantages, such as: (1) NLC dispersions with higher solid content can be produced, (2) drug loading capacity is better than that of SLNs, (3) drug release profile can be easily modulated, (4) drug leakage during storage is lower than with SLNs, and (5) production of final dosage forms (e.g., tablets, capsules) is feasible [90]. These technologies are applied to many herbal compounds. A pharmacokinetic study in male rats at an oral dose equivalent to 10 mg kg^{-1} demonstrated that α-asarone-loaded SLNs improve oral bioavailability and tissue uptake of the α-asarone compared to pure α-asarone group [91]. In vivo pharmacokinetics following oral administration of curcumin-loaded SLNs (50, 25, 12.5, and 1 mg kg^{-1} dose) and curcumin solution (50 mg kg^{-1}) demonstrated significant improvement in oral bioavailability (39, 32, 59, and 155 times at 50, 25, 12.5, and 1 mg kg^{-1} dose, respectively) after administration of SLNs compared to curcumin solution [92]. In another pharmacokinetic study with quercetin SLNs in rats following oral administration of quercetin (50 mg kg^{-1}) in the form of either SLNs or suspension showed that the relative bioavailability of quercetin−SLNs to quercetin suspension was 571.4%. The T_{max} and mean residence time for quercetin in plasma were delayed. The study suggested that SLNs may be potential carrier systems to enhance the absorption of poorly soluble drugs [93].

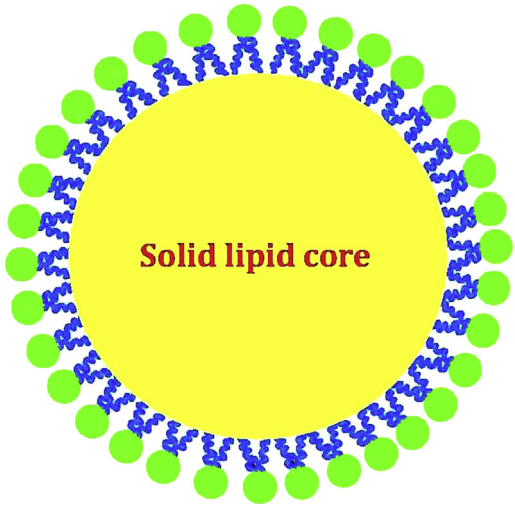


FIGURE 10.6 Schematic representation of solid lipid nanoparticle (SLN).

10.5.9 Microemulsion and NE

Microemulsions and NEs are isotropic mixtures of oils, surfactants, and cosolvents/cosurfactants [94]. NEs are thermodynamically stable systems that consist of emulsifier-coated oil droplets dispersed within an aqueous medium [95]. The size of the droplets (o/w: oil in water and w/o: water in oil) produced depends on the composition of the system and the

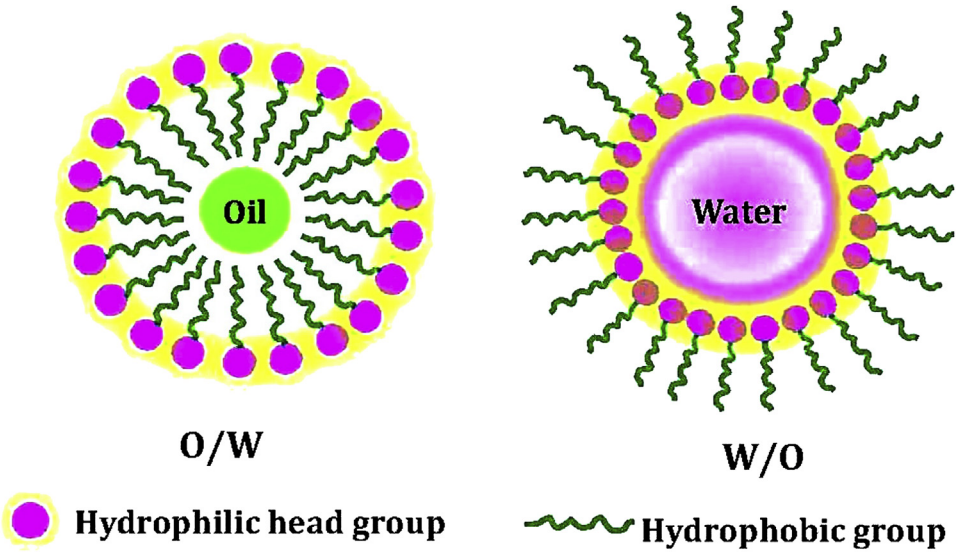


FIGURE 10.7 Structure of microemulsion or nanoemulsion.

homogenization method used. The structure of microemulsions/NEs is represented in Figure 10.7.

NEs when orally administered quickly disperse in the GI fluid through self-emulsification and form nanodroplets in the range of 20–200 nm with the digestive motility of the stomach and the intestine [96]. Incorporation of herbal drugs into these systems strengthens the stability, improves the permeability to the skin and mucous, and produces sustained-release effect. So far, herbal drugs, like *Brucea javanica* oil, camptothecin, coixenolide oil, and zedoary oil have been explored with these systems. Choudhury et al. (2014) developed the NEs (o/w) to improve the oral bioavailability of paclitaxel. The terminal elimination $t_{1/2}$ was increased in oral NE up to 5.79 h as compared to the IV formulation ($t_{1/2}$, 1.22 h) thereby indicating a sustained release profile of paclitaxel-loaded NE. Moreover, the absolute oral bioavailability and sustained-release profile of the paclitaxel NE evaluated in mouse model is found to improve up to 55.9% [97].

10.5.10 Micelles

Micelles are lipid molecules that set themselves in a spherical form in aqueous solutions. Polymeric micelles range from 10 to 100 nm in size, and they are usually very narrow. They increase the drug solubility, stability, and hence its biomembrane permeability and bioavailability through micellar surroundings. Drug release from micelles is governed by various factors, such as micelle stability, the rate of drug diffusion, the partition coefficient, and the rate of copolymer biodegradation. Polymeric micelles designed from amphiphilic block copolymers have been found to hold a significant potential as drug delivery vehicles for a variety of anticancer drugs due to unique properties, such as high solubility and low toxicity. They can also lessen the P-gp efflux effect and, consequently, show a different mechanism of action from the entrapped drugs [98]. The high toxicity of potent chemotherapeutic drugs like paclitaxel, doxorubicin, and many others limit the therapeutic window in which they can be applied, which can be expanded by using this form of delivery system. Preclinical studies revealed that in colon-26-bearing CDF1 mice, a more than 50 times higher AUC was obtained, while the maximum plasma concentration (C_{max}) in tumors was three times higher compared to paclitaxel alone [99]. In summary, polymeric micelle systems have become increasingly important in oncology, and so far, the evidence points to an increasing hope for use in cancer therapy. The structure of micelle is shown in Figure 10.8.

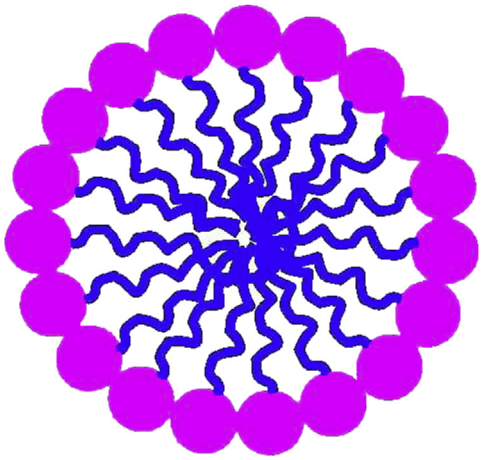


FIGURE 10.8 Structure of micelle.

10.5.11 Phytosome

Phytosome is a novel technology that emerged in 1989. The term "phyto" means plant/herb while "some" means cell-like structure [100]. Phytosome is a technology used as controlled- and sustained-release delivery systems consisting of phospholipid complex system of herbal extract or phytoconstituents in the nanosize range (<100 nm) of particles [101]. Phytosomes result from the reaction of a stoichiometric amount (1:1−1:3) of the phospholipid (phosphatidylcholine) with the standardized extract or phytoconstituents in a nonpolar solvent [100]. It is a patented technology to encapsulate standardized extracts or phytoconstituents into phospholipids to fabricate molecular complexes for enhancing their permeation and bioavailability, especially for those which have poor aqueous solubility and strong tendency to self-aggregate [102]. The schematic illustration of phytosome is shown in Figure 10.9.

The backbone of phytosomal system is only phospholipid [102], several phospholipids such as hydrogenated soy phosphatidylcholine (HSPC), dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine have been employed for this purpose [103]. Phospholipid-based delivery systems have been developed to improve the bioavailability of phytoconstituents and herbal extracts. Phytosome has been successfully applied to many plant extracts (milk thistle, *Ginkgo*, green tea, *Boswellia*, etc.) as well as phytochemicals (silybin, curcumin), with significant results in both animals and human pharmacokinetic studies [104]. Several studies on phytoconstituent−phopholipid complex have been reported, namely, quercetin [105], curcumin [106], EA [59], naringenin [101], AP [39], resveratrol [107], mangiferin [52], and GA [62], have they been successfully exploited for better therapeutic efficacy as well as bioavailability.

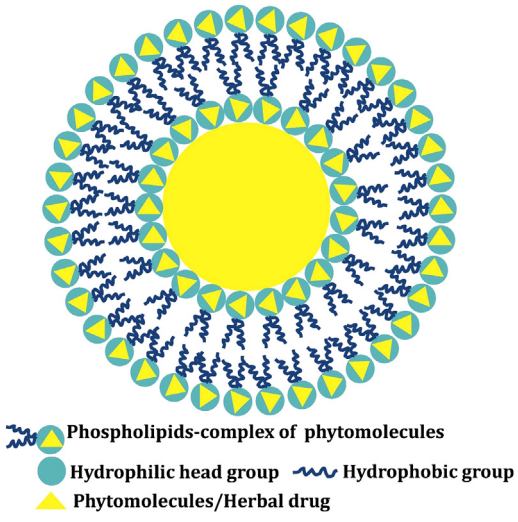


FIGURE 10.9 Schematic diagram of phytosome.

10.6 PHOSPHOLIPID COMPLEX OF HERBS—MODIFICATION OF BIOAVAILABILITY AND EFFICACY

Most of the biologically active constituents of plants are lipophilic or hydrophilic. However, hydrophilic phytoconstituents are poorly absorbed either due to their large molecular size which does not allow them to be absorbed by passive diffusion, or due to their poor lipid solubility, severely limiting their ability to pass across the biological membranes and resulting in poor bioavailability [108]. It has often been observed that the isolation and purification of the constituents of an extract may lead to the partial or total loss of specific bioactivity for the purified constituent; the natural constituent synergy is lost. Very often the chemical complexity of the crude or partially purified extract seems to be essential for the bioavailability of the active constituents. When extracts are taken orally, some constituents may be destroyed in the gastric environment.

Phytosome is produced by binding individual components of herbal extracts to phosphatidylcholine, resulting in a dosage form that is better absorbed and thus produces better results than the conventional herbal extracts. Encapsulating drugs can improve the solubility and pharmacokinetics of drugs, and in some cases, enable further clinical development of NCEs that have been stalled because of poor pharmacokinetic properties. The phytosome technology has been applied to many popular phytomedicines including GB, grape seed, milk thistle, green tea, naringenin, hesperetin, resveratrol, and ginseng [108].

10.6.1 Physical and Chemical Properties of Phytosomes

Phospholipids are complex molecules that are used in all known life forms to make cell membranes. Phospholipids are small lipid molecules where glycerol is bonded to two fatty acids, with the third hydroxyl, normally one of the two primary methylenes, bearing a phosphate group. Phospholipids from soy, mainly phosphatidylcholine, are lipophilic substances and readily complex polyphenolics. In this context, phosphatidylcholine, the major molecular building block of cell membranes and a compound miscible in both water and oil/lipid environments, is well absorbed orally, and has the potential to act as a chaperon for polyphenolics, accompanying them through biological membranes. In humans and other higher animals, the phospholipids are also employed as natural digestive aids and as carriers for both fat-miscible and water-miscible nutrients. They are miscible both in water and in lipid environments and are well absorbed orally. Phytosomes are

more bioavailable as compared to conventional herbal extracts owing to their enhanced capacity to cross the lipidic biomembrane and finally reaching the systemic circulation. Compared to liposomes, phytosomes are characterized by a high bioactive/lipid ratio [109] with an overall stoichiometry in the range of 1:1 to 1:3 between the constituents and the phospholipid formulation aid [110]. The complex can somewhat be compared to an integral part of the lipid membrane, where the polar functionalities of the lipophilic guests interact via hydrogen bonds with the polar head of a phospholipid (i.e., phosphate and ammonium groups), forming a unique arrangement that can be evidenced by spectroscopic analysis. Thus, Fourier transform infrared spectroscopic and multi–nuclear magnetic resonance studies show that a phytosome is not a mechanical mixture of two constituents, but a specific complex between a hydrophilic guest and a lipophilic host characterized by specific dipolar interactions, in accordance with the spectroscopic differences between a phytosome and a mechanical mixture of its two constituents [109]. Phytosomal carrier systems are promising techniques for the oral route of natural polyphenols as an effective delivery system. This scheme would be effective for phytomolecules to overcome the troubles concerned with the conventional systems.

10.6.2 The Impact of Phytosome Formulation on Bioavailability of Herbal Products

A phytosome is generally more bioavailable than a simple herbal extract due to its enhanced capacity to cross the lipid-rich biomembranes and reach circulation. The characteristics of phytosome are as follows: (1) the small size of the particles offers large interfacial area from which molecules can be quickly released; (2) it improves the solubility and biomembrane permeability and hence the bioavailability; (3) the phospholipids form a complex structure between groups of phytoconstituents so it improves the stability; (4) these are biocompatible, biodegradable, and nonmutagenic so it is clinically safe; (5) it offers the site-specific and prolonged-release profile; (6) it minimizes dose-associated toxicities and provides better therapeutic effects; (7) it is a value-added delivery system of nutraceutical and dietary supplements; and (8) it facilitates patient compliance.

This technology has now been utilized to improve the bioavailability and pharmacokinetic parameters of several phytomolecules like curcumin, quercetin, naringenin, hesperetin, and resveratrol. Among different strategies, a phytosome delivery system has emerged as a promising nanocarrier for oral and topical applications [100]. The impact of phospholipid complexation in the pharmacokinetics of herbal products is enumerated in Table 10.2.

The bioavailability of ginkgolides A and B and bilobalide in healthy subjects after oral administration of 160 mg extract (24% flavonol glycosides, 6% total terpene lactones) was greatly increased when it was administered as a phospholipid complex [111]. The dose was administered in the form of free extract or its phospholipid complex. The C_{max} of total ginkgolides and bilobalides were about two- to threefold higher when administered by the phospholipid complex. The mean elimination half-life of each terpene lactone was in the range of 120–180 min. To improve the oral bioavailability of silybin, silybin–phosphatidylcholine complex was developed [112].

The pharmacokinetics of silybin–phosphatidylcholine complex was studied in healthy subjects after a single dose of 80 mg (silybin equivalent). Free and conjugated silybin concentrations peaked at 2.4 and 3.8 h, respectively. The half-life of free and conjugated silybin was 1.6 and 3.4 h, respectively [113]. After administration of silybin–phosphatidylcholine complex (80 mg) both C_{max} and AUC were increased by two- and threefold, respectively, after intake of the soft gelatin compared to the hard gelatin capsule. The concentration of silybin in bile after oral administration of silybin–phosphatidylcholine complex and silymarin (120 mg, silybin equivalent) in surgical patients requiring T-tube biliary drainage was studied [114]. The amount of silybin recovered in bile in the free and conjugated forms within 48 h accounted for 11 and 3% of the dose after silipide and siliymarin administration, respectively. Based on the comparison of the biliary excretion values, the bioavailability of silybin from silipide was 4.2 times higher than that from silymarin [136].

The bioavailability of curcumin has been enhanced by phospholipid complex system. Liu et al. (2006) showed a significant improvement in curcumin bioavailability due to curcumin–phospholipid complex formation. In this study, curcumin (100 mg/kg) and curcumin–phospholipid complex (~100 mg/kg of curcumin) were administered orally to Sprague Dawley male rats. Curcumin–phospholipid complex showed a maximum plasma curcumin level of 600 ng/mL after 2.33 h of oral administration, as opposed to that of free curcumin having maximum plasma concentration of 267 ng/mL after 1.62 h of oral dosing. About 1.5-fold increase in curcumin half-life was found in rats in this study of the curcumin–phospholipid complex over free curcumin. These results indicate that the curcumin–phospholipid complex can significantly increase circulating levels of presumably active curcumin in rats [115]. Another study conducted by Maiti et al. (2007) showed a threefold increase in aqueous solubility and a better hepatoprotective effect of curcumin–phospholipid

TABLE 10.2 The Effect of Phospholipid Complex on the Pharmacokinetics of Different Herbal Products (Mean ± SD)

Phytomolecules		Subject	Oral dose	C_{max} (μg/mL)	T_{max} (h)	AUC_{0-t} (μgh/mL)	$AUC_{0-\infty}$ (μgh/mL)	$t_{1/2el}$ (h)	K_{el} (h^{-1})	C_L (Lh^{-1})	(V_d) (L)	Relative Bioavailability	Reference
Quercetin	Pure	Rats	20 mg/kg	6.56 ± 0.42	3.0	27.87 ± 2.02	28.05 ± 1.95	1.40 ± 0.05	0.493	0.11	0.224	–	[105]
	Complex			9.86 ± 0.52	6.0	112.87 ± 5.23	113.52 ± 6.23	3.22 ± 0.23	0.215	0.034	0.159	125.56%	
Naringenin	Pure	Rats	100 mg/kg	6.32 ± 0.41	5.0	318.07 ± 2.12	38.45 ± 2.44	2.43 ± 0.11	0.28	0.40	1.44	–	[102]
	Complex			9.35 ± 0.62	8.0	104.48 ± 5.64	107.48 ± 6.10	3.78 ± 0.15	0.18	0.17	0.95	118.55%	
Curcumin	Pure	Rats	1000 mg/kg	0.50	0.75	1.32	1.68	1.45	0.48	92.26	192.21	–	[106]
	Complex			1.20	1.50	5.90	8.73	1.96	0.35	22.33	63.82	125.80%	
Hesperetin	Pure	Rats	100 mg/kg	6.1 ± 0.30	4.0	30.47 ± 2.34	31.24 ± 2.54	1.78 ± 0.09	0.380	0.490	1.310	–	[116]
	Complex			9.2 ± 0.41	6.0	109.72 ± 6.72	151.90 ± 8.43	3.86 ± 0.13	0.140	0.130	0.970	133.31%	
Andrographolide	Pure	Rats	25 mg/kg	6.7 ± 0.54	2.50	26.24 ± 1.23	26.74 ± 1.42	1.20 ± 0.05	0.57	0.17	0.30	–	[103]
	Complex			9.6 ± 0.72	4.0	85.50 ± 2.77	87.30 ± 2.35	4.01 ± 0.12	0.21	0.055	0.26	104.24%	
EA	Pure	Rats	80 mg/kg	0.21	0.5	0.72	0.89	6.11	0.11	89,640.03	789,766.58	–	[59]
	Complex			0.54	0.5	2.05	2.53	8.32	0.08	31,681.15	380,301.38	2.84 fold	
Resveratrol	Pure	Rats	20 mg/kg	5.9	0.17	3.307	4.474	1.156	0.6	4.47	7.454	–	[107]
	Complex			7.2	0.17	4.693	10.0985	2.584	0.27	1.98	7.383	2.26 fold	
Genistein	Pure	Rats	20 mg/kg	1680	12	36,740	40,284.61	9.10	0.08	0.5	6.52	–	Unpublished data
	Complex			2405	12	57,066	62,049.10	11.63	0.06	0.32	5.41	1.54 fold	
Ginkgolide A	Extract	Human	160 mg	0.0418	2	–	–	2.63 ± 0.45	–	–	–	–	[111]
	Complex			0.108	4	–	–	1.88 ± 0.13	–	–	–	–	
Ginkgolide B	Extract	Human	160 mg	0.0056	2	–	–	2.34 ± 0.38	–	–	–	–	[111]
	Complex			0.0134	3	–	–	1.69 ± 0.30	–	–	–	–	
Bilobalide	Extract	Human	160 mg	0.0376	2	–	–	2.30 ± 0.24	–	–	–	–	[111]
	Complex			0.0603	3	–	–	3.16 ± 0.35	–	–	–	–	

complex compared to free curcumin. Curcumin–phospholipid complex significantly protected the liver from carbon-tetrachloride-induced acute liver damage in rats by restoring enzyme levels of the liver glutathione system and those of superoxide-dismutase-, catalase-, and thiobarbituric-acid-reactive substances [106]. Marczylo et al. (2007) explored whether the formulation with phosphatidylcholine increases the oral bioavailability or affects the metabolite profile of curcumin in vivo. Male Wistar rats received 340 mg/kg of either unformulated curcumin or curcumin formulated with phosphatidylcholine by oral gavage. Curcumin, the accompanying curcuminoids desmethoxycurcumin and bisdesmethoxycurcumin, and the metabolites tetrahydrocurcumin, hexahydrocurcumin, curcumin glucuronide, and curcumin sulfate were identified in plasma, intestinal mucosa, and liver of rats that had received the phospholipid complex. Peak plasma levels for parent curcumin after administration of the complex were fivefold higher than the equivalent values seen after unformulated curcumin dosing. Similarly, in the liver, the levels of curcumin were higher after administration as a complex as compared to unformulated curcumin. In contrast, curcumin concentrations in the GI mucosa after ingestion of Meriva were somewhat lower than those observed after administration of unformulated curcumin [50].

The bioavailability and pharmacokinetic parameters of AP–phospholipid complex were determined in the male Wistar rats after oral administration of pure AP 25 mg/kg and AP–phospholipid complex equivalent to 25 mg/kg. The pharmacokinetics was determined in a noncompartmental model. C_{max} and T_{max} were increased in the case of the complex. The elimination half-life of AP was increased when it was in phospholipid complex form, and eventually the clearance of the molecule in this form was also lowered. The phospholipid complex persisted for a longer period of time in the rat body, with a higher relative bioavailability of 104.24% [103].

In another study, the effect on the phospholipid complex on the pharmacokinetic parameters of hesperetin was determined in a noncompartmental model [116]. Male albino Wistar rats were divided into two groups, one group for oral administration of hesperetin at a dose of 100 mg/kg and the other group for oral administration of the complex at a dose equivalent to 100 mg/kg of hesperetin. The phospholipid complexation increased the oral absorption of hesperetin and provided a sustained-release effect. C_{max} as well as T_{max} increased in case of the complex. The AUC of hesperetin was increased from the complex compared to the free form. The elimination half-life of hesperetin was increased when it was in the complex form with phospholipids, and eventually the clearance of the molecule in complex form was also lowered. The complex persisted for a longer period of time in the body with a higher relative bioavailability of 133.31%.

The bioavailability and pharmacokinetics of EA, a poorly water-soluble phytochemical were improved by the use of phospholipid complex. EA at a dose of 80 mg/kg was administered orally to male albino Wistar rats. The other group of rats was administered of EA–posphatidylcholine complex at a dose equivalent to 80 mg/kg of EA. Peak serum concentration (0.21 μg/mL) was attained at 0.5 h when pure EA was administered. Also, in the case of complex, the peak concentration (0.54 μg/mL) appeared at 2 h, and concentration was maintained significantly for a longer period of time. The pharmacokinetic parameters of the EA–phospholipid complex and pure EA were determined in a noncompartmental model. C_{max} and T_{max} were increased in the case of the complex. The elimination half-life of EA was increased when it was in the complex form with phospholipids, and eventually the clearance of the molecule in complex form was also lowered. The $AUC_{0-\infty}$ for the EA–phospholipid complex was 2.53 units, whereas that of pure EA was 0.89 units. Thus, the EA–phospholipid complex has a relative bioavailability of 2.84 compared to that of pure EA. The results indicate that the relative bioavailability of the EA–phospholipid complex is 2.84-fold compared to normal EA [59].

In a similar study conducted by Mukherjee et al. (2011), the bioavailability and pharmacokinetics of resveratrol–HSPC complex were determined. Male albino Wistar rats were orally administered resveratrol at a dose of 20 mg/kg or resveratrol–HSPC complex at a dose equivalent to 20 mg/kg of resveratrol. Peak plasma concentration (5.9 μg/mL) was attained at 10 min when pure resveratrol was administered, whereas in case of complex, the peak concentration (7.2 μg/mL) appeared at 10 min and the concentration was maintained significantly for a longer period of time. The pharmacokinetic parameters calculated through a noncompartmental model showed that C_{max} and T_{max} were increased in the case of the complex. The elimination half-life of resveratrol was increased when it was in the complex form with phospholipids, and eventually the clearance of the molecule in complex form was also lowered. The $AUC_{0-\infty}$ for resveratrol–HSPC complex was 605.91 units, while that of pure resveratrol was 268.44 units. Thus, the complex has a relative bioavailability of 2.26 compared to pure resveratrol. The results indicate that the relative bioavailability of the complex is 2.26-fold compared to the normal resveratrol [107].

In an unpublished study, the effect of phospholipid complextion on the pharmacokinetic parameters of genistein was determined in our laboratory by a noncompartmental model. Male albino Wistar rats were divided into

two groups, one group for oral administration of genistein at a dose of 20 mg/kg and the other group for oral administration of the complex at a dose equivalent to 20 mg/kg of genistein. The phospholipid complexation increased the oral absorption of genistein and provided a sustained-release effect. C_{max} was increased in the case of the complex, so did the T_{max}. The elimination half-life of genistein was increased when it was in the complex form with phospholipids, and eventually the clearance of the molecule in complex form was also lowered. The $AUC_{0-\infty}$ for the genistein−phospholipid complex was 62,049.1 units, while that of pure genistein was 40,284.61 units. Thus, the complex has a relative bioavailability of 1.54-fold compared to that of pure genistein.

10.7 CONCLUSION

Use of phytomedicines is limited because they are poorly absorbed due to several physicochemical problems. The goal of novel delivery systems is to enhance permeation and bioavailability of lipophilic plant secondary metabolites with low bioavailability by improving its solubility using NDDS. The effectiveness of any herbal medicine depends upon delivering an effective level of therapeutically active compounds. Lipid solubility and molecular size are the major rate-limiting factors for molecules to cross the biological membrane. The improvement of bioavailability of drugs with such properties presents one of the greatest challenges in drug formulations. Oral lipid-based formulations are attracting considerable attention due to their capacity to increase the solubility, facilitate GI absorption, and reduce the effect of food on the absorption of low-water-soluble, lipophilic drug and therefore increase the bioavailability. Moreover, most of the potential molecules from the herbs have low bioavailability and pharmacokinetic profile. The half-life of phytomolecules plays an important role in the therapeutic effect and availability of drug concentration in blood/plasma. This has resulted in greater dependence on herbal medicine. With the growth of novel delivery systems, newer formulations of herbal medication can be produced with the intent to change the physical attributes of the phytochemicals, which is responsible for its reduced bioavailability. Phospholipid-based delivery systems, i.e. phytosome, liposome, niosome, transfersome, SLN, and NE, are found to be very effective for the delivery of herbal drugs in contrast to conventional delivery systems. This approach may be useful to accomplish the desired therapeutic effect even at a lower dosage of the same drug and hence with reduced side effects. So a novel attempt in the area of herbal drug delivery system can be successfully exploited to increase the bioavailability as well as pharmacological activity.

Acknowledgment

The authors wish to express their gratitude to Department of Biotechnology, Govt. of India, New Delhi, for providing financial support through senior research fellowship to Ranjit K. Harwansh.

References

[1] Rahman MA, Harwansh R, Mirza MA, Hussain S, Hussain A. Oral lipid based drug delivery system (LBDDS): formulation, characterization and application: a review. Curr Drug Deliv 2011;8:1−16.
[2] Kesarwani K, Gupta R. Bioavailability enhancers of herbal origin: an overview. Asian Pac J Trop Biomed 2013;3:253−66.
[3] Mukherjee PK, Venkatesh M, Maiti K, Mukherjee K, Saha BP. Value added herbal drug delivery systems−perspectives and developments. Indian J Pharm Educ Res 2009;43:329−37.
[4] Ajazuddin SS. Applications of novel drug delivery system for herbal formulations. Fitoterapia 2010;81:680−9.
[5] Li Y, Zheng J, Xiao H, McClements DJ. Nanoemulsion-based delivery systems for poorly water-soluble bioactive compounds: influence of formulation parameters on polymethoxyflavone crystallization. Food Hydrocolloids 2012;27:517−28.
[6] Silva HD, Cerqueira MÂ, Vicente AA. Nanoemulsions for food applications: development and characterization. Food Bioprocess Technol 2012;5:854−67.
[7] Sessa M, Balestrieri ML, Ferrari G, Servillo L, Castaldo D, D'Onofrio N, et al. Bioavailability of encapsulated resveratrol into nanoemulsion-based delivery systems. Food Chem 2014; 147:42−50.
[8] Aqil F, Munagala R, Jeyabalan J, Vadhanam MV. Bioavailability of phytochemicals and its enhancement by drug delivery Systems. Cancer Lett 2013;334:133−41.
[9] Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 2001;46:3−26.
[10] Huang Q, Yu H, Ru Q. Bioavailability and delivery of nutraceuticals using nanotechnology. J Food Sci 2010;75:R50−7.
[11] Brown JE, Khodr H, Hider RC, Rice-Evans CA. Structural dependence of flavonoid interactions with Cu21 ions: implications for their antioxidant properties. Biochem J 1998;330:1173−8.
[12] Walle T. Absorption and metabolism of flavonoids. Free Radical Biol Med 2004;36:829−37.
[13] Espín JC, García-Conesa MT, Tomás-Barberán FA. Nutraceuticals: facts and fiction. Phytochemistry 2007;68:2986−3008.
[14] Passamonti S, Terdoslavich M, Franca R, Vanzo A, Tramer F, Braidot E, et al. Bioavailability of flavonoids: a review of their membrane transport and the function of bilitranslocase in animal and plant organisms. Curr Drug Metab 2009;10:369−94.
[15] Hollman PC, Bijsman MN, VanGameren Y, Cnossen EP, DeVries JH, Katan MB. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. Free Radical Res 1999;31:569−73.
[16] Harborne JB. The flavonoids: advances in research since 1986. London, UK: Chapman & Hall; 1994.
[17] Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J Nutr 2000;130:2073S−85S.
[18] Walle T, Hsieh F, DeLegge MH, Oatis Jr JE, Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 2004;32:1377−82.
[19] Wenzel E, Somoza V. Metabolism and bioavailability of trans-resveratrol. Mol Nutr Food Res 2005;49:472−81.
[20] Bhattaram VA, Graefe U, Kohlert C, Veit M, Derendorf H. Pharmacokinetics and bioavailability of herbal medicinal products. Phytomedicine 2002;9:1−33.

[21] Fourtillan JB, Brisson AM, Girault J, Ingrand I, Decourt JP, Drieu K, et al. Pharmacokinetic properties of bilobalide and ginkgolides A and B in healthy subjects after intravenous and oral administration of *Ginkgo biloba* extract (EGb 761). Therapie 1995;50:137–44.
[22] Kleijnen J, Knipschild P. Ginkgo biloba. Lancet 1992;340: 1136–9.
[23] Biber A, Koch E. Bioavailability of ginkgolides and bilobalide from extracts of *Ginkgo biloba* using GC/MS. Planta Med 1999; 65:192–3.
[24] Biber A, Fischer H, Römer A, Chatterjee SS. Oral bioavailability of hyperforin from *Hypericum* extracts in rats and human volunteers. Pharmacopsychiatry 1998;31:36–43.
[25] Staffeldt B, Kerb R, Brockmöller J, Ploch M, Roots I. Pharmacokinetics of hypericin and pseudohypericin after oral intake of the *Hpericum perforatum* extract LI 160 in healthy volunteers. J Geriatr Psychiatry Neurol 1994;1:S47–53.
[26] Fox E, Murphy RF, McCully CL, Adamson PC. Plasma pharmacokinetics and cerebrospinal fluid penetration of hypericin in nonhuman primates. Cancer Chemother Pharmacol 2001;47: 41–4.
[27] Yoshikawa M, Murakami T, Yamahara J, Matsuda H. Bioactive saponins and glycosides: XII. Horse chestnut. (2) Structures of escins IIIb, IV, V, and VI and isoescins Ia, Ib, and V, acylated polyhydroxyoleanene triterpene oligoglycosides, from the seeds of horse chestnut tree (*Aesculus hippocastanum* L. Hippocastanaceae). Chem Pharm Bull 1998;46:1764–9.
[28] Loew D, Schrodter A, Schwankl W, Marz RW. Measurement of the bioavailability of aescin-containing extracts. Methods Find Exp Clin Pharmacol 2000;22:537–42.
[29] Lang W, Mennicke WH. Pharmacokinetic studies on tritiated aescin in the mouse and rat. Arzneimittelforschung 1972;22: 1928–32.
[30] Steinegger E, Hövel H. Analytische und biologische untersuchungen an saliceen-wirkstoffen, insbesondere an salicin: i. identifizierungs-, isolierungs- und bestimmungsmethoden. Pharm Acta Helv 1972;47:133–41.
[31] Pentz R, Busse HG, König R, Sioegers CP. Bioavailability of salicylic acid and caffeine from a combination preparation phytoanalgetischen Lübeck, Med Univ, Diss. 1989.
[32] Schmid B, Kötter I, Heide L. Pharmacokinetics of salicin after oral administration of a standardised willow bark extract. Eur J Clin Pharmacol 2001;57:387–91.
[33] Weyhenmeyer R, Mascher H, Birkmayer J. Study on dose-linearity of the pharmacokinetics of silibin diastereomers using a new stereospecific assay. Int J Clin Pharmacol Ther Toxicol 1992;30:134–8.
[34] Erlund I, Kosonen T, Alfthan G, Maenpaa J, Perttunen K, Kenraali J, et al. Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. Eur J Clin Pharmacol 2000;56:545–53.
[35] Graefe EU, Wittig J, Mueller S, Riethling AK, Uehleke B, Drewelow B, et al. Pharmacokinetics and bioavailability of quercetin glycosides in humans. J Clin Pharmacol 2001;41: 492–9.
[36] Morand C, Manach C, Crespy V, Remesy C. Respective bioavailability of quercetin aglycone and its glycosides in a rat model. Biofactors 2000;12:169–74.
[37] Suo XB, Zhang H, Wang YQ. HPLC determination of andrographolide in rat whole blood: study on the pharmacokinetics of andrographolide incorporated in liposomes and tablets. Biomed Chromatogr 2007;21:730–4.
[38] Ye L, Wang T, Tang L, Liu W, Yang Z, Zhou J, et al. Poor oral bioavailability of a promising anticancer agent andrographolide is due to extensive metabolism and efflux by P glycoprotein. J Pharm Sci 2011;100:5007–17.
[39] Delmas D, Aires V, Limagne E, Dutartre P, Mazué F, Ghiringhelli F, et al. Transport, stability, and biological activity of resveratrol. Ann N Y Acad Sci 2011;1215:48–59.
[40] Goldberg DM, Yan J, Soleas GJ. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem 2003;36:79–87.
[41] Boocock DJ, Faust GE, Patel KR, Schinas AM, Brown VA, Ducharme MP, et al. Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. Cancer Epidemiol Biomarkers Prev 2007;16:1246–52.
[42] Brown VA, Patel KR, Viskaduraki M, Crowell JA, Perloff M, Booth TD, et al. Repeat dose study of the cancer chemopreventive agent resveratrol in healthy volunteers: safety, pharmacokinetics, and effect on the insulin-like growth factor axis. Cancer Res 2010;70:9003–11.
[43] Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. Bioavailability of curcumin: problems and promises. Mol Pharmaceutics 2007;4:807–18.
[44] Holder GM, Plummer JL, Ryan AJ. The metabolism and excretion of curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6- heptadiene-3,5-dione) in the rat. Xenobiotica 1978;8:761–8.
[45] Wahlstrom B, Blennow G. A study on the fate of curcumin in the rat. Acta Pharmacol Toxicol 1978;43:86–92.
[46] Ravindranath V, Chandrasekhara N. Absorption and tissue distribution of curcumin in rats. Toxicology 1980;16:259–65.
[47] Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, Srinivas PS. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med 1998;64:353–6.
[48] Pan MH, Huang TM, Lin JK. Biotransformation of curcumin through reduction and glucuronidation in mice. Drug Metab Dispos 1999;27:486–94.
[49] Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, et al. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clin Cancer Res 2004;10:6847–54.
[50] Marczylo TH, Verschoyle RD, Cooke DN, Morazzoni P, Steward WP, Gescher AJ. Comparison of systemic availability of curcumin with that of curcumin formulated with phosphatidylcholine. Cancer Chemother Pharmacol 2007;60: 171–7.
[51] Yang KY, Lin LC, Tseng TY, Wang SC, Tsai TH. Oral bioavailability of curcumin in rat and the herbal analysis from *Curcuma longa* by LC-MS/MS. J Chromatogr B Anal Technol Biomed Life Sci 2007;853:183–9.
[52] Bhattacharyya S, Ahmmed SM, Saha BP, Mukherjee PK. Soya phospholipid complex of mangiferin enhances its hepatoprotectivity by improving its bioavailability and pharmacokinetics. J Sci Food Agric 2014;94:1380–8.
[53] Han D, Chen C, Zhang C, Zhang Y, Tang X. Determination of mangiferin in rat plasma by liquid-liquid extraction with UPLC–MS/MS. J Pharm Biomed Anal 2010;51:260–3.
[54] Hou S, Wang F, Li Y, Li Y, Wang M, Sun D, et al. Pharmacokinetic study of mangiferin in human plasma after oral administration. Food Chem 2012;132:289–94.
[55] Zhang Y, Wang GJ, Song TT, Murphy PA, Hendrich S. Urinary disposition of the soybean isoflavones daidzein, genistein and glycitein differs among humans with moderate fecal isoflavone degradation activity. J Nutr 1999;129:957–62.
[56] Coldham NG, Sauer MJ. Pharmacokinetics of [14C]genistein in the rat: gender-related differences, potential mechanisms of biological action, and implications for human health. Toxicol Appl Pharmacol 2000;164:206–15.
[57] Kwon SH, Kang MJ, Huh JS, Ha KW, Lee JR, Lee SK, et al. Comparison of oral bioavailability of genistein and genistin in rats. Int J Pharm 2007;337:148–54.

[58] Anupongsanugool E, Teekachunhatean S, Rojanasthien N, Pongsatha S, Sangdee C. Pharmacokinetics of isoflavones, daidzein and genistein, after ingestion of soy beverage compared with soy extract capsules in postmenopausal Thai women. BMC Clin Pharmacol 2005;3:5-−2.
[59] Murugan V, Mukherjee K, Maiti K, Mukherjee PK. Enhanced oral bioavailability and antioxidant profile of ellagic acid by phospholipids. J Agric Food Chem 2009;57:4559−65.
[60] Stoner GD, Sardo C, Apseloff G, Mullet D, Wargo W, Pound V, et al. Pharmacokinetics of anthocyanins and ellagic acid in healthy volunteers fed freeze-dried black raspberries daily for 7 days. J Clin Pharmacol 2005;45:1153−64.
[61] Seeram NP, Lee R, Heber D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. Clin Chim Acta 2004;348: 63−8.
[62] Bhattacharyya S, Ahammed SM, Saha BP, Mukherjee PK. The gallic acid-phospholipid complex improved the antioxidant potential of gallic acid by enhancing its bioavailability. AAPS PharmSciTech 2013;14:1025−33.
[63] Gao S, Zhan Q, Li J, Yang Q, Li X, Chen W, et al. LC-MS/MS method for the simultaneous determination of ethyl gallate and its major metabolite in rat plasma. Biomed Chromatogr 2010;24:472−8.
[64] Ferruzzi MG, Lobo JK, Janle EM, Cooper B, Simon JE, Wu QL, et al. Bioavailability of gallic acid and catechins from grape seed polyphenol extract is improved by repeated dosing in rats: implications for treatment in Alzheimer's disease. J Alzheimers Dis 2009;18:113−24.
[65] Konishi Y, Kobayashi S. Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabolites in intestinal caco-2 cell monolayers. J Agric Food Chem 2004;5:2518−26.
[66] Chen WC, Liou SS, Tzeng TF, Lee SL, Liu IM. Effect of topical application of chlorogenic acid on excision wound healing in rats. Planta Med 2013;79:616−21.
[67] Ren J, Jiang X, Li C. Investigation on the absorption kinetics of chlorogenic acid in rats by HPLC. Arch Pharmacal Res 2007;30: 911−6.
[68] Yang H, Yuan B, Li L, Chen H, Li F. HPLC determination and pharmacokinetics of chlorogenic acid in rabbit plasma after an oral dose of *Flos Lonicerae* extract. J Chromatogr Sci 2004;42:173−6.
[69] Qin SH, Liu HG. Pharmacokinetics study on yinhuang compound microenema in rabbits. Zhongguo Zhongyao Zazhi 2006;31:54−6.
[70] Sultana S, Verma K, Khan R. Nephroprotective efficacy of chrysin against cisplatin-induced toxicity via attenuation of oxidative stress. J Pharm Pharmacol 2012;64:872−81.
[71] Walle T, Otake Y, Brubaker JA, Walle UK, Halushka PV. Disposition and metabolism of the flavonoid chrysin in normal volunteers. Br J Clin Pharmacol 2001;51:143−6.
[72] Tong L, Wan M, Zhang L, Zhu Y, Sun H, Bi K. Simultaneous determination of baicalin, wogonoside, baicalein, wogonin, oroxylin A and chrysin of *Radix scutellariae* extract in rat plasma by liquid chromatography tandem mass spectrometry. J Pharm Biomed Anal 2012;70:6−12.
[73] Zhu Y, Tong L, Zhou S, Sun H, Bi K, Zhang Boli. Simultaneous determination of active flavonoids and alkaloids of Tang-Min-Ling-Pill in rat plasma by liquid chromatography tandem mass Spectrometry. J Chromatogr B Anal Technol Biomed Life Sci 2012;904:51−8.
[74] Melo CLLD, Queiroz MGR, Filho ACVA, Rodrigues AM, Sousa DFD, Almeida JGL, et al. Betulinic acid, a natural pentacyclic triterpenoid, prevents abdominal fat accumulation in mice fed a high-fat diet. J Agric Food Chem 2009;57:8776−81.
[75] Udeani GO, Zhao GM, Shin YG, Cooke BP, Graham J, Beecher CWW, et al. Pharmacokinetics and tissue distribution of betulinic acid in CD-1 mice. Biopharm Drug Dispos 1999;20: 379−83.
[76] Rahman MA, Hussain A, Iqbal Z, Harwansh RK, Singh LR, Ahmad S. Nanosuspension: a potential nanoformulation for improved delivery of poorly bioavailable drug. Micro Nanosyst 2013;5:273−87.
[77] Mishra B, Patel BB, Tiwari S. Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery. Nanomedicine 2010;6:9−24.
[78] Braithwaite MC, Tyagi C, Tomar LK, Kumar P, Choonara YE, Pillay V. Nutraceutical-based therapeutics and formulation strategies augmenting their efficiency to complement modern medicine: an overview. J Funct Foods 2014;68:2−99.
[79] El-Samaligy MS, Afifi NN, Mahmoud EA. Evaluation of hybrid liposomes-encapsulated silymarin regarding physical stability and in vivo performance. Int J Pharm 2006;319:121−9.
[80] Benson HAE. Transdermal drug delivery: penetration enhancement techniques. Curr Drug Deliv 2005;2:23−33.
[81] Bavarsad N, Bazzaz BSF, Khamesipour A, Jaafari MR. Colloidal, in vitro and in vivo anti-leishmanial properties of transfersomes containing paromomycin sulfate in susceptible BALB/c mice. Acta Trop 2012;124:33−41.
[82] Mahale NB, Thakkar PD, Mali RG, Walunj DR, Chaudhari SR. Niosomes: novel sustained release nonionic stable vesicular systems−an overview. Adv Colloid Interface Sci 2012;183:46−54.
[83] Junyaprasert VB, Singha P, Suksiriworapong J, Chantasart D. Physicochemical properties and skin permeation of Span 60/ Tween 60 niosomes of ellagic acid. Int J Pharm 2012;423: 303−11.
[84] Paolino D, Lucania G, Mardente D, Alhaique F, Fresta M. Ethosomes for skin delivery of ammonium glycyrrhizinate: in vitro percutaneous permeation through human skin and *in vivo* anti-inflammatory activity on human volunteers. J Controlled Release 2005;106:99−110.
[85] Jain NK, Gupta U. Application of dendrimer-drug complexation in the enhancement of drug solubility and bioavailability. Expert Opin Drug Metab Toxicol 2008;4:1035−52.
[86] Abderrezak A, Bourassa P, Mandeville JS, Sedaghat-Herati R, Tajmir-Riahi HA. Dendrimers bind antioxidant polyphenols and cisplatin drug. PLoS ONE 2012;7:1−12.
[87] Tiyaboonchai W, Tungpradit W, Plianbangchang P. Formulation and characterization of curcuminoids loaded solid lipid nanaparticles. Int J Pharm 2007;337:299−306.
[88] Arica YB, Benoit JP, Lamprecht A. Paclitaxel-loaded lipid nanoparticles. Drug Dev Ind Pharm 2006;32:1089−94.
[89] Mainardes RM, Evangelista RC. PLGA nanoparticles containing praziquantel: effect of formulation variables on size distribution. Int J Pharm 2005;290:137−44.
[90] Das S, Chaudhury A. Recent advances in lipid nanoparticle formulations with solid matrix for oral drug delivery. AAPS PharmSciTech 2011;12:62−76.
[91] Wang D, Wang X, Li X, Ye L. Preparation and characterization of solid lipid nanoparticles loaded with α-asarone. PDA J Pharm Sci Technol 2008;62:56−65.
[92] Hu L, Jia H, Luo Z, Liu C, Xing Q. Improvement of digoxin oral absorption in rabbits by incorporation into solid lipid nanoparticles. Pharmazie 2010;65:110−3.
[93] Li H, Zhao X, Ma Y, Zhai G, Li L, Lou H. Enhancement of gastrointestinal absorption of quercetin by solid lipid nanoparticles. J Controlled Release 2009;133:238−44.
[94] Harwansh RK, Patra KC, Pareta SK. Nanoemulsion as potential vehicles for transdermal delivery of pure phytopharmaceuticals and poorly soluble drug. Int J Drug Delivery 2011;3:209−18.
[95] Harwansh RK, Patra KC, Pareta SK, Singh J, Rahman MA. Nanoemulsions as vehicles for transdermal delivery of glycyrrhizin. Braz J Pharm Sci 2011;47:769−78.

[96] Porter CJH, Pouton CW, Cuine JF, Charman WN. Enhancing intestinal drug solubilization using lipid-based delivery systems. Adv Drug Delivery Rev 2008;60:673–91.
[97] Choudhury H, Gorain B, Karmakar S, Biswas E, Dey G, Barik R, et al. Improvement of cellular uptake, in vitro antitumor activity and sustained release profile with increased bioavailability from a nanoemulsion platform. Int J Pharm 2014;460:131–43.
[98] Mikhail AS, Allen C. Block copolymer micelles for delivery of cancer therapy: transport at the whole body, tissue and cellular levels. J Controlled Release 2009;138:214–23.
[99] Hamaguchi T, Matsumura Y, Suzuki M, Shimizu K, Goda R, Nakamura I, et al. NK105, a paclitaxel incorporating micellar nanoparticle formulation, can extend in vivo antitumour activity and reduce the neurotoxicity of paclitaxel. Br J Cancer 2005;92: 1240–6.
[100] Kidd PM. Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. Altern Med Rev 2009;14:226–46.
[101] Freag MS, Elnaggar YSR, Abdallah OY. Lyophilized phytosomal nanocarriers as platforms for enhanced diosmin delivery: optimization and ex vivo permeation. Int J Nanomed 2013;8: 2385–97.
[102] Maiti K, Mukherjee K, Gantait A, Saha BP, Mukherjee PK. Enhanced therapeutic potential of naringenin-phospholipid complex in rats. J Pharm Pharmacol 2006;58:1227–33.
[103] Maiti K, Mukherjee K, Murugan V, Saha BP, Mukherjee PK. Enhancing bioavailability and hepatoprotective activity of andrographolide from Andrographis paniculata, a well-known medicinal food, through its herbosome. J Sci Food Agric 2010; 90:43–51.
[104] Hüsch J, Bohnet J, Fricker G, Skarke C, Artaria C, Appendino G, et al. Enhanced absorption of boswellic acids by a lecithin delivery form (Phytosome®) of *Boswellia* extract. Fitoterapia 2013;84: 89–98.
[105] Maiti K, Mukherjee K, Gantait A, Ahamed KFH, Saha BP, Mukherjee PK. Enhanced therapeutic benefit of quercetin-phospholipid complex in carbon tetrachloride-induced acute liver injury in rats: a comparative study. Iran J Pharmacol Ther 2005;4:84–90.
[106] Maiti K, Mukherjee K, Gantait A, Saha BP, Mukherjee PK. Curcumin-phospholipid complex: preparation, therapeutic evaluation and pharmacokinetic study in rats. Int J Pharm 2007;330:155–63.
[107] Mukherjee K, Venkatesh M, Venkatesh P, Saha BP, Mukherjee PK. Effect of soy phosphatidyl choline on the bioavailability and nutritional health benefits of resveratrol. Food Res Int 2011;44: 1088–93.
[108] Pifferi G. Silipide: a new bioavailable complex of silybin. Planta Med 1991;57:A12.
[109] Semalty A, Semalty M, Riwat MSM, Franceschi F. Supramolecular phospholipids-polyphenolics interaction: the PHYTOSOME® strategy to improve the bioavailability of phytochemicals. Fitoterapia 2010;81:306–14.
[110] Hüsch J, Dutagaci B, Glaubitz C, Geppert T, Schneider G, Harms M, et al. Structural properties of so-called NSAID-phospholipid-complexes. Eur J Pharm Sci 2011;44:103–16.
[111] Mauri P, Simonetti P, Gardana C, Minoggio M, Morazzoni P, Bombardelli E, et al. Liquid chromatography/atmospheric pressure chemical ionization mass spectrometry of terpene lactones in plasma of volunteers dosed with *Ginkgo biloba* L. extracts. Rapid Commun Mass Spectrom 2001;15:929–34.
[112] Gatti G, Perucca E. Plasma concentrations of free and conjugated silybin after oral intake of a silybin-phosphatidylcholine complex (silipide) in healthy volunteers. Int J Clin Pharmacol Ther 1994;32:614–7.
[113] Savio D, Harrasser PC, Basso G. Softgel capsule technology as an enhancer device for the absorption of natural principles in humans, a bioavailability cross-over randomised study on silybin. Arzneimittelforschung 1998;48:1104–6.
[114] Schandalik R, Gatti G, Perucca E. Pharmacokinetics of silybin in bile following administration of silipide and silymarin in cholecystectomy patients. Arzneimittelforschung 1992;42:964–8.
[115] Liu A, Lou H, Zhao L, Fan P. Validated LC/MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin. J Pharm Biomed Anal 2006;40:720–7.
[116] Maiti K, Mukherjee K, Murugan V, Saha BP, Mukherjee PK. Exploring the effect of hesperetin-HSPC complex - a novel drug delivery system on the in vitro release, therapeutic efficacy and pharmacokinetics. AAPS PharmSciTech 2009;10:943–50.
[117] Jacobson JM, Feinman L, Liebes L, Ostrow N, Koslowski V, Tobia A, et al. Pharmacokinetics, safety, and antiviral effects of hypericin, a derivative of St. John's wort plant, in patients with chronic hepatitis-C virus infection. Antimicrob Agents Chemother 2001;45:517–24.
[118] Brockmöller J, Reum T, Bauer S, Kerb R, Hübner WD, Roots I. Hypericin and pseudohypericin: pharmacokinetics and effects on photosensitivity in humans. Pharmacopsychiatry 1997;30: 94–101.
[119] Kerb R, Brockmöller J, Staffeldt B, Ploch M, Roots I. Single-dose and steady-state pharmacokinetics of hypericin and pseudohypericin. Antimicrob Agents Chemother 1996;40: 2087–93.
[120] Howells LM, Berry DP, Elliott PJ, Jacobson EW, Hoffmann E, Hegarty B, et al. Phase I randomized, double-blind pilot study of micronized resveratrol (SRT501) in patients with hepatic metastases-safety, pharmacokinetics, and pharmacodynamics. Cancer Prev Res 2011;4:1419–25.
[121] Amiot MJ, Romier B, Dao TM, Fanciullino R, Ciccolini J, Burcelin R, et al. Optimization of trans-Resveratrol bioavailability for human therapy. Biochimie 2013;95:1233–8.
[122] Muzzio M, Huang Z, Hu SC, Johnson WD, McCormick DL, Kapetanovic IM. Determination of resveratrol and its sulfate and glucuronide metabolites in plasma by LC–MS/MS and their pharmacokinetics in dogs. J Pharm Biomed Anal 2012; 59:201–8.
[123] Liang L, Liu X, Wang Q, Cheng S, Zhang S, Zhang M. Pharmacokinetics, tissue distribution and excretion study of resveratrol and its prodrug 3,5,4′-tri-O-acetylresveratrol in rats. Phytomedicine 2013;20:558–63.
[124] Zhang J, Tang Q, Xu X, Li N. Development and evaluation of a novel phytosome-loaded chitosan microsphere system for curcumin delivery. Int J Pharm 2013;448:168–74.
[125] Arya P, Pathak K. Assessing the viability of microsponges as gastro retentive drug delivery system of curcumin: optimization and pharmacokinetics. Int J Pharm 2014;460:1–12.
[126] He B, Li Q, Jia Y, Zhao L, Xiao F, Lv C, et al. A UFLC-MS/MS method for simultaneous quantitation of spinosin, mangiferin and ferulic acid in rat plasma: application to a comparative pharmacokinetic study in normal and insomnic rats. J Mass Spectrom 2012;47:1333–40.
[127] Cai F, Xu W, Wei H, Sun L, Gao S, Yang Q, et al. Simultaneous determination of active xanthone glycosides, timosaponins and alkaloids in rat plasma after oral administration of Zi-Shen Pill extract for the pharmacokinetic study by liquid chromatography-tandem mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci 2010;878:1845–54.
[128] Liu Y, Xu F, Zeng X, Yang L, Deng Y, Wu Z, et al. Application of a liquid chromatography/tandem mass spectrometry method to pharmacokinetic study of mangiferin in rats. J Chromatogr B Anal Technol Biomed Life Sci 2010;878:3345–50.
[129] Setchell KD, Brzezinski A, Brown NM, Desai PB, Melhem M, Meredith T, et al. Pharmacokinetics of a slow-release formulation of soybean isoflavones in healthy postmenopausal women. J Agric Food Chem 2005;53:1938–44.
[130] Hou P, Zeng Y, Ma B, Wang X, Liu Z, Li L, et al. A fast, sensitive, and high-throughput method for the simultaneous

quantitation of three ellagitannins from *Euphorbiae pekinensis* Radix in rat plasma by ultra-HPLC-MS/MS. J Sep Sci 2013;36: 2544−51.
[131] Shahrzad S, Aoyagi K, Winter A, Koyama A, Bitsch I. Pharmacokinetics of gallic acid and its relative bioavailability from tea in healthy humans. J Nutr 2001;131:1207−10.
[132] Zhou W, Liu S, Ju W, Shan J, Meng M, Cai B, et al. Simultaneous determination of phenolic acids by UPLC-MS/MS in rat plasma and its application in pharmacokinetic study after oral administration of *Flos Lonicerae* preparations. J Pharm Biomed Anal 2013; 86:189−97.
[133] Sun H, Dong T, Zhang A, Yang J, Yan G, Sakurai T, et al. Pharmacokinetics of hesperetin and naringenin in the Zhi Zhu Wan, a traditional Chinese medicinal formulae, and its pharmacodynamics study. Phytother Res 2013;27:1345−51.
[134] Yang CP, Liu MH, Zou W, Guan XL, Lai L, Su WW. Toxicokinetics of naringin and its metabolite naringenin after 180-day repeated oral administration in beagle dogs assayed by a rapid resolution liquid chromatography/tandem mass spectrometric method. J Asian Nat Prod Res 2012;14:68−75.
[135] Wen J, Qiao Y, Yang J, Liu X, Song Y, Liu Z, et al. UPLC-MS/MS determination of paeoniflorin, naringin, naringenin and glycyrrhetinic acid in rat plasma and its application to a pharmacokinetic study after oral administration of Si-Ni-San decoction. J Pharm Biomed Anal 2012;66:271−7.
[136] Tong L, Zhou D, Gao J, Zhu Y, Sun H, Bi K. Simultaneous determination of naringin, hesperidin, neohesperidin, naringenin and hesperedin of *Fractus aurantii* extract in rat plasma by liquid chromatography tandem mass spectrometry. J Pharm Biomed Anal 2012;58:58−64.
[137] Jin MJ, Kim U, Kim IS, Kim Y, Kim DH, Han SB, et al. Effects of gut microflora on pharmacokinetics of hesperidin: a study on non-antibiotic and pseudo-germ-free rats. J Toxicol Environ Health 2010;73:1441−50.

LIST OF ABBREVIATIONS

ABC ATP binding cassette
ADME Absorption, distribution, metabolism, and elimination
AG Ammonium glycyrrhizinate
AP Andrographolide
APE *Andrographis paniculata* extract
API Active pharmaceutical ingredients
ATP Adenosine triphosphate
AUC Area under the curve
BA Betulinic acid
CA Chlorogenic acid
COMT Catechol-O-methyltransferase
CSF Cerebrospinal fluids
CYP450 Cytochrome P450
DPPC Dipalmitoylphosphatidyl choline
DSPC Distearoylphosphatidyl choline
EA Ellagic acid
FA Ferulic acid
GA Gallic acid
GB *Ginkgo biloba*
GI Gastrointestinal
GIT Gastrointestinal tract
GLUT-2 Glucose transporter-2
GSH Glutathione
HP *Hypericum perforatum*
HSPC Hydrogenated soy phosphatidylcholine
I.P. Intraperitoneal
ISM Indian system of medicine
mPEG Poly (ethylene glycol) moiety'
MRT Mean residence time
NCEs New chemical entities
NDDS Novel drug delivery system
NE Nanoemulsion
NLC Nanostructured lipid carrier
NPs Nanoparticles
ODMA O-desmethylangolensin
PAMAM Poly(amidoamine)
PC Phosphatidylcholine
PEG400 Polyethylene glycol 400
PG Propylene glycol
P-gp P-glycoprotein
PLA Polylactic acid
PLGA Copolymer of lactic acid and glycolic acid
ROS Reactive oxygen species
SC Stratum corneum
SGLT1 Sodium-dependent glucose transporter-1
SLN Solid lipid nanoparticle
TCM Traditional Chinese medicine
TDDS Targeted drug delivery system

This page intentionally left blank

CHAPTER

11

Good Quality and Clinical Practices for the Future Development of Herbal Medicines

Gail B. Mahady

Department of Pharmacy Practice, College of Pharmacy, PAHO/WHO Collaborating Centre for Traditional Medicine, University of Illinois at Chicago, Chicago, IL USA

OUTLINE

11.1 INTRODUCTION

11.1.1 Increasing Use of Traditional and Complementary Medicines

According to the World Health Organization (WHO), the term Traditional medicine (TM) is defined as "the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement, or treatment of physical and mental illness" [1]. The WHO contends that TMs including herbal medicines (HMs), where proven to be of sufficient quality, safety, and efficacy can "contribute

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00011-8

to the goal of ensuring healthcare for all people" [1]. WHO estimates that a large percentage of the global population relies on TM as a normal part of healthcare [1]. The interest in TMs and HMs has increased and not decreased, as billions of people worldwide are using HMs and other traditional (complementary and alternative) treatments such as acupuncture, massage therapy, and traditional practitioners (healers), as part of their daily healthcare services (Figures 11.1 and 11.2; adapted from Refs [1,2]).

TMs are culturally acceptable and trusted by many populations worldwide. HMs are often easily accessible and more affordable than is Western medicine [1,3]. It is the accessibility and affordability of most HMs that make their use very practical in light of excessive healthcare costs. HMs are also useful for the treatment of conditions that are chronic noncommunicable diseases, such as chronic back pain [1,3]. Thus, it is not surprising that during 1994–2014, there has been an increased interest in TMs and herbal medicinal products (HMPs) worldwide. This interest is in response to the healthcare needs of not only developing countries but also of developed nations, where the use of HMs and acupuncture is widely recognized as complementary and alternative medicines (CAMs) and are now readily accepted by large segments of the population [4,5]. For example, a 2007 survey by the National Center for Complementary and Alternative Medicine/National Institutes of Health suggested that approximately 38% of the US population (~100 M people) were using CAM therapies, including HMs [5]. However, more recent data suggest that as high as 68% (>200 M; Figure 11.2) of the US population are using dietary supplements (DSs), including HMs and spending approximately $11.5 billion USD on these products, including the $5.6 billion USD spent on herbal supplements [4,6]. In fact, while acupuncture is a well-recognized aspect of traditional Chinese medicine, it is now used worldwide in approximately 80% of 129 countries [1].

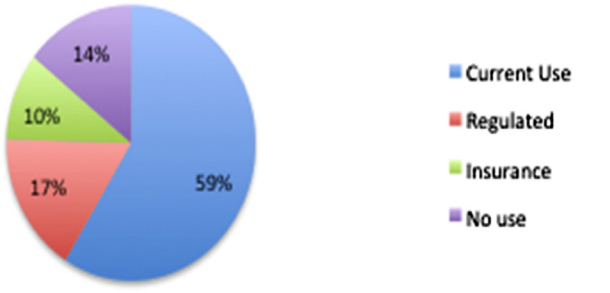


FIGURE 11.1 Use of acupuncture by 129 WHO member states 2012.

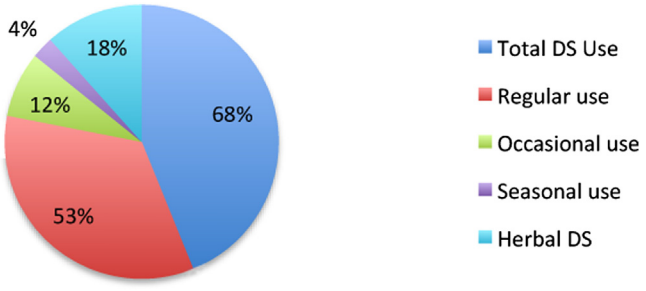


FIGURE 11.2 Dietary supplement use in the United States in 2013.

11.1.2 Global HMs Market

Although the diversity of regulations and regulatory categories for TM products and services make it difficult to accurately assess the size of the total market, available data suggest that the TM market is substantial. For example, the output of Chinese Materia Medica was estimated to amount to $83.1 billion USD in 2012, an increase of >20% from the previous year's value [1]. In addition, to facilitate the flow of traditional Chinese medicines (TCMs) into the United States, the Chinese Pharmacopoeia has begun an international collaboration with the US Pharmacopoeia to monograph approximately 100 TCMs for inclusion as quality monographs in the United States Pharmacopoeia's (USP's) Herbal Compendium. These monographs will contain quality information only, and do not serve to provide information on the medical use of these products [7]. Another good example is the Republic of Korea, whose annual expenditures on TM were $4.4 billion USD in 2004 that had increased to $7.4 billion USD by 2009 [1]. Out-of-pocket spending for natural products in the United States was estimated at $14.8 billion USD in 2008 [8]. As suggested by the Nigeria Natural Medicine Development Agency (Nigeria), the global market for HM is currently estimated at $160 billion USD [9], compared with the estimated $19 billion USD in 2006 [10]. What is clear from these data is that the use of TMs and HMs has not declined in popularity but in fact has seen considerable growth despite the dismal economic climate, which may in fact have contributed to their popularity due to lower costs. With prevailing current global financial constraints, the use of TM for health promotion, self-healthcare, and disease prevention where appropriate has the potential to reduce some healthcare costs. One good example of this is St. John's wort (SJW; *Hypericum perforatum* L. Clusiaceae) [11]. Indigenous to northern Africa, South Africa, South America, Asia, Australia, Europe, and New Zealand, SJW is naturalized in the United States [11]. Clinically, SJW is used for the symptomatic treatment of mild to moderate depressive episodes (classified as F32.0 and F32.1, respectively, in the International statistical

classification of diseases and related health problems [11]). Reviews and meta-analyses of randomized controlled trials (RCTs) have found SJW preparations to be superior to that of placebo and similarly as effective as standard antidepressants in the acute treatment of mild to moderate depression [11–15]. Several meta-analyses and a clinical trial have shown that the rates of adverse events are comparable to those of placebo and are less than that of standard antidepressant treatments [11–15]. Thus, a clear advantage of SJW over antidepressants has been demonstrated by a reduced frequency of adverse effects and lower treatment withdrawal rates, low rates of side effects, and good compliance, key variables affecting the cost effectiveness of a given form of therapy [16]. The most important risk associated with SJW is the possibility of drug interactions that can be somewhat mitigated by using extracts with lower hyperforin concentrations. Considering that the indirect costs of depression are five times greater than direct treatment costs, and given the increasing cost of pharmaceutical antidepressants as compared with the comparatively low cost of SJW extracts, it is worthy of consideration as first line therapy of mild to moderate depression [16]. Thus, SJW is a very good example of how an HM may eventually be considered as first-line therapy when proven safety and efficacy have been established in clinical trials, along with good quality assurance.

11.1.3 Role of HMs in Economic Development

While HMs are growing and expanding worldwide, particularly with respect to products bought over the Internet, the possibility that they can play a major role in the economic development in all countries is significant. However, major stumbling blocks still exist in terms of quality, safety, and efficacy for herbal products in most countries, including the United States. Several factors hamper the full-scale application of traditional HMs including lack of implementation of effective quality assurance/control in the manufacturing process; lack of traceability in the supply chain and associated value additions; and improper identification of botanical species, as well as the lack of characterized chemical constituents that can affect the therapeutic efficacy of the final product [17,18]. Thus, improving the overall quality of HMs worldwide will have a positive impact on both the safety and efficacy of these products, and facilitate their acceptance and thereby enhance commerce.

Quality, safety, and efficacy are still the primary issues that need to be addressed before HMs can reach their full potential of *contributing to the goal of ensuring healthcare for all people*.

11.2 QUALITY ISSUES: LACK OF GMP OR FAILURE TO COMPLY WITH cGMP GUIDELINES

11.2.1 Issues with GMP of HMs in the United States

The quality of any therapeutic product whether it is a prescription drug or HMs is the foundation upon which safety and efficacy of any treatment is based (Figure 11.3). Good quality and batch-to-batch consistency ensure the efficacy, safety, and clinical reproducibility of any medicine [18]. While the quality of many medicinal herbs has been monographed in pharmacopoeias such as the US, Chinese, European, Japanese, Indian, and African pharmacopoeias, these quality standards are often times voluntary and not mandatory for manufacturers. In the United States, HMs are regulated as botanical DSs under the Dietary Supplements Health and Education Act (DSHEA) of 1994, and herbal quality is not based on a monograph system. Under DSHEA, the manufacturer alone is responsible for the quality and safety of a DS before it goes on the market, but once on the market, the US. Food and Drug Administration (FDA) must prove that a supplement is unsafe for general consumption before it can be removed from the marketplace. Under DSHEA, the manufacturers are not required to register DS products with the FDA or obtain FDA approval, before producing or selling DSs unless it is a New Dietary Ingredient, or to verify the acceptability of structure–function claims [17]. In 2007, the FDA published current Good manufacturing guidelines (cGMP) for DSs in the US Federal Register [19]. The new GMP rules require manufacturers to manufacture all DSs under cGMP. The 815 page rule "establishes the minimum cGMPs necessary for activities related to manufacturing, packaging, labeling, or holding DSs to ensure the quality of the dietary supplements," and documentation of the entire manufacturing process is required [19]. The final rules for cGMP require that

FIGURE 11.3 Quality control and GMP are important for safety and efficacy.

proper controls are in place for the entire DS manufacturing process so that DSs are processed consistently, and meet quality standards [17,18]. These cGMP regulations apply to all domestic and foreign companies that manufacture, package, label, or hold DSs, and include those involved with the activities of testing, quality control, packaging and labeling, and distribution in the United States [19]. The final rules contend that cGMPs are necessary to ensure that DSs are manufactured consistently to ensure identity, purity, strength, and composition ([19]; extensively reviewed in Refs [17,18]). However, even though cGMPs were instituted by the US FDA and all DS manufacturers should have come into compliance by 2010, four years later in 2014, the quality of herbal products continues to be problematic in the United States [20]. The Director of the Dietary Supplements Program at the FDA has stated that many companies are still not compliant even on basic compliance issues and encourages all companies to come into compliance or face consequences [20]. This is a serious problem because a lack of quality compliance on the part of the manufacturers can impact public perception of HMs, as well as sales. Lack of quality control and assurance can also affect both the efficacy and/or safety of the herbal products being used. If the product is of poor quality due to adulteration with prescription drugs, contamination with heavy metals or microbes, or prepared with misidentified plants, both safety and efficacy are jeopardized. Thus, while public perception of the quality of herbal supplements is high in the United States, the truth is that herbal product quality in the United States ranges from very high to very low, despite the institution of cGMPs.

11.2.2 The Need for GMP in Developing Countries

Rather than following the US example, where possible all countries should develop, institute, and enforce their own GMPs for HMs that would ensure good quality products and facilitate clinical trials and safety studies. By doing so, the acceptability of these herbal products worldwide will increase dramatically. For countries without current GMPs for herbal products and needing guidance, the WHO's Traditional Medicine Program (TRM) has published two books "Quality control methods for herbal materials" [21] and "WHO guidelines on Good agricultural and collection practices for medicinal plants" [22] to establish internationally recognized guidelines for assessing herbal quality and facilitate the production of HMs. WHO-TRM has published these guidelines to promote the safety of HMs as many of the adverse events associated with HMs have been attributed to poor quality of the product [21]. However, for any manufacturer or company wishing to enter their products into the US market, the US cGMP guidelines apply, and all companies must be compliant with these regulations. To assist companies in complying with cGMP for the United States, the USP also has a program of independent testing to certify legally marketed DSs in the United States. This is a voluntary program for DS manufacturers and involves the review of manufacturing and quality control processes and testing of product samples, including off-the shelf-testing follow-up. In addition to testing for contamination, adulteration, and good manufacturing processes, the USP also examines products for pharmacologic properties, dissolution, and breakdown in the stomach. Companies that use this service obtain a USP verification mark for products that meet the program's rigorous standards and a statement on the label saying "USP has tested and verified ingredients, product, and manufacturing process." The USP sets official standards for DSs. See www.uspverified.org. Postverification, off-the-shelf testing to monitor conformance to program requirements is also performed to ensure postmarketing quality. The results of the USP evaluations can be found free of charge at www.uspverified.org to assist consumers in finding good quality DS products. This program is highly regarded by pharmacists, physicians, and other healthcare professionals in the United States.

11.3 SAFETY OF HMS IN THE UNITED STATES, EUROPE, AND ASIA

11.3.1 Patient Safety and the Widespread Use of HMs

As we have previously mentioned in the Introduction, the use of HMs has been increasing steadily in the United States for several years. However, this is true for many other countries in Europe and Asia as well. One multinational study looked at the use of HMs in 9459 pregnant women from 23 countries in Europe, North America, South America, and Australia [23]. Of the women surveyed, 28.9% reported the use of HMs during pregnancy. Most HMs were used for pregnancy-related ailments such as the common cold or nausea and vomiting. The highest rate of using HMs was reported in Russia at 69%. Women from Eastern Europe (51.8%) and Australia (43.8%) were twice as likely to use HMs as were those in other regions [23]. Considering that there is a significant lack of clinical trials demonstrating safety and efficacy of many HMs in general, and almost no information on their safety during pregnancy, these new statistics are alarming. Beyond the use in pregnancy, the use of HMs in pediatric populations is also rising worldwide.

A recent study in Italy showed that the use of HMs for the treatment of neurological conditions is increasing [24]. In this study, CAMs (including HMs) were used by 76% patients in a cohort of 124 children affected by headaches. These treatments were being used preventively in 80% cases because the parents were trying to avoid the chronic use of drugs with their related side effects, the reported inefficacy of prescription medicines, and the wish for a more integrated approach to treatment [24]. Interestingly, herbal remedies such as *Valeriana*, *Ginkgo biloba*, *Boswellia serrata*, *Vitex agnus-castus*, passion flower, and Linden tree made up 64% of these CAM therapies, and showed that HMs from traditional systems of medicine from Europe, China, and India are all being used in this pediatric population [24]. Again the significant issues here are that with the exception of Valarian, most of these HMs have not been tested in clinical trials in children, so we do not have data for safety or efficacy in children. In another study from Italy, the use of CAM in children with recurrent acute otitis media (RAOM) was investigated [25]. Eight hundred and forty Italian children with RAOM ($\geq$3 episodes in six months) for the prevalence, determinants, reasons, cost, and perceived safety and efficacy of CAM. About one-half (46%) of the children used CAM therapies, including HMs. The use was associated with CAM use in the family, a fear of the adverse effects of conventional medicines (40%) and to increase host defenses (20%). CAM was widely seen as safe (95%) and highly effective (68%), and CAM prescribers were pediatricians in 50.7% of cases [25]. A similar study in Germany looked at CAM use in 500 children in outpatient clinics [26]. Of the 405 respondents 229 (57%) reported lifetime CAM use. Among CAM users, the most prevalent therapies were homeopathy (25%), herbal remedies (8%), anthroposophic medicine (7%), vitamin preparations (6%), and acupuncture (5%) [26]. Similar reports have been published from the United States where CAM and HM use in children is high and continuing to increase. There is an absolute dearth of clinical information concerning HM safety and efficacy in pediatric populations, and thus, there is now an urgent need for these studies.

11.3.2 The Safety of TCMs

TCM, including TCM drugs, has played an important role in health protection and disease control and prevention in China for thousands of years [27,28]. Relying on natural drug products, primarily herbal, that are used either as raw materials for decoction, or prepared as formulated TMs, are widely accepted by the Chinese, especially for chronic disease treatment [27]. However, because of the global rise in the use of TCMs and Chinese Materia Medica (medicinal materials), there is a great deal of concern about their evidence base, safety/possible toxicity, questionable quality, and use of endangered species of both animals and plants in other countries [29]. The potential toxicity of many TCMs is well recognized, and to reduce risks, the use of some herbs is restricted while for others specific processing methods have been developed to modify the activities/toxicity of these plants [30]. However, many adverse reactions and drug interactions have been reported. Many of these problems have been associated with the misuse or abuse of Chinese medicine; however, there are also many cases where these products have been adulterated with pharmaceuticals for weight loss or erectile dysfunction [30]. As the use of TCM worldwide continues to grow, improved pharmacovigilance and pharmacoepidemiology are needed to contribute valuable safety information that will be relevant to clinical use. The utility and risk factors associated with these drugs require both a proper understanding and control of the risk by strengthening standardization of clinical applications, basic science research, quality control in manufacturing, exploration of the active monitoring methodology, and enhancement of international communication and cooperation [27].

11.3.3 New Safety Measures in the United States for DSs

In December 2006, the Dietary Supplement and Nonprescription Drug Consumer Safety Act S.2546 was passed in the United States [31]. This act establishes a mandatory reporting system of serious adverse events for nonprescription "over-the-counter" (OTC) drugs and DSs that are sold for consumption in the United States [17]. As part of the requirements of the Act, all manufacturers, packers, or distributors of DSs are required to submit serious adverse event reports associated with use of their DS in the United States to the FDA, through the adverse reaction reporting program (MedWatch). The Act contains five major provisions: (1) It requires manufacturers and distributors of DSs and OTC drugs to report all serious adverse events to FDA. (2) The reporting requirements are limited to the information FDA really needs: reports of death; a life-threatening experience; hospitalization; a persistent or significant disability or incapacity; a congenital anomaly or birth defect. (3) The bill recognizes the need for reports to be accurate and valid. It does so in two ways—by prohibiting false reporting, and by authorizing the FDA to issue guidance on the information that is needed for a report to be deemed complete [32]. The bill sets a 15-day timeframe for responsible persons to submit the serious reports they receive to the FDA and requires that manufacturers

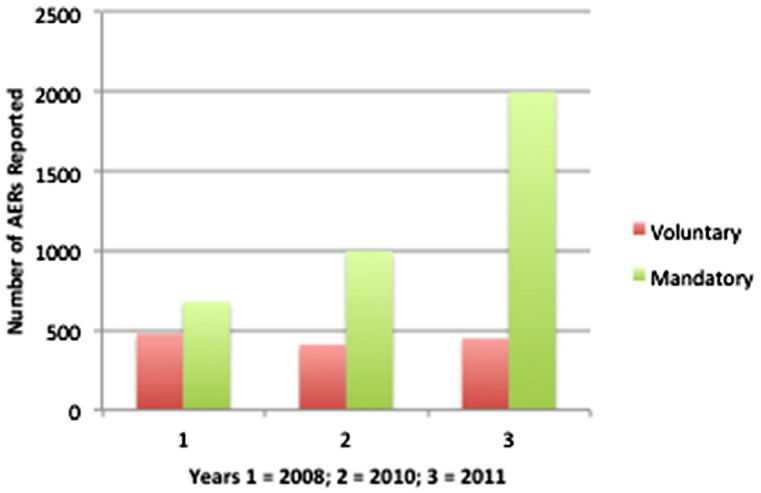


FIGURE 11.4 Adverse events reports (AERs) reported from 2008 to 2011 after FDA mandatory reporting requirements. *Adapted from GAO 2013.*

keep these for 6 years. Finally, the new Federal reporting requirement supersedes state reporting [33]. Thus, all manufacturers and companies selling DS in the United States must comply with this law. From 2008 to 2011, the FDA received 6307 reports of adverse events reports (AERs) related to DSs ([34]; Figures 11.4 and 11.5). Approximately 70% of these reports came from industry for serious adverse events, and most were linked to DSs containing multiple ingredients. The number of adverse events reported in the United States has doubled from 2008 to 2011 mainly due to the mandatory reporting by industry, while voluntary reporting of adverse events remained stable [35]. The increase in mandatory adverse event reporting has been driven by stricter FDA enforcement efforts including warning letters, and second by lawsuits against companies that did not report this information and that have publicized the consequences of adverse events [34]. Despite the mandatory reporting, AERs for herbal supplements in the United States remain very low.

While HMs have the perception of being relatively safe as compared with prescription medications, it is well known that some HMs have adverse events, contraindications, drug interactions, and toxicities [17,32,33,35–47]. While the percentage of HMs with intrinsic toxicity is low, one good example of a toxic plant is *Aristolochia*, which contains artistolochic acid, a compound know to be toxic [44,47,48]. The toxicity of Aristolochia came to light in 1992, when female consumers ingesting a herbal weight-loss preparation sold in Belgium developed a severe renal disease called aristolochic acid nephropathy (ANN) [44,47,48]. The problem was attributed to the misidentification of *Stephania tetrandra* S. Moore, Menispermaceae that should have been present in the weight-loss preparation, which was found to be an *Aristolochia* species. Batch testing of the weight-loss product showed that it did not contain *S. tetrandra's* chemical constituent tetrandrine, but it rather contained aristolochic acids instead [17,44,48]. This mistake in plant identification led to an outbreak of renal failure and resulting kidney transplants, and the patients that developed ANN from ingesting the product are at a higher risk for developing bladder urothelial cancers [32]. This incident highlights

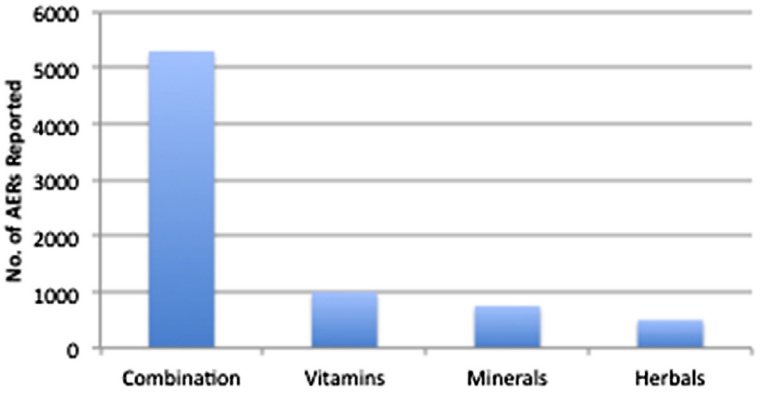


FIGURE 11.5 Number of adverse events reports (AERs) associated with dietary supplements from 2008 to 2011. *Adapted from GAO 2013.*

how important quality assurance and control for herbal products impact the safety of these products.

Other potential causes of toxicity from HMs in addition to the plant itself include contamination with toxic metals, adulteration with prescription drugs, misidentification of the plant materials, or substitution of herbal ingredients, as well as improperly processed or prepared products [33,35–39]. Herb–drug interactions are more often being reported between prescription drugs and herbs or as herb–herb interactions as well as food–herb interactions [38,40,45,46].

11.3.4 Protocols and Guidance Documents for Safety and Toxicity of Herbals

Recently, there has been increasing concern that the reporting of adverse events and drug interactions for HMs is underestimated, and thus, safety assessments of HMs are urgently needed [17]. Thus, during 2004–2014, a number of protocols and guidance documents outlining safety and toxicity testing of herbal have been published by multiple groups [49–54], including the International Life Sciences Institute, the Institute of Medicine (IOM)/National Research Council, the Union of Pure and Applied Chemistry, the European Medicines Agency, and the European Food Safety Authority [50–54]. An excellent review of these documents, the impact of quality on the safety of HMs, adverse reactions, and causality assessments can be found in Ref. [17]. These various guidance documents illustrate examples of the types of information that are needed to adequately define the toxicity of a specific herb or a finished herbal product [17]. Obviously, global harmonization of the methods would be ideal; however, because the international systems regulating herbs varies considerably from country to country in terms of safety requirements and toxicity testing, harmonization of these practices is difficult and expensive. In countries where extensive toxicity testing for herbals is not required, such as the United States, there is little in the way of premarketing testing. While in European countries where herbals are regulated as HMPs, they are assessed for quality, safety, and efficacy prior to market authorization, and pharmacovigilance is actively used to promote the postmarketing safety of HMPs [17].

11.3.5 IOM Guidelines for the Safety of HMs

In light of the fact that the global use of HMs continues to increase and not decrease, the identification of safety signals becomes of increased importance [53]. The IOM at the National Academies of Sciences developed guidelines for the assessment of safety for herbals [53]. Under this model, safety assessments should begin with preexisting data, including historical and traditional use. Clinical assessment of adverse reaction reports should be considered along with any available toxicological and pharmacological information to fully characterize potential safety concerns [53]. It is essential that quality information of the product being assessed is included, so that judgments made as to the hazard and risk of herbal products can be made with increased certainty [53]. However, the identification and investigation of safety signals for HMs are often hampered by deficiencies in both the quantity of information (e.g., underreporting of adverse reactions, general lack of toxicological information on herbs) and the quality of information (e.g., poor quality of adverse reaction case reports or lack of an adequate description of the herbal products that is associated with case reports submitted to regulatory authorities or published in the scientific literature [17,55]). Thus, quality standardized adverse event reporting systems containing sufficient details to properly identify the product and herbs associated with the event are essential for postmarketing surveillance. From a regulatory perspective, a well-performed safety assessment can significantly impact if certain herbal products should be restricted, removed from the market, or have augmented safety information placed on the label [17].

11.4 EFFICACY: THE IMPORTANCE OF RANDOMIZED CONTROLLED CLINICAL TRIALS

11.4.1 Clinical Data for HMs Need to be Improved

Although HMs have played an integral role in medicine and healing for thousands of years, and there is much in the way of in vitro and in vivo experiential data, as well as observational “evidence” for the more common herbs, clinical data to support the use of HMs remain poor. Since most countries now practice evidence-based medicine (EBM), the lack of good quality clinical data for most herbal causes a great deal of skepticism concerning their efficacy by many healthcare professionals. Evidence concerning the efficacy of botanicals ranges from historical data, observational case studies, uncontrolled clinical trials to randomized double-blind, placebo controlled clinical trials, and meta-analyses [56–61]. Randomized controlled clinical trials provide the best evidence for efficacy, and are well recognized as the gold standard for allopathic research. HMs, while not recognized as an integrative part of conventional care in most Western countries, are still used by many patients in their healthcare

management [56]. Thus, the evaluation of efficacy of HMs using good clinical practice guidelines and the principles of EBM is of paramount importance to having these therapies accepted by mainstream medicine, and integrated into medical practice. Historical, pharmacological, and observational studies can provide good preclinical support, as well as mechanisms of action, and chemical studies in combination with biological and pharmacological studies can provide details of active constituents, which are needed for pharmacokinetic and pharmacodynamic investigations [18,56,57]. Observational and uncontrolled studies are useful for hypothesis generation and identification of potential adverse events. However, they are not without bias and confounding, and thus should be considered supporting evidence rather than primary [18,56]. The "gold standard" of evidence for treatment efficacy within EBM is considered to be a systematic review or meta-analysis of RCTs with double blinding and a comparator group [56–58] that are used to determine the efficacy or lack thereof of a specific treatment intervention [56]. As the use of herbal products continues to increase over 2014–2024, clinical investigation of HMs becomes increasingly important, because once efficacy is proven, treatments can be endorsed and integrated into medical practice.

11.4.2 WHO Operational Guidelines for Clinical Trials

While there are many issues involved with clinical trial research as it relates to HMs, there are also many guidelines that are available to assist with the product quality and the clinical trial design. The WHO has published an operational guideline regarding these regulatory requirements needed to support clinical trials of herbal products [60]. The evaluation of the quality of clinical research in HM uses the same approach as that of clinical trials of prescription drugs, with additional components specifically needed for HMs. Determination of the quality of a clinical trial is complex; however, the RCT study design provides the best level of protection against bias that is seen in other study designs. The RCT design protocol should always include the details of the product being tested (as much detail as possible so that the study may be repeated): the baseline subject details, how randomization was performed; how the blinding was done; and accounting of all participants, even dropouts [56–61]. Randomization needs to be explained in detail, as it is this process that assigns similar subjects to receive or not receive the specific intervention (treatment). Allocation of treatments may be either computer generated, or achieved by the use of a table of random numbers. The use of delineators, such as date of birth, date of admission, or similar, is not recommended. Every participant must have an equal opportunity of receiving the intervention, and the investigators must not be able to predict which treatment the participant will receive [56]. Proper randomization significantly reduces bias when compared with trials that do not.

For clinical trials involving HMs, blinding is critical and maybe the most difficult problem to address. Blinding helps to separate the placebo effect and the observer bias in the trial. The most reliable RCT is double blind meaning that neither the prescriber nor the patient knows about the treatment allotment [56]. These precautions will ensure that the clinical data are collected in an unbiased method, and that neither the patient's nor the clinician's responses will be influenced by their attitudes or own biases.

HM trials are intrinsically difficult to blind, particularly when the treatments are multifaceted, and involve counseling, listening, explaining, lifestyle, and dietary advice as well as prescribing HMs [56]. In addition, blinding the product means that the placebo and the test product are identical in appearance, feel, taste, smell, and dosage form. For most HMs with a strong taste, odor, or color, this is difficult as masking these characteristics can require considerable work and is sometimes expensive. Masking of the treatment received by the subjects is acceptable only when the either the participants or the investigators involved in the study are able to identify which intervention is being assessed. A properly blinded study must report a detailed description of both the placebo and the treatment being tested.

In the analysis of the finished study, all participants who started the trial must be accounted for regardless of whether they dropped out or were not included in the final analysis [56–61]. The number and percent of dropouts and the reasons for withdrawal must be detailed. If participants in the active arm drop out due to adverse events or because they perceive that the treatment is not working, this information still needs to be reported. Intention to treat (ITT) analysis should be included in all RCTs, which is a strategy for analyzing data in which all participants are included in the group in which they were assigned, whether or not they completed the intervention [56]. The ITT preserves the randomization by fully accounting for all the participants in all arms of the study [56].

In addition to randomization, blinding, and accounting for all participants, RCTs must state the estimated size effect of the intervention on the outcome measures [56–61]. The treatment effect of the clinical trial is called the point estimate and provides the best estimate of the size effect. Sample size also needs to be calculated in response to the size effect. Further more, there needs

to be an adequate description of statistical methods used to analyze the data generated by the study and how they were applied [56–61]. In terms of the final effect, appropriate inclusion and exclusion criteria are important for the overall outcomes of the study, and must be clearly explained and relevant to the clinical condition being studied. Outcomes and endpoints must be measured using validated and accepted methods, as using methods that have not been validated or old assessment measures that are no longer accepted will reduce the overall quality of the study. Outcome measures should be able to be objectively measured and clinically relevant, and ones that meet a diverse array of needs and values of patient populations [56–61].

11.4.3 Herbal Product Descriptions in Clinical Trials Must be Detailed

Botanical and product descriptions of the HM being used in the clinical trial must be given clearly so that they are sufficient for another investigator to reproduce the study with the same or similar product. This is critical to determine the overall effect and safety of the HM being tested. Latin binomials and authorities, plant part used, type of preparation, identity of the solvent for extracts and liquids, ratio of solvent to plant material, and chemical standardization are all needed where available. The details of the placebo are especially important: how it was prepared and with what. If it is a matching placebo, all critical details are needed not only to ascertain the quality of the study and compare it with other previously published studies but also in order for researchers to be able to repeat the study with the appropriate herbal product. It is relatively impossible to compare the results of clinical trials for HMs in terms of safety and efficacy if the products used are not similar in strength and dose. There are too many examples in the current literature to indicate that one herbal formulation is effective as a given treatment, and that another formulation of the same HMs is not. The clinical trials for Echinacea are a very good example of this problem, to show that efficacy in clinical trials is linked to specific products.

11.4.4 Guidelines for Clinical Trials for TCM

Recently, as part of the GP-TCM project, a team of experienced clinical researchers and Chinese medical practitioners have developed clinical trial guidelines for TCM that combines an appreciation for traditional methods of practice with detailed and practical advice on research methodology. These guidelines are published and emphasize the importance of identifying best practice, and then developing and applying appropriate and rigorous research methodologies to investigate TCM as a whole system [62]. While the guidelines were developed to enhance the robustness of medical and scientific investigation of TCM, and clarify the contribution that TCM can make to our future healthcare, similar approaches could also be used for Ayurveda, Unani, or other traditional systems of medicine. Innovative new approaches are urgently needed, including the application of transcriptomic, metabolomic, and proteomic technologies and systems biology as a way of enhancing research and the understanding of TM [62].

11.5 FUTURE OUTLOOK FOR HMS

The interest and use of HMs and botanical DSs (United States) continues to increase, despite the adverse economic circumstances worldwide. It is estimated that in the United States, herbal supplement sales will continue to rise during 2015–2019 [4,6]. The global herbal market is roughly estimated at $160 billion USD and will also continue to increase; however, it will be HMs with proven quality, safety, and efficacy that will lead the way in global markets. TCMs are now making their way to the United States, and many are backed by safety and efficacy clinical trials from China where they are viewed as medicines to treat serious conditions such as congestive heart failure, high blood pressure, and many other diseases. How these products will fit into the DSHEA and regulatory framework of the United States is yet to be determined, as in China, TCMs are medicines for the treatment and prevention of disease, and DSHEA strictly prohibits this. The output of Chinese Materia Medica was estimated to amount to $83.1 billion USD in 2012, alone and continues to increase at double-digit rates [1]. India has now moved into the second position after China, as one of the leading exporters of herbal products, and it will soon become mandatory for all new TMs to undergo clinical trials before entering the market [53]. However, this includes only the newly patented TMs and not the classical formulations from India's ancient texts, some of which are 5000 years old [53]. India will now start to impose strict enforcement of quality and safety of herbal drugs. This is a very strategic move by India as the quality of India's herbal drugs has always been in question, especially in the Western countries. But, where good quality and clinical data exist, the sky is the limit in terms of sales of these products. The European Union's Traditional HMPs Directive has also stated that companies making herbal products will have to provide clinical data to demonstrate their safety through the use of those products within the EU for a minimum of 30 or 15 years within the EU, and 30 years outside the

Union. Thus, by implementing mandatory clinical trials for HMs, India is moving forward toward making these products available in both Europe and the United States.

11.5.1 Clinical Trials are Essential for Market Growth and Integration into Medical Practice

It is estimated that there are thousands of HMs available on the market in the United States alone with little or no scientific data available for these products. Thus, with the influx of new products from China, India, and other countries that are associated with good quality standards and clinical trials supporting safety and efficacy, herbal products with little or no scientific documentation of either their safety or efficacy will eventually be abandoned for products with proven safety and efficacy and be integrated into Western medicine. During 1994–2014, the efficacy of specific herbal products has been demonstrated including Andrographis [*Andrographis paniculata* (Burm. f) Nees (Acanthaceae)] for common cold, turmeric, or curcuma rhizome [*Curcuma longa* L. (Zingiberaceae)], Echinacea herb (*Echinacea pupurea*) for common cold, SJW (*H. perforatum*) for mild to moderate depression, plantago seed and husk (*Plantago* species) for reducing cholesterol and cardiovascular risk, and *B. serrata* for use as an antiinflammatory drug, to name just a few. Many of these HMs are used to treat conditions that Western medicine cannot, such as the common cold, chronic pain, and inflammation. Clinical validations of these products represent a significant step forward in the scientific integration of herbal preparations into modern therapy. However, these represent only a small number of HMs, and while a positive step forward, there is a significant amount of clinical data still needed.

11.5.2 The Need of Good Quality and Clinical Practices for HMs

As in the case of any therapeutic agent, clinical trials for safety, efficacy, and/or effectiveness are the ultimate demonstration of therapeutic usefulness of herbal products [63,64]. The general accepted use of HMs will only make take place when the tested herbal products are authentic, standardized, and quality controlled. Only when good practice guidelines of EBM are followed, and relevant controls and outcome measures are scientifically defined, will herbal products flourish. Overall, the future of HM looks very bright as global consumer interest for these products is strong and is predicted to increase in the coming years. However, with this being said, there is an absolute need for high-quality clinical trials data to integrate HM into modern medical practices. *The stumbling blocks that prevent this from occurring are the interrelated issues of quality, safety, and efficacy, and while some advances have been made in these areas, much more work needs to be done.* The present lack of uniform quality in herbal products is an impediment to both safety and efficacy, as the lack of consistency from product to product and from batch to batch may lead to negative outcomes in clinical trials [64]. Since both Europe and the United States now have mandatory GMPs in place for all products coming onto the market, there is significant recognition by the industry for the absolute need for standardization of HMs to ensure batch-to-batch consistency [64]. Fortunately, there are many reference guidelines available from the EU, FDA, and the WHO to assist manufacturers in complying with GMPs. Also available are numerous research guidelines for the chemical, biological, and clinical research on HMs available from the WHO. Where the safety and efficacy of herbal products have been validated in scientific and clinical research, HMs will become accepted and take their rightful place as fully integrated into modern medical practices.

References

[1] World Health Organization. In: WHO traditional medicine strategy 2014–2023. Geneva (Switzerland): WHO Press; 2013.
[2] Daniels S. CRN survey: 85% of US adults confident in the safety, quality and effectiveness of dietary supplements. Nutraingredients (Nutraingredients-use.com); Sept. 23, 2013. www.nutraingredients-usa.com.
[3] Anon. World Health Organization, 2nd WHO TRM global survey. 2012 [accessed 11.06.12], WHO.org.
[4] Council of Responsible Nutrition. Consumer survey on dietary supplements. 2013. crnusa.org/CRNPR13-ConsumerSurvey093013.html.
[5] National Institutes of Health. The use of complementary and alternative medicine in the United States. National Center for Complementary and Alternative Medicine; 2007. NIH.gov.
[6] Schultz H. Herbal supplements sales rose 5.5% in US in 2102, ABC says. 2013. Nutraingredients-usa.com. website [accessed 05.02.14].
[7] USP and Chinese Pharmacopoeia Collaboration. Usp.org
[8] Nahin RL. Costs of complementary and alternative medicine (CAM) and frequency of visits to CAM practitioners: United States, 2007. National health statistics reports No. 18. Hyattsville (Maryland): National Center for Health Statistics; 2009. NIH.gov.
[9] Anon. The guardian Nigeria, 'Herbal medicine market hits $160 billion globally' - Guardian Mobile. 2013.
[10] heguardianmobile.com/readNewsItem1.php?nid=13411, [accessed 10.05.13].
[11] China Post. (Taiwan) Herbal medicine market to top US$26 bil. in 2011. China Post; 2007.
[12] Farnsworth NR, Fong HHS, Mahady GB. Herba Hyperici. In: WHO model monographs on selected medicinal plants, vol. II. Geneva (Switzerland): World Health Organization, Traditional Medicine Programme; 2002. p. 149–71.
[13] Gaster B, Holroyd J. St John's wort for depression: a systematic review. Arch Intern Med 2000;160:152–6.

[14] Linde K, Berner MM, Kriston L. St John's wort for major depression. Cochrane Database Syst Rev 2008;4:CD000448.
[15] Linde K, Ramirez G, Mulrow CD, Pauls A, Weidenhammer W, Melchart D. St John's wort for depression—an overview and meta-analysis of randomized clinical trials. Br Med J 1996;313: 253–8.
[16] Whiskey E, Wernecke U, Taylor D. A systematic review and meta-analysis of *Hypericum perforatum* in depression: a comprehensive clinical review. Int Clin Psychopharmacol. 2001;16:239–52.
[17] Solomon D, Ford E, Adams J, Graves N. Potential of St John's wort for the treatment of depression: the economic perspective. Aust N Z J Psychiatry 2011;45:123–30.
[18] Jordan SA, Cunningham DG, Marles RJ. Assessment of herbal medicinal products: challenges, and opportunities to increase the knowledge base for safety assessment. Toxicol Appl Pharmacol 2010;243:198–216.
[19] Zhang AL, Changli Xue C, Fong HHS. Integration of herbal medicine into evidence-based clinical practice: current status and issues. In: Benzie IFF, Wachtel-Galor S, editors. Source herbal medicine: biomolecular and clinical aspects. 2nd ed. Boca Raton (FL): CRC Press; 2011 [chapter 22].
[20] FDA. Dietary supplement current manufacturing practice (CGMP) final rule 21 CFR Part 111. US Federal Register 2007;72: 34751–958.
[21] Daniel Fabricant interview. Nutraingredients.
[22] WHO. In: Quality control methods for herbal materials. Geneva (Switzerland): WHO Press; 2011.
[23] WHO. WHO guidelines on "good agricultural and collection practices (GACP) for medicinal plants". Geneva (Switzerland): WHO Press; 2003.
[24] Kennedy DA, Lupattelli A, Koren G, Nordeng H. Herbal medicine use in pregnancy: results of a multinational study. BMC Complement Altern Med 2013;13:355–6.
[25] Dalla Libera D, Colombo B, Pavan G, Comi G. Complementary and alternative medicine (CAM) use in an Italian cohort of pediatric headache patients: the tip of the iceberg. Neurol Sci 2014;35: 145–8.
[26] Marchisio P, Bianchini S, Galeone C, Baggi E, Rossi E, Albertario G, et al. Use of complementary and alternative medicine in children with recurrent acute otitis media in Italy. Int J Immunopathol Pharmacol 2011;24:441–9.
[27] Gottschling S, Gronwald B, Schmitt S, Schmitt C, Längler A, Leidig E, et al. Use of complementary and alternative medicine in healthy children and children with chronic medical conditions in Germany. Complement Ther Med 2013;21(Suppl. 1):S61–9.
[28] Zhang L, Yan J, Liu X, Ye Z, Yang X, Meyboom R, et al. Pharmacovigilance practice and risk control of traditional Chinese medicine drugs in China: current status and future perspective. J Ethnopharmacol 2012;140:519–25.
[29] Xu J, Liu M, Xia Z. Will the Europe Union's traditional herbal medicinal products directive (Directive 2004/24/EC) be against traditional Chinese medicine in EU market? J Evid Based Med 2013;6:104–8.
[30] Williamson EM, Lorenc A, Booker A, Robinson N. The rise of traditional Chinese medicine and its materia medica: a comparison of the frequency and safety of materials and species used in Europe and China. J Ethnopharmacol 2013;149:453–6.
[31] Shaw D. Toxicological risks of Chinese herbs. Planta Med 2010;76: 2012–8.
[32] Tsai HH, Lin HW, Pickard AS, Tsai HY, Mahady GB. Evaluation of documented drug interactions and contraindications associated with herbs and dietary supplements: a systematic literature review. Int J Clin Pract 2012;66:1056–78.
[33] GAO. United States government accountability office. Dietary supplements. FDA may have opportunities to expand its use of reported health problems to oversee products. GAO; 2013. pp. 13–244, www.gao.gov/assets/660/653113.pdf.
[34] FDA. Dietary supplement and nonprescription drug consumer safety act S.2546. Adverse Event Reporting; 2006. FDA.gov.
[35] Posadzki P, Watson L, Ernst E. Contamination and adulteration of herbal medicinal products (HMPs): an overview of systematic reviews. Eur J Clin Pharmacol 2013;69:295–307.
[36] Ernst E. Adulteration of Chinese herbal medicines with synthetic drugs: a systematic review. J Intern Med 2002;252:107–13.
[37] Ernst E. Risks of herbal medicinal products. Pharmacoepidemiol Drug Saf 2004;13:767–71.
[38] Cooper K, Noller B, Connell D, Yu J, Sadler R, Olszowy H, et al. Public health risks from heavy metals and metalloids present in traditional Chinese medicines. J Toxicol Environ Health Part A Curr Issues 2007;70:1694–9.
[39] Hui H, Lin M, Pickard S, Mahady GB. A review of potential harmful interactions between anticoagulant and antiplatelet agents and Chinese herbal medicines. PLoS One 2013;8(5):e64255.
[40] Fu PP, Chiang HM, Xia Q, Chen T, Chen BH, Yin JJ, et al. Quality assurance and safety of herbal dietary supplements. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2009;27:91–119.
[41] Van Breemen RB, Fong HHS, Farnsworth NR. The role of quality assurance and standardization in the safety of botanical dietary supplements. Chem Res Toxicol 2007;20:577–82.
[42] Van Breemen RB, Fong HHS, Farnsworth NR. Ensuring the safety of botanical dietary supplements. Am J Clin Nutr 2008;87:21–33.
[43] Vanherweghem JL, Depierreux M, Tielemans C, Abramowicz D, Dratwa M, Jadoul M, et al. Rapidly progressive interstitial renal fibrosis in young women: association with slimming regimen including Chinese herbs. Lancet 1993;341:387–91.
[44] Foster BC, Arnason JT, Briggs CJ. Natural health products and drug disposition. Annu Rev Pharmacol Toxicol 2005;45:203–26.
[45] Fugh-Berman A. Herb–drug interactions. Lancet 2000;355:134–8.
[46] Posadzki P, Watson L, Ernst E. Herb–drug interactions: an overview of systematic reviews. Br J Clin Pharamcol 2012;75:603–18.
[47] Corns C, Metcalfe K. Risks associated with herbal slimming remedies. J R Soc Promot Health 2002;122:213–9.
[48] Cosyns JP. Aristolochic acid and "Chinese herbs nephropathy": a review of the evidence to date. Drug Saf 2003;26:33–48.
[49] Lemy A, Wissing KM, Rorive S, Zlotta A, Roumeguere T, Muniz Martinez MC, et al. Late onset of bladder urothelial carcinoma after kidney transplantation for endstage aristolochic acid nephropathy: a case series with 15-year follow-up. Am J Kidney Dis 2008;51:471–7.
[50] Schilter B, Andersson C, Anton R, Constable A, Kleiner J, O'Brien J, et al. Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements. Food Chem Toxicol 2003;41:1625–49.
[51] Mosihuzzaman M, Choudhary MI. Protocols on safety, efficacy, standardization, and documentation of herbal medicine (IUPAC technical report). Pure Appl Chem 2008;80:2195–230.
[52] EMEA. In: Committee on Herbal Medicinal Products, editor. Guidelines on the assessment of genotoxic constituents in herbal substances/preparations (Draft). London: European Medicines Evaluation Agency (EMEA); 2007.
[53] EMEA. In: Committee on Herbal Medicinal Products, editor. Guidelines on selection of test materials for genotoxicity testing for traditional herbal medicinal products/herbal medicinal products (Draft). London: EMEA; 2009.
[54] Institute of Medicine. Dietary supplements. A framework for evaluating safety. (Washington, DC): National Academies Press; 2004.
[55] EFSA. European food safety authority, guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements. EFSA J 2009;7:1249.

[56] LowDog T, Marles R, Mahady G, Gardiner P, Ko R, Barnes J, et al. Assessing safety of herbal products for menopausal complaints: an international perspective. Maturitas 2010;66:355–62.
[57] Bansal D, Hota D, Chakrabarti A. Research methodological issues in evaluating herbal interventions. J Clinical Trials 2010;2:15–21.
[58] Jonas WB, Linde K. Conducting and evaluating clinical research on complementary and alternative medicine. In: Gallin JI, editor. Principles and practice of clinical research. San Diego (CA): Academic Press; 2002. p. 401–26.
[59] Leung PC. Complementary medicine. In: Machin D, Day S, Green S, editors. Textbook of clinical trials. 1st ed. Chichester (UK): John Wiley & Sons; 2004. p. 63–84.
[60] Mills S. Herbal medicine. In: Lewith GT, Jonas WB, Walach H, editors. Clinical research in complementary therapies: Principles, problems and solutions. New York: Elsevier Science; 2003. p. 211–27.
[61] World Health Organization. Operational guidance: information needed to support clinical trials of herbal products. 2005 (Document reference who/TDR/GEN/Guidance/05.1).
[62] Sinha K. Mandatory clinical trials for herbal drugs soon. The Times of India; Aug. 9, 2012.
[63] Werner SM. Patient safety and the widespread use of herbs and supplements. Front Pharmacol 2014;5:142–4.
[64] Pelkonen O, Xu Q, Fan TP. Why is research on herbal medicinal products important and how can we improve its quality? J Tradition Complement Med 2014;4:1–7.

LIST OF ABBREVIATIONS

AER Adverse events reports
ANN Aristolochic acid nephropathy
CAM Complementary and alternative medicines
DS Dietary supplement
DSHEA Dietary Supplements Health and Education Act
EMEA European Medicines Agency
FDA Food and Drug Administration (USA)
cGMP current Good manufacturing guidelines
GMP Good manufacturing guidelines
HM Herbal medicines
IOM Institute of Medicine
ITT Intention to treat
NCCAM National Center for Complementary and Alternative Medicine
NIH National Institutes of Medicine (US)
PAHO Pan American Health Organization
RAOM Recurrent acute otitis media
RCT Randomized controlled clinical trials
SJW St John's wort
TCM Traditional Chinese medicines
THMPD Traditional herbal medicinal products directive
USP United States Pharmacopoeia
WHO World Health Organization
WHO-TRM WHO's TM Program

CHAPTER

12

Traditional Medicine-Inspired Evidence-Based Approaches to Drug Discovery

Bhushan Patwardhan

Interdisciplinary School of Health Sciences, Savitribai Phule Pune University, Maharashtra, India

OUTLINE

12.1 INTRODUCTION

The drug discovery and development has undergone several transitions during last twenty years. The advances in biomedical sciences, biotechnology, vaccinology and in computer-aided drug design, genomics, and molecular technologies have brought mind boggling changes leading to a more powerful, broad spectrum of chemical and biological drugs for many diseases. Still today the rate at which new drugs are discovered has decreased, and many existing drugs continue to be withdrawn by regulators for safety reasons. Chemical drugs are rapidly getting replaced by biologicals. With the advent of pharmacogenomics and systems biology concepts, pharmaceutical medicine is becoming more personalized. Industry leaders and pharmaceutical and biomedical scientists are desperately looking for novel approaches, new ideas, and innovations to disrupt this discovery bottleneck. Undoubtedly, the pharmaceutical sector is facing a severe innovation deficit.

The pharmaceutical sector seems to be going around in circles doing more of the same but powerfully hoping to disrupt the present impasse [1]. Several strategies have been discussed and practiced including open-innovations, industry–academia and industry–industry collaborations, and many recent initiatives to expedite lead generation are only indicative of desperation and aspirations [2]. The drug discovery in 2014 is not limited to serendipity or availability of technology. It is rightly indicated that the strategies of the past may not guarantee success in the future [3]. The pharmaceutical industry has historically seen an incredible growth primarily due to the discovery of a blockbuster; however, recent trends indicate that this

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00012-X

model may no longer be sustainable. The average cost and time of discovering, developing, and launching a new drug is consistently increasing without an expected corresponding increase in the number of newer, safer, and better drugs. The situation is progressively deteriorating, and analysts predict that the worst as predicted is yet to come.

The Galen era of the use of botanical crude extracts for therapeutic purposes is long over. We now need to evolve new technologies and strategies in the field of drug discovery. The enormous indigenous knowledge from Indian systems such as Ayurveda may serve as rich sources of leads not only for drug and vaccine discovery but also offer entirely new sets of multitarget formulations and treatment regimes. The era of decoctions, extractions, and attenuated vaccines can be transformed through "innovations" in the development of drugs and vaccines for affordable, accessible, safe, and effective medicines. Therefore, herbal drugs, ethnopharmacology, and evidence-based traditional medicine-inspired drug discovery seem to be a promising way ahead.

Traditional knowledge-inspired natural product drug discovery is reemerging as an attractive option. Traditional medicine, Ethnopharmacolgy, pharmacognosy, herbal drugs, and natural product drug discovery are considered as attractive options to revitalize and fast forward the discovery process. Disciplines such as ethnopharmacology and pharmacognosy are no more restricted to crude drugs, microscopy, macroscopy, organoleptic studies, and animal pharmacology. Advances in pharmaceutical chemistry, network pharmacology, genetics, molecular biology, and biotechnology offer many opportunities in drug discovery [4]. The knowledge available from traditional medicine and natural products libraries with significant chemical diversity may bring a new hope in depressed discovery scenario [5]. New drug discovery requires the power of technology and benefits of ancient wisdom to bring better safety and efficacy in natural products [6].

Any drug whether chemical, botanical, or biological will have inherent limitations if it is focused only on a single target. The high specificity to specific target may actually turn out to be a limitation. It is important to address multiple targets from a syndrome-related metabolic cascade so that a holistic management can be effectively achieved. Therefore, it is necessary to move from a single target new chemical entity such as a "drug" to multiple target, synergistic "formulation" discovery approach. In this chapter, we give few examples of Ayurveda-inspired approaches to natural product drug discovery.

12.2 AYURVEDA INSPIRATION

In the Indian subcontinent, documentation and use of medicinal plants started during the Vedic period, when over 100 medicinal plants are found. *Charaka Samhita* and *Sushruta Samhita* remain the main classics of Ayurveda and contain detailed descriptions of >700 medicinal herbs and >8000 formulations. The Indian traditional health system known as Ayurveda deals with a healthy life style, health promotion and sustenance, disease prevention, diagnosis, and treatment. The prolonged use of Ayurveda by people has also led to the development and use of time-tested home remedies for common ailments, which continue to be local health traditions. Traditional systems of medicines need more evidence-based studies on both crude drugs and purified phytomolecules [7]. Researchers have also proposed a way forward to address the changing scenario in the development and promotion of Ayurveda [8].

With strong philosophical foundations from *Sankhya* and *Vaishesika* knowledge systems, Ayurveda diagnosis and treatment involve combinations of variables such as five *mahabhootas* (primordial elements), three *doshas* (humors known as *vata*, *pitta*, and *kapha*), seven *dhatu*s (tissues), six *rasa* (tastes), 20 pairs of *guna*s (attributes), and many *dravaya*s (substances). Ayurveda has sophisticated knowledge and its own concepts known as *dravya guna vigyan* (pharmacology), *rasashastra* (study of metal preparations), and *bhaishaja kalpana* (pharmaceutics). The complex interrelationships between Ayurvedic concepts and modern parameters, such as cellular and molecular biology, physiology, pathology, diagnostics, and therapeutics, are schematically presented in Figure 12.1. The ancient treatises of Ayurveda furnish the original principles on which drugs were presumed, derived, and understood. Charaka classifies drugs according to their actions on body systems, their roles in health and diseases, according to parts used, formulation types, dietary and therapeutic roles, methods of processing and indications as internal, external therapies as well as uses for palliative therapies and *Panchakarma*s (cleansing processes including medical emesis, purgation, oleation, steam, massage, and bloodletting).

The Ayurvedic drug delivery system is different and special because it upholds the concept of a "target organ" or "target therapeutic function," rather than active principles and single or a few biochemical reactions by a given herb. Ayurveda believes that "drugs do not act against diseases only because of their physical nature or properties. They function only in a specific time and after reaching their target tissue, when they make specific therapeutic action." Such traditional wisdom may

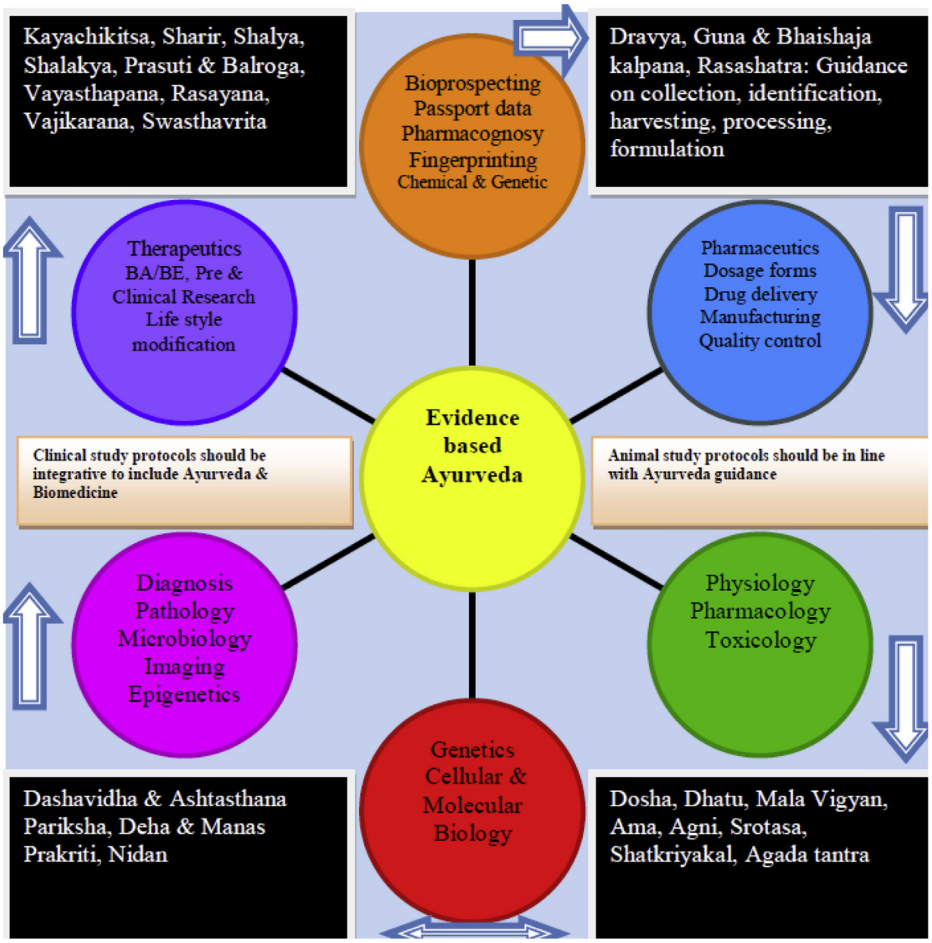


FIGURE 12.1 Complex relationships between Ayurvedic and modern concepts related to physiology, pharmacology, and therapeutics. The colored circles show modern terminology and the rectangles give related Ayurvedic concepts. The arrows show the circular flow consisting of various steps from materials to medicine. *Ref. [90]*

play an important role in guiding evidence-based scientific research on herbal medicines.

Philosophical principles are wisely applied for health promotion, disease prevention, and treatment by practicing physicians known as *Vaidyas*. However, it may be admitted that today, clinical practice of classical Ayurveda is rare. Ayurvedic practitioners are reported to exclusively adopt allopathic practices for better acceptance in an urban setting [9]. The practice of Ayurveda draws significantly from the three classic books known as *Samhitas* including *Charaka*, *Sushruta*, and *Vagbhata*; however, significant variations in clinical practice exist in different parts of India. Admittedly, huge knowledge resource and wisdom are available from Ayurveda classic books; however, systematic data on the actual use and evidence of reproducible outcomes are not available in the public domain. Standard treatment protocols for practitioners are not available.

Despite a considerably large infrastructure at the disposal of the Department of Ayurveda, Yoga, Unani, Siddha, Homoeopathy (AYUSH) consisting of 495 UG and 106 PG Colleges; 3277 Hospitals; 62,649 Beds; 8644 Manufacturing units; and 785,185 registered Practitioners; and an over Rs 1000 crore yearly budget, hardly any systematic clinical data or analysis are available. Systematic documentation and reliable data on pharmacoepidemiology and pharmacovigilance for clinical practice, safety, and adverse drug reactions are not available as open access, although a modest beginning has been made [10]. The status of professional [11] and continuing education [12] and attitudes of practitioners toward safety [13] are also worrying. As per the regulations existing in 2014 in India, no scientific or clinical data are required for manufacture and sale of classical Ayurvedic medicines. Technically sound Pharmacopoeia, Good Manufacturing Practices, quality control [14], and pharmaceutical technologies for Ayurvedic medicine are still evolving [15,16]. Issues related to appropriate research methodologies and treatment protocols for Ayurveda have not been properly addressed [17]. Many critiques are demanding better coordination between stakeholders, continuous dialog with the scientific community [18] and total overhaul of the curriculum and pedagogy along with

the need for crosstalks between different streams [19]. Thus, the evidence base to support good clinical practice, guidelines, and documentation in Ayurvedic medicine remains scant and grossly inadequate. It should be made clear that scientific research and evidence of clinical practice are not necessary for the purpose to offer new ideas and leads to drug discovery, but it is required more to justify the present form of clinical practice of Ayurveda. Evidence-based Ayurveda is actually in the interest of the sector, profession, and science [20].

Therefore, scientific research on Ayurvedic medicine is needed to bring its benefits to mainstream drug discovery. The conventional drug discovery pipelines follows the path from targets, leads to preclinical and clinical phases. However, Ayurveda-inspired discovery follows a reverse path since these medicines are already prescribed by clinicians and consumed by people for hundreds of years. These observational data and clinical experiences may be valuable to offer new resources and understanding of target and lead relationships. A large number of molecules have come out of the Ayurvedic clinical base, including Rauwolfia alkaloids for hypertension, Psoralens in Vitiligo, Holarrhena alkaloids in Amebiasis, Guggulsterones as hypolipidemic agents, Piperidines as bioavailability enhancers, Baccosides in mental retention, Picrosides in hepatic protection, Curcumines in inflammation, Withanolides, and many other steroidal lactones and glycosides as immunomodulators. Using this knowledge amounts to traditional knowledge-inspired or Ayurveda-inspired drug discovery. This discovery path is also known as reverse pharmacology approach.

12.3 REVERSE PHARMACOLOGY

A new strategy known as "reverse pharmacology" has been proposed by senior clinical pharmacologist Ashok Vaidya. Ayurveda knowledge allows drug researchers to start from time tested and safe botanical material. The conventional drug discovery path from "Laboratory to Clinics" flows from "Clinics to Laboratories"—as a true "Reverse Pharmacology" approach (Figure 12.2). In this process, safety remains the most important starting point, and efficacy becomes a matter of validation [21,22]. The best example of reverse pharmacology-based bioprospecting using traditional knowledge is Reserpine, the antihypertensive alkaloid from *Rauwolfia serpentine*. Reserpine as a modern medicine pharmaceutical product became available as a result of work carried out by CIBA in India in close collaboration with Ayurveda experts. Traditional medicine-inspired drug discovery and development are therefore considered to be an efficient, faster, and affordable strategy. In a process of scientific evidence in support of herbal medicine, the discipline of pharmacoepidemiology plays an important role through systematic documentation and analysis [23].

Our research group has been experimenting on these approaches for several years. In the following sections, few examples and case studies are presented to indicate the importance of these approaches.

12.4 *SEMECARPUS ANACARDIUM* CASE

Semecarpus anacardium popularly known as Bhallataka, Bhilva, or Marking Nut is routinely used in Indian

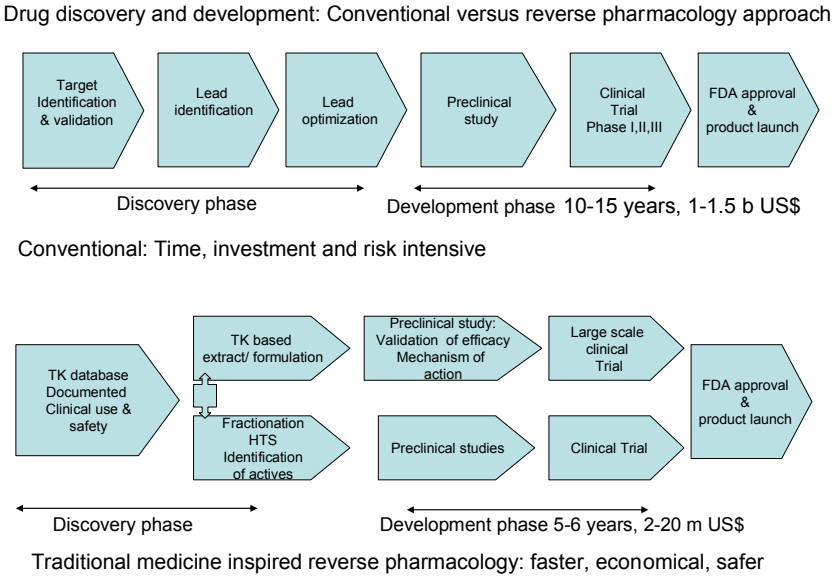


FIGURE 12.2 Reverse pharmacology. *Ref. [22]*

families as a home remedy. It has activities including antimicrobial, analgesic, anabolic, etc. It can cause severe allergy or anaphylactic reactions if one does not take proper precautions. A series of studies on the pharmacological activity of *S. anacardium* presents a good case to understand issues related to evidence-based herbal medicine [24,25]. During the early 1970s, B. G. Vad, a renowned physician from Mumbai, developed a product for the treatment of cancer from Bhallataka named Anacarcin Forte. This was a gelatinous preparation made from chloroform extracts of nut and seed of *S. anacardium* blended in peanut oil. Vad and colleagues had reported the complete regression of acute myeloblastic leukemia by the administration of Anacarcin forte without the use of any single or combined antileukemic chemotherapeutic drugs [26]. Senior physician scientists from the Tata Memorial Hospital had observed the beneficial effects of Anacarcin among cancer patients. This led to a pioneering interdisciplinary, multiinstitutional collaborative network projects.

Traditionally, the nut shell oil of Bhallataka is supposed to have analgesic, antiinflammatory, antimicrobial, and anticancer activities. Several experiments involving animal pharmacology to study traditional claims and mechanisms of action for the antiinflammatory and antiarthritis properties were done to obtain the evidence in support of traditional claims [27]. Bhallataka oil showed significant antitumor activities in animal models of sarcoma and adenomas. There was a statistically significant increase in life span in treatment groups. Attempts were made to isolate the active principle by using preparative high-performance liquid chromatography coupled with anticancer activity testing of pure fractions; however, none of these were active. The researchers could not identify any anticancer compound in a pure form even after four years of intensive effort. The crude extracts and nut shell oil had significant anticancer activity, which was lost during the process of purification. These studies indicate the possibility of synergistic activities and the importance of processing and vehicles used in delivery of drugs.

Ayurveda offers detailed instructions on how to take a particular drug to enhance potency and reduce toxicity. This is known as *Anupana*. Ayurveda suggests vehicles such as honey, milk, and warm water, during administration. For example, Bhallataka is considered to be potentially toxic, and needs to be processed and consumed along with a suitable oil. In acute and subacute toxicity studies, fractions emulsified using Tween 80 saline produced considerable toxicity leading to 100% mortality at a dose of 25 mg/kg. Interestingly, the same dose in the same experimental conditions yielded no mortality in a group that received drug fractions with peanut oil; on the contrary, signs of anabolic activity were observed indicating a typical Rasayana effect. Researchers also showed that traditional use involving peanut oil as a delivery vehicle was safe while significant toxicity was observed at the same dose when the test material was converted into an emulsion [28]. These results only strengthen an interesting relationship of peanut oil and Bhallataka for better safety, which was intelligently used by Vad while preparing Anacarcin forte. These results make us think more about the value of traditional knowledge and demonstrate how experiential wisdom and experimental rigor can be complementary to each other.

Traditionally, Bhallataka is used as a first aid for deep wounds caused by thorns or nails mainly to prevent pain and infection. This observation prompted us to study its activity on selected anaerobes responsible for infections in cases involving a threat of tetanus or gangrene. In systematic activity directed fractionation, we were able to isolate three compounds known as monoene, diene, and triene bhilawanols, which were shown to be responsible for specific anaerobic antibacterial activity against *Clostridium* Spp [29–31]. These observations offer unique learning and changed attitude of involved researchers toward traditional knowledge-inspired research. Systematic work on animal pharmacology and possible mechanistic studies were also performed involving toxicology, immunology, and was exposed to the basics of pharmaceutical medicine and drug development.

12.5 FORMULATION DISCOVERY

The Semecarpus case suggests that drug discovery need not be always confined to the discovery of a single molecule. Many analysts believe that the current assumption of one drug can fit all will be unsustainable in the future. Moreover, we are dealing with polygenic syndromes and not just with isolated diseases. Multitarget approaches are in the mainstream with renewed interest in multiingredient synergistic formulations [32]. Due to the diversity of structures, herbal extracts can deal with multiple targets simultaneously and may give a synergistic effect. Therefore, the development of standardized, synergetic, safe, and effective herbal formulations with sufficient robust scientific evidence support can also offer a faster and much economical alternative.

For instance, Ayurvedic texts include a few thousands of single or polyherbal formulations. These are rationally designed and are in therapeutic use for several years. Sufficient pharmacoepidemiological evidence could be generated to support their safety and efficacy. Systematic data mining of the huge existing formulations database can certainly expedite drug

discovery processes where real effective and safe candidates could be identified. One of the pioneering series of clinical studies on Ayurvedic antiarthritis formulation known as Rheumayog concluded confirmed the disease-modifying activity, which was comparable with that of modern drug Auranofin [33]. Many more examples based on traditional medicines even for nutraceutical [34] and veterinary [35] applications seem promising. The US Food and Drug Administration (FDA) and few other regulators do have very practical guidelines for botanical "drug" development, and herbals are no more restricted to nutraceuticals.

Presumably, any drug whether chemical, botanical, or biological will have inherent limitations if it is focused only on a single target. It is important to address multiple targets from a syndrome-related metabolic cascade so that a holistic management can be effectively achieved. Therefore, it is necessary to move from a single target new chemical entity such as a s"drug" to a multiple target, synergistic "formulation" discovery approach. It is possible that polybotanical complex formulations from traditional medicines, such as Ayurveda and Traditional Chinese Medicines, have a similar logic. Such traditional knowledge-inspired discovery attempts have shown a promising potential in several chronic diseases, such as cancer, diabetes and arthritis, through the modulation of multiple targets. Several single or multi-botanical formulas are widely used globally; however, their rational and scientific evidence for pharmacodynamic actions remain insufficient [36].

12.6 PHYTOPHARMACEUTICALS AS DRUGS

Recently, the Government of India has published a draft amendment to Drugs and Cosmetics Act, and Rules by defining phytopharmaceuticals as botanical-based drugs. This amendment provides requirements of scientific data on quality, safety, and efficacy to evaluate and marketing of plant-based substances as drugs. This will create a new category on similar lines to synthetic, chemical drugs presently covered under Schedule Y of the D & C Act. This initiative is expected to give a boost to scientific research-based drug development from traditional medicine. Earlier, the approval of Guggulu tablets developed by Indian scientists took almost a decade. This was done as a special case by the Drug Control General of India. The new regulations for phytopharmaceuticals as a new category are required because many provisions for synthetic drugs are not relevant to botanical-based products or phytopharmaceuticals. DBA Narayana, former scientist from Unilever, played a significant role in convincing and pursuing the government authorities for creation of new category of phytopharmaceuticals [37].

Experts feel that new amendments will not adversely affect the AYUSH sector. In fact, they may be helpful in facilitating traditional knowledge-inspired drug discovery and extensive evaluation through biomedical sciences. This may formally encourage the use of scientifically studied herbal products by modern medical practitioners not only in India but also in the rest of the world.

Traditional herbal formulations may also follow this route to create a scientific evidence base with robust chemistry, manufacturing, and controls. Department of AYUSH has established a research center at the University of Mississippi Oxford to facilitate scientific investigations on Indian herbal drugs. In such a process, there may be exciting spin-offs where promising molecular entities could be discovered.

Thus, if safe and effective herbal formulations are developed in accordance with stringent regulatory requirements on par with any modern drug, we hope that the conventional skepticism against herbals may slowly wane. However, 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 seems much more suited to study the therapeutic efficacy and pharmacodynamics of traditional medicine-based drug development [38]. It is also argued that instead of 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 [39].

12.7 CLINICAL RESEARCH

Designing and implementing proper research on traditional clinical practice is a major challenge. Several initial hurdles in documentation, data retrieval, and standardization and analysis are presently faced. However, it is important to initiate the process and start moving in the right direction. The few exemplary efforts in this direction include pioneering studies by Saravu Narahari in the field of integrative dermatology. Other notable pioneering efforts include a study on whole system trials by Ram Manohar from Arya Vaidya Pharmacy, Coimbatore in collaboration with Daniel Furst of University of California at Los Angeles and systematic drug development effort through robust RCTs in rheumatology by Arvind Chopra and colleagues from Pune. Recent efforts to develop Consolidated Standards of Reporting Trials-like reporting standards for Ayurveda undertaken by renowned biostatistician Ashwini Mathur

with the help of Prathap Tharyan, Director of the South Asian Cochrane Network; efforts of Pratik Debnath to establish Gananath Sen Institute of Ayurvidya and Research in Kolkata. The efforts of Darshan Shankar Vice Chancellor of new University known as "Institute for Transdisciplinary Research in Health Science and Technology" also need to be recognized for promoting scientific research on traditional medicine. Few relevant case studies where herbal formulations were successfully developed using these strategies in a much lesser time and resources are discussed here as examples.

12.8 THE ARTREX STORY

The Ayurvedic formulary gives thousands of such multiingredient preparations and an excellent rationale for such formulations in the Ayurvedic classics. One such attempt to design a multiingredient formulation (Artrex) for the treatment of rheumatoid arthritis and osteoarthritis (OA) has been successfully completed and the formulation tested in a well-designed randomized, double-blind, placebo-controlled clinical trial. This formulation provides therapeutic benefits in acute conditions of pain and inflammation, and it also addresses immunopathological interventions required for the long-term management of slow progressive degenerative diseases such as rheumatoid arthritis [40,41]. It has ingredients with analgesic and antiinflammatory activities similar to those of nonsteroidal antiinflammatory drugs (NSAIDs) and also includes ingredients with immunomodulatory, anabolic, disease-modifying, and free radical scavenging activities. Thus, the formulation as a whole acts as a combination of NSAIDs and disease-modifying antirheumatic drugs. The product was codeveloped with BioVed Pharmaceuticals, and has been patented in India and in the United States. It is available in the market in a few countries [42].

12.9 FORMULATIONS FOR ARTHRITIS

The Council for Scientific and Industrial Research (CSIR) supported an Ayurveda-based herbal drug development project under the New Millennium Indian Technology Leadership Initiative (NMITLI) program of the Government of India. This project was a teamwork comprising six research institutes, five clinical centers, and six industry collaborators. The clinical team developed integrative protocols and appropriate research methodologies for evidence-based Ayurveda and botanical drug development [43]. Two formulations, which performed better than placebo and glucosamine in exploratory trials, were then taken up for further mechanistic studies [44]. All the Formulations prepared for clinical trials were manufactured and labeled generally in accordance with US FDA Guidance to Industry for botanical drugs. Most of the required tests were performed during the entire process starting from passport data of raw material, botanical identification, chemical profile and DNA analysis, and stability of finished products [45–49]. In vitro studies using suitable Cell and Tissue Culture models on these formulations revealed significant chondroprotection, proteoglycan release, nitric oxide release, aggrecan release, and hyaluronidase inhibition as markers in an explant model of OA cartilage damage [50–53]. A patent describing innovative process, formulation, and use has been filed by the CSIR [54].

The OA herbal drug development NMITLI project involved a network of 16 national research institutions, modern medicine hospitals, and pharmaceutical industries from India (Figure 12.3). Following prior art [41] and several rounds of national level consultations with Ayurvedic scholars few botanicals were, short-listed. These selected botanicals were subjected to animal pharmacology and open label observational studies by clinicians. The project used a traditional knowledge-guided platform where the base formulation was optimized with additional ingredients to obtain desired therapeutic activities. All the formulations were manufactured under Good Manufacturing Practices in accordance with US FDA guidance to industries for botanical drugs. Preclinical evaluation was designed on the basis of systems approach, wherein the assay battery involved targets relevant to inflammation, pain, immunomodulation, and chondroprotection (proteoglycan release, nitric oxide release, aggrecan release, and hyaluronidase inhibition as markers) in a human explant model of OA cartilage damage. This led to the design of synergistic polyherbal formulations that were found to be safe and devoid of any genotoxicity or mutagenic activity. Short-listed formulations entered a series of randomized clinical trials compared with the known drugs glucosamine and celecoxib. Finally, one best formulation was selected, which led to one Indian and one Patent Cooperation Treaty application with a dossier of necessary data required for possible regulatory submissions [55]. Thus, this project was completed using reverse pharmacology approach in five years with an expenditure of about two million US dollars. This treatment may cost just 25 US dollars a month for patients, with much better therapeutic benefits including chondroprotection that no other modern drug offers. Currently, the CSIR is in the process of identifying a suitable industrial partner for further development, optimization, manufacturing, registrations, and marketing.

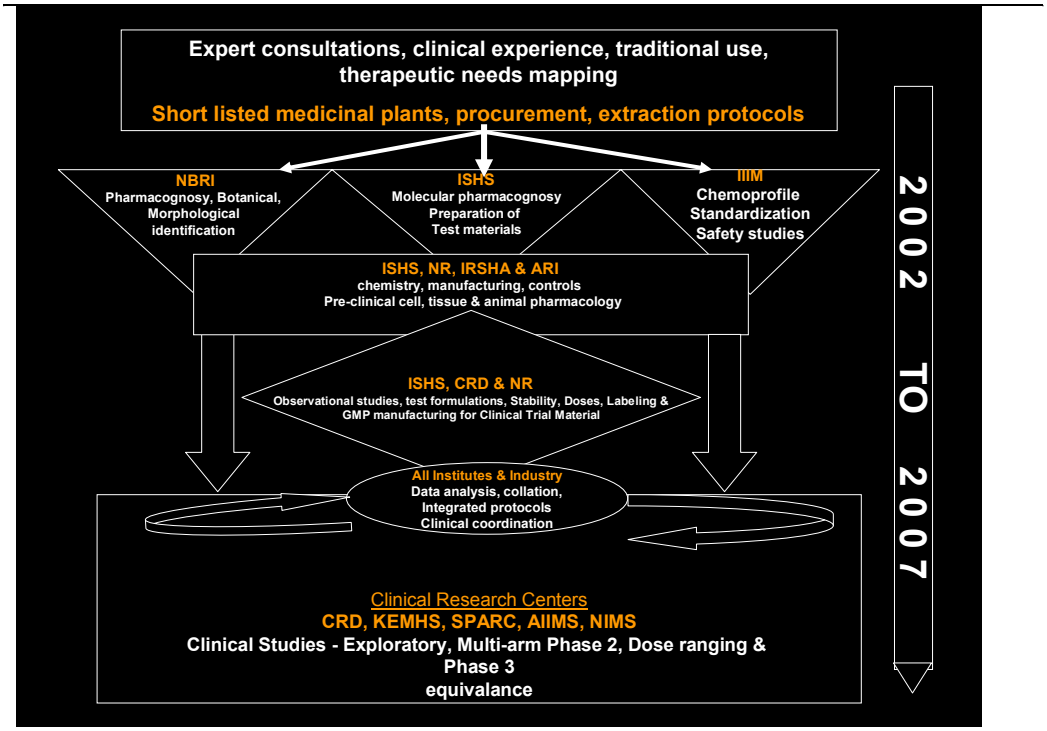


FIGURE 12.3 New Millennium Indian Technology Leadership Initiative Osteoarthritis project: National institutional network, steps, and responsibilities. *Ref. [6]*

12.10 RASAYANA AND IMMUNOADJUVANTS

Rasayanas are a group of medicinal plants described in Ayurveda for rejuvenation, antiaging, and immunomodulatory properties. Several studies on Rasayana have made important contributions to ethnopharmacology especially to inflammation and immunopharmacology, and some interesting research projects and publications on obesity [56], anxiety [57], arthritis [58,59], inflammation [60,61], immunomodulators [62], and natural product drug discovery [63]. These studies clearly show the importance of traditional knowledge systems and ethnopharmacology in bioprospecting safer and effective medicines and treatments [64].

Reviews of the current literature available on *Rasayanas* indicate that immunomodulation is the most studied property/activity [65]. Researchers have studied a few selected *Rasayana* plants, including *Withania somnifera* (Ashwagandha), *Asparagus racemosus* (Shatavari), *Tinospora cordifoloia* (Guduchi), *Phyllanthus embellica* (Amalaki), and *S. anacardium* (Bhallataka), and reported immunomodulatory activity for various standardized extracts and formulations prepared from them. Researchers also evaluated their potential as antistress [66], anxiolytic [57], adaptogenic [67], immune [68], and myeloprotectants [69]. In one particular study, it has been reported that Ashwagandha is a better and safer drug than Ginseng [70]. Work on antiaging activities of Ayurvedic medicines in topical application forms has also been encouraging [71]. Such evidence base investigations are important to properly position Ayurvedic herbal medicine in the competitive international market.

Researchers have studied the pharmacodynamics of Ashwagandha, Shatavari, and Guduchi in experimentally induced tumors and infection mouse models for immunomodulation and Th1−Th2 balance [72]. Studies on in vivo cytokine modulation using flow cytometry showed that a100-mg/kg dose resulted in a significant Th1 response (interleukin-2, interferon-g) in comparison to that of Levamisole and Cyclosporin. In immunosuppressed animals, Ashwagandha exhibited a significant dose-dependent potentiation of cellular and humoral immune response comparable to that of Levamisole and a faster recovery of $CD4^+$ T cells percentages compared to that in control and cyclosporine [73]. The study indicated immunostasis activity and suggests its use where Th1−Th2 modulation is required. This activity has also led to significant benefits as immunoadjuvants when studied on mortality and morbidity associated with diphtheria, pertussis, tetanus (DPT) and potentiating protective effects of vaccines (Figure 12.4).

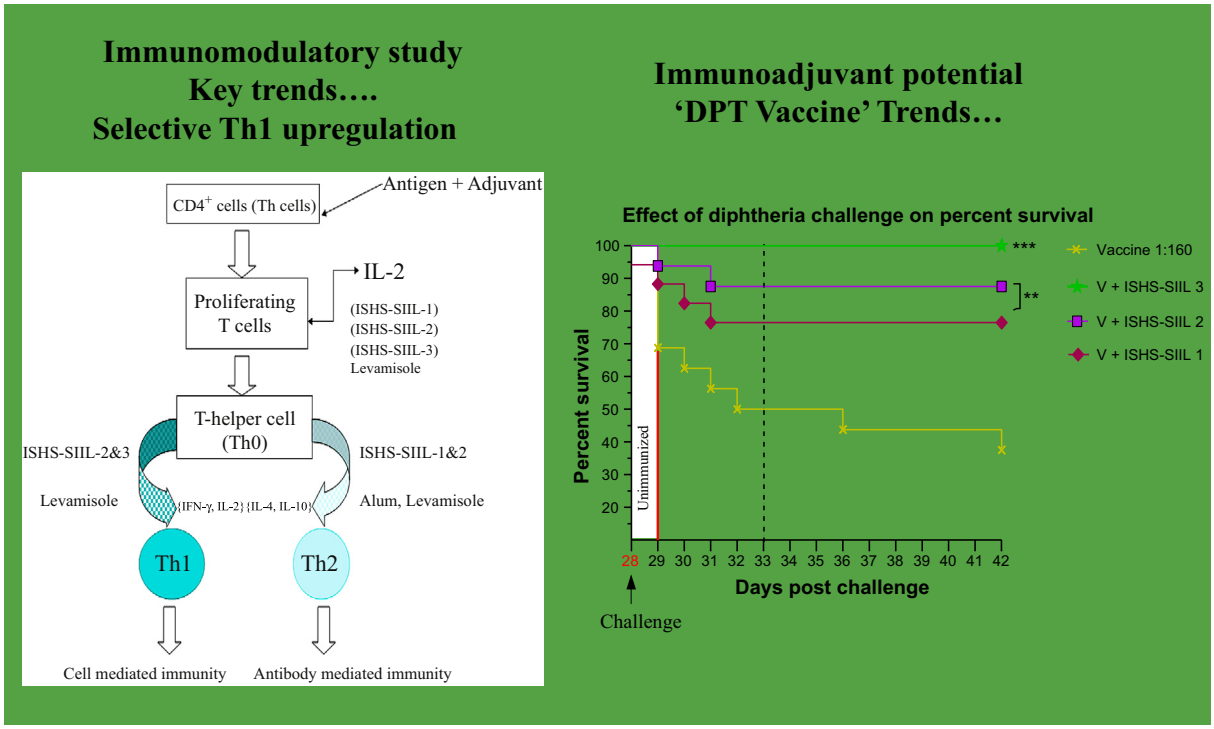


FIGURE 12.4 Selective stimulation of Th1 response produced by the active fraction from Ashwagandha. This is useful to potentiate the activity of diphtheria, pertussis, tetanus (DPT) vaccines, where a higher degree of immunity is provided through the immune–adjuvant activity of Ashwagandha.

Newer vaccines such as subunits and DNA vaccines are weakly immunogenic and require adjuvants. We hypothesized that Ayurveda-based *Rasayanas* may offer better and safer immunodrugs that can be used as adjuvants in vaccines and cancer treatments [74]. Researchers from our group used a modified Kendrick test that involved a challenge of live Pertussis cells intracerebrally, where a significant increase in the antibody titer, reduced mortality, and improvement in overall health was observed [75]. This observation has immense importance in the vaccine industry to obtain more efficient and sustained immunostimulation resulting in an increased yield of immune sera and immunobiologicals [76]. These studies indicate applications of *Rasayanas* to be potential immunoadjuvants that also offer direct therapeutic benefits and result in lower morbidity and mortality [77]. A project to develop a vaccine adjuvant was successfully completed in collaboration with the Pune University and the Serum Institute of India and has led to four Indian and one US Patents in the area of vaccine adjuvant [78–80]. Using the Ayurveda-inspired reverse pharmacology approach, bioactive fractions have been developed as potential vaccine adjuvants (Figure 12.5).

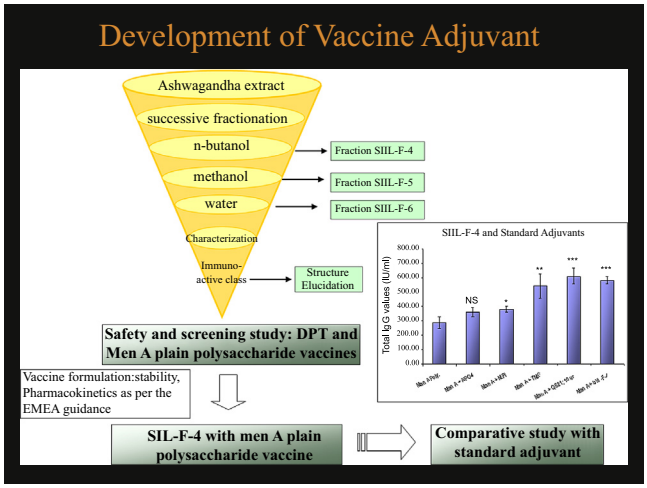


FIGURE 12.5 Identification of the best active component by starting from the crude extract of medicinal plant "Ashwagandha" through systematic activity-directed extraction and fractionation.. This process may be useful in phytopharmaceutical discovery.

Most cancer chemotherapeutic agents are immunosuppressants and cytotoxic. Researchers have used cyclophosphamide-induced immunosuppression to screen plant-derived drugs for anticancer and cytoprotective potential and to demonstrate myeloprotective and immunoprotective activities in ascitic sarcoma-bearing animals [81]. Researchers carried out

activity-related extractions to identify best performing candidate drugs. This work resulted in a US patent in the area of cancer adjuvants. This product will have importance in cancer therapeutics especially to counter untoward effects of chemotherapy without compromising their anticancer activity [81]. These examples to attempt scientific evidence base for herbal medicines remain important and exemplary.

12.11 DISCOVERY APPROACHES

The traditional medicine-inspired drug discovery and development has two main approaches, and it is important to differentiate them for bringing better clarity. First, the traditional medicines as they are used either in single, crude, or processed form of botanicals or minerals can be standardized by using modern methods. Here, their original nature is not changed. This approach is relatively easier because these medicines are used for many years in patients. Therefore, safety and efficacy have been experienced. Here, the role of modern science is to ensure quality and reproducibility.

The second approach is to use the traditional knowledge for bioprospecting and use natural product chemistry extensively. This may lead to isolation of single chemical entities, which can be developed as new drugs for use of modern medicine. This approach is very relevant to the pharmaceutical industry, which is facing a severe innovation deficit.

There may be a third approach that draws best from both the approaches. This is termed as "formulation discovery." It is felt that drug discovery that targets one target with one molecule is not sustainable. Instead a multitarget multiingredient "formulations may be a smarter approach." Here, the formulation may follow the same path as that of phytopharmaceuticals. Such evidence-based formulation discovery seems to be a rational approach for the future.

12.12 HERB–DRUG INTERACTIONS

Medical pluralism is a common phenomenon all over the world. The concurrent use of prescription drugs and herbal drugs especially in chronic diseases has been recognized. Several diseases such as diabetes, hypertension, and cancer may incur situations where modern drugs and botanical drugs are likely to be consumed concurrently. In such cases, drug–herb interactions become very important, yet very few studies are available in this field (Figure 12.6). Several such studies on many commonly used synthetic drugs and botanical extracts are needed, which may have the possibility of concurrent consumption [82,83].

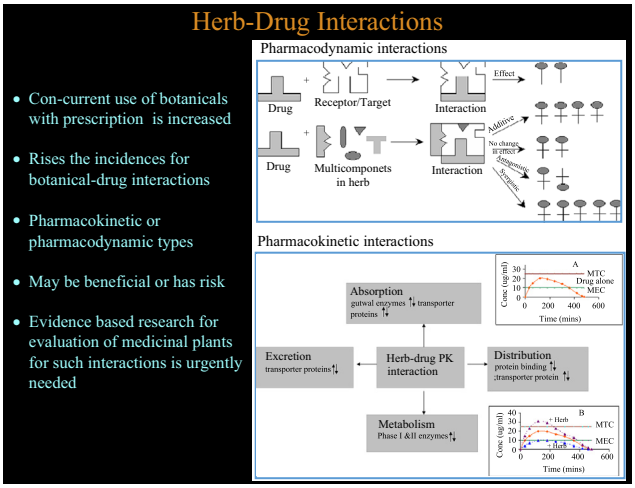


FIGURE 12.6 Importance of herb–drug interactions when modern prescription drugs and botanical drugs are consumed together. These interactions can be adverse, neutral, or beneficial. Therefore, detailed pharmacokinetic studies are advocated.

Many botanical drugs are frequently used as adjuvants or as dietary supplements during cancer chemotherapy. Sufficient data are not available on possible herb–drug interaction through CYP3A4 inhibition potential. Ayurvedic botanicals, such as Ashwagandha, Shatavari, and Guduchi, prepared as per traditional procedures did not show significant CYP3A4 inhibition after oral administration, even at a concentration equivalent to the highest clinical dose. This suggests that they may be safely used as adjuvants concurrently with cancer chemotherapeutic agents linked to CYP3A4 substrates. Interestingly, nonpolar fractions containing berberine, palmatine, and jatrorrhizine from Terminalia extract showed significant inhibition, which needs to be monitored for safety when concurrently used with such substrates. More studies especially in vivo are necessary to confirm these observations [84].

12.13 QUALITY CONTROL AND STANDARDIZATION

Both the approaches based on traditional medicine-inspired drug discovery use natural resources. It is crucial to ensure the quality and standardization of the entire cycle from raw materials to finished products. Botanical drugs are subjected to processes involving harvesting, drying, and storage. Due to environmental and seasonal variations quantities and proportions of active ingredients can change.

Therefore, a detailed knowledge of their quantity, quality, and consistency is very important. Various aspects of quality assurance, stability, and regulatory issues are relevant to botanical drugs from preformulation stages and are a crucial part of botanical drug development. Researchers have studied the physicochemical stability and biological activity of few extracts under real-time and accelerated storage conditions [85,86]. Similar studies on the chemical quality control of Ayurvedic botanicals may help in understanding the stability of extracts and formulations [87,88].

One of the major challenges faced by the herbal drug industry is the quality assurance of raw materials. Inconsistency in the quality of herbal raw material is one of the common problems that the herbal industry is facing. Few studies have reported the presence of potentially harmful levels of lead, mercury, and arsenic in some herbal medical products.

Standardization of herbal medicines is the process of establishing quality and identity profiles as well as unshared, unique features that can be used for the purpose of safety monitoring and overall quality assurance. Lack of standardization has hindered the regulation and control of herbal medicine despite its existence over many centuries and its expanding use in most countries. Adequate control measures to ensure reproducibility and repeatability in terms of quality, safety, dosing, and toxicity at all stages of herbal medicine production are necessary.

The process of collection, drying, extraction, formulation, and packaging must be standardized so that the materials in use may be classified, quantified, and volumes estimated at any given time. We need standardization to define precise directions for use of medicinal plants. A set of such standards should be compiled in the form of a monograph for each herbal medicine. Provision of a monograph in a Herbal Pharmacopoeia is evidence of standardization, for that particular herbal medicine. Such standards are helpful for quality assurance, safety, efficacy, and reproducibility, which are essential parameters in any dossier as drug regulatory requirements. Many international authorities including the World Health Organization have proposed new mechanisms to introduce quality control and standardization for herbal medicine. The US FDA guidance for botanical drugs is useful in quality assurance. The Government of India has separate regulations for AYUSH products and has recently amended Drugs and Cosmetics Act, and Rules to introduce phytopharmaceuticals as a new category. Several issues related to Good Manufacturing Practices, quality control, standardization, and regulations are discussed in detail by many experts [16,89].

12.14 EVIDENCE-BASED AYURVEDIC MEDICINE

Especially, when drug discovery is facing innovation deficit, it is important to study herbal drugs and ethnopharmacology, which offer rich sources of natural products [4]. Evidence-based traditional knowledge-inspired herbal drug discovery may provide natural products libraries with significant chemical diversity [5]. More scientific research and evidence for herbal drugs and Ayurvedic medicines are undoubtedly needed. However, it is important to undertake studies using proper models suitable to the epistemological aspects of traditional systems [90]. The process to generate evidence requires the power of technology and benefits of ancient wisdom to bring better safety and efficacy in natural products. Botanicals and herbals or poly herbal formulations may emerge as affordable and safer therapeutic options, provided the scientific evidence related to quality, standardization, safety, and efficacy is supported by scientific studies. In this process, the role of traditional knowledge and wisdom from systems such as Ayurveda must be seriously explored [6]. Few examples discussed in this chapter may help to affirm the importance of adopting suitable protocols and methods for traditional knowledge-inspired evidence-based medicines.

12.15 CONCLUSION

In conclusion, traditional medicine-inspired evidence-based approaches are very valuable in drug discovery and development to overcome many bottlenecks. The reverse pharmacology approach may offer effective, safer, and affordable medicines in relatively less time in an economical and ecofriendly manner. Effective quality control and standardization of traditional medicines as either traditional formulations or new medicines is necessary. New emerging categories such as phytopharmaceuticals may offer new opportunities to scientists, practitioners, and industries to develop research-based drugs from botanical sources, which can get global acceptance from modern medicine. The present approach of single drug-single target approach needs to shift toward multitarget–multiingredient formulations. Drug discovery should move toward formulation discovery. It is hoped that scientific research-supported evidence-based traditional medicine may be a good strategy for new drugs and formulation discovery and development.

References

[1] Patwardhan B. The new pharmacognosy. Comb Chem High Throughput Screen 2014;17(2):97.

[2] Simpson PB, Reichman M. Opening the lead generation toolbox. Nat Rev Drug Discovery 2014;13(1):3–4.
[3] Schmid EF, Smith DA. Is pharmaceutical R&D is just a game of chance or can strategy make a difference? Drug Discovery Today 2004;9(1):18–26.
[4] Bauer A, Brönstrup M. Industrial natural product chemistry for drug discovery and development. Nat Prod Rep 2014;31(1): 35–60.
[5] Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 2012;75(3): 311–35.
[6] Patwardhan B, Mashelkar RA. Traditional medicine-inspired approaches to drug discovery: can Ayurveda show the way forward? Drug Discovery Today 2009;14(15):804–11.
[7] Mukherjee PK, Venkatesh P, Ponnusankar S. Ethnopharmacology and integrative medicine—Let the history tell the future. J Ayurveda Integr Med 2010;1(2):100.
[8] Mukherjee PK, Nema NK, Venkatesh P, Debnath PK. Changing scenario for promotion and development of Ayurveda—way forward. J Ethnopharmacol 2012;143(2):424–34.
[9] Nisula Tapio. In the presence of biomedicine: ayurveda, medical integration and health seeking in Mysore, South India. Anthropol Med 2006;13(3):207–24.
[10] Chaudhary A, Singh N, Kumar N. Pharmacovigilance: boon for the safety and efficacy of ayurvedic formulations. J Ayurveda Integr Med 2010;1(2):51–6.
[11] Patwardhan K, Gehlot S, Singh G, Rathore, HCS. The Ayurveda education in India: how well are the graduates exposed to basic clinical skills?. Evidence-Based Complementary and Alternative Medicine, Evidence-Based Complementary and Alternative Medicine, vol. 2011, Article ID 197391, 6 pages, 2011. http://dx.doi.org/10.1093/ecam/nep113.
[12] Patwardhan Bhushan, Joglekar Vishnu, Pathak Namyata. Vaidya-scientists: catalysing Ayurveda renaissance. Curr Sci 2011;100(4): 476–83.
[13] Rastogi Sanjeev. Identifying attitudes about drug safety: a sample survey of Ayurvedic physicians. Int J Risk Saf Med 2010;22(2): 93–101.
[14] Sahoo N, Manchikanti P, Dey SH. Herbal drugs standards and regulation. Fitoterapia 2010;81:462–71.
[15] Narayana DBA. Approaches to pre-formulation R & D for phytopharmaceuticals emanating from herb based traditional Ayurvedic processes. J Ayurveda Integr Med 2013;4:4–8.
[16] Bhutani KK. Natural products: bench to bedside, an Indian perspective. Planta Med 2012;78(05):OP20.
[17] Singh RH. Exploring issues in the development of Ayurvedic research methodology. J Ayurveda Integr Med 2010;1:91–5.
[18] Valiathan MS. Putting house in order. Editorial Curr Sci 2006; 90(1):5–6.
[19] Patwardhan K. Medical education in India: time to encourage cross-talk between different streams. J Ayurveda Integr Med 2013;4:52–5.
[20] Patwardhan B. Time for evidence-based Ayurveda: a clarion call for action. J Ayurveda Integr Med 2013;4:63–6.
[21] Patwardhan B, Vaidya ADB, Chorghade M, Joshi SP. Reverse pharmacology and systems approaches for drug discovery and development. Curr Bioact Compd 2008;4(4):201–12.
[22] Patwardhan B, Vaidya ADB. Natural products drug discovery: accelerating the clinical candidate development using reverse pharmacology approaches. Indian J Exp Biol 2010;48(3):220–7.
[23] Vaidya RA, Vaidya ADB, Patwardhan B, Tillu G, Rao Y. Ayurvedic pharmacoepidemiology: a proposed new discipline. J Assoc Physicians India 2003;51:528.
[24] Patwardhan B, Francis RP, Kapre SV, Sharma KD. Antibacterial properties of *S. anacardium*. Bull Haffkine Inst 1982;10:27.
[25] Rajan A, Sreekumaran T, Joseph CD, Varghese PR. Immunopathological response to Anacarcin Forte in dogs. Indian J Pathol Microbiol 1987;30(2):181–7.
[26] Vad BG, Kulkarni DR. Acute myeloblastic subleukemic leukemia treatment with Anacarcin. Indian Pract 1975;28:513–9.
[27] Saraf MN, Ghooi RB, Patwardhan BK. Studies on mechanism of action of *S. anacardium* in rheumatoid arthritis. J Ethnopharmacol 1989;25:159–64.
[28] Patwardhan B, Saraf MN, David SB. Toxicity of *Semecarpus anacardium* extract. Ancient Sci Life 1988;8(2):106.
[29] Patwardhan B, Ghooi RB. Chemical and biological properties of *Semecarpus anacardium*. Bull Haffkine Inst 1982;10:87.
[30] Patwardhan B, Phadke UR, Ghooi RB. Binding of monoene bhilwanol on *Clostridium tetani*. Bull Haffkine Inst 1983;11:14.
[31] Patwardhan B, Ghooi RB, David SB. A new anaerobic inhibitor of herbal origin. Indian J Pharm Sci 1988;50(2):130–2.
[32] Zimmermann GR, Lehar J, Keith CT. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discovery Today 2007;12(1/2):34–42.
[33] Chandrasekaran AN, Porkodi R, Radhamadhavan, Parthiban M, Bhatt NS. Studies on Ayurvedic drugs in rheumatoid arthritis—comparison with Auranofin. Indian Pract 1994; XLVII(6):489–502.
[34] Chennaiah S, Qadri SSYH, Reddy CVK, Rao SV, Shyamsunder G, Raghuramulu N. Incorporation of *Cestrum diurnum* leaf improves intestinal Ca transport in broilers. J Steroid Biochem Mol Biol 2007;103(3–5):645–50.
[35] Bansod KV. Efficacy of topicure on wounds in canines. Indian Vet J 2003;80(12):1300–1.
[36] Patwardhan B. Death of drugs and rebirth of health Care: Indian response to discovery impasse. Collaborative innovation in drug discovery. USA: John Wiley & Sons Inc; 2014. p 173–194.
[37] Narayana DB, Katiyar CK. Draft amendment to drugs and cosmetics rules to license science based botanicals, phytopharmaceuticals a drugs in India. J Ayurveda Integr Med 2013;4: 245–6.
[38] Verpoorte R. Ethnopharmacology and systems biology: a perfect holistic match. J Ethnopharmacol 2005;100(1–2):53–6.
[39] Fønnebø V, Grimsgaard S, Walach H, Ritenbaugh C, Norheim AJ, MacPherson H, et al. Researching complementary and alternative treatments—the gatekeepers are not at home. BMC Med Res Methodol 2007;7:7.
[40] Chopra A, Lavin P, Patwardhan B, Chitre D. Randomized double blind trial of an ayurvedic plant derived formulation for treatment of rheumatoid arthritis. J Rheumatol 2000;27(6):1365–72.
[41] Chopra A, Lavin P, Patwardhan B, Chitre D. A 32-week randomized, placebo-controlled clinical evaluation of RA-11, an ayurvedic drug, on osteoarthritis of the knees. J Clin Rheumatol 2004; 10(5):236–45.
[42] Patwardhan B. Method of treating musculoskeletal disorders and a novel composition therefore. United States Patent No. 5494668, issued on February 27, 1996. Licensed to BioVed Inc, San Jose, CA, USA.
[43] Chopra A, Saluja M, Tillu G. Ayurveda-modern medicine interface: a critical appraisal of studies of Ayurvedic medicines to treat osteoarthritis and rheumatoid arthritis. J Ayurveda Integr Med. 17(1): 190–198.
[44] Chopra A, Saluja M, Tillu G, Venugopalan A, Sarmukaddam S, Raut AK, et al. A randomized controlled exploratory evaluation of standardized ayurvedic formulations in symptomatic osteoarthritis knees: a government of India NMITLI project. Evidence-Based Complementary Altern Med 2011;2011.
[45] Joshi K, Chavan P, Warude D, Patwardhan B. Molecular markers in herbal drug technology. Curr Sci 2004;87(2):159–65.

[46] Chavan P, Warude D, Joshi K, Patwardhan B. Development of SCAR markers as a complementary tool for identification of ginger from crude drugs and multicomponent formulations. Biotechnol Appl Biochem 2008;50(1):61–9.
[47] Shinde VM, Dhalwal K, Mahadik KR, Joshi KS, Patwardhan BK. RAPD analysis for determination of components in herbal medicine. Evidence-Based Complementary Altern Med 2007; 4(Suppl. 1):21–3.
[48] Chavan P, Joshi K, Patwardhan B. DNA microarrays in herbal drug research. Evidence-Based Complementary Altern Med 2006;3(4):447–57.
[49] Dnyaneshwar W, Preeti C, Kalpana J, Bhushan P. Development and application of RAPD-SCAR marker for identification of *Phyllanthus emblica* linn. Biol Pharm Bull 2006;29(11):2313–6.
[50] Sumantran VN, Joshi AK, Boddul S, Koppikar SJ, Warude D, Patwardhan B, et al. Antiarthritic activity of a standardized, multiherbal, ayurvedic formulation containing *Boswellia serrata*: in vitro studies on knee cartilage from osteoarthritis patients. Phytother Res 2011;25(9):1375–80.
[51] Sumantran VN, Kulkarni A, Chandwaskar R, Harsulkar A, Patwardhan B, Chopra A, et al. Chondroprotective potential of fruit extracts of phyllanthus emblica in osteoarthritis. Evidence-Based Complementary Altern Med 2008;5(3):329–35.
[52] Sumantran VN, Kulkarni A, Boddul S, Chinchwade T, Koppikar SJ, Harsulkar A, et al. Chondroprotective potential of root extracts of *Withania somnifera* in osteoarthritis. J Biosci 2007; 32(2):299–307.
[53] Sumantran VN, Chandwaskar R, Joshi AK, Boddul S, Patwardhan B, Chopra A, et al. The relationship between chondroprotective and antiinflammatory effects of *Withania somnifera* root and glucosamine sulphate on human osteoarthritic cartilage in vitro. Phytother Res 2008;22(10):1342–8.
[54] Patwardhan B., et al. A synergistic herbal composition for treatment of rheumatoid and musculoskeletal disorders. CSIR Patent, International Application Number PCT/IN2008/000462, dated July 18, 2008.
[55] Chopra A, Patwardhan B. Validating safety & efficacy of Ayurvedic derived botanical formulations: a clinical arthritis model of NMITLI. In: 5th Oxford international conference on the science of botanicals (ICSB). USA: University of Mississippi at Oxford; 2006.
[56] Paranjpe P, Patki P, Patwardhan B. Ayurvedic treatment of obesity: a randomised double-blind, placebo-controlled clinical trial. J Ethnopharmacol 1990;29(1):1–11.
[57] Jadhav RB, Patwardhan B. Anti-anxiety activity of *Celastrus paniculatus*. Indian J Nat Prod 2003;19(3):16–9.
[58] Kulkarni RR, Patki PS, Jog VP, Gandage SG, Patwardhan B. Treatment of osteoarthritis with a herbomineral formulation: a double-blind, placebo-controlled, cross-over study. J Ethnopharmacol 1991;33(1–2):91–5.
[59] Kulkarni RR, Jog V, Gandage S, Patki P, Patwardhan B. Efficacy of ayurvedic formulation in rheumatoid arthritis: a randomized, double blind, placebo controlled crossover study. Indian J Pharmacol 1992;24:98–101.
[60] Saraf MN, Patwardhan B. Pharmacological studies on *S. brevistgma* part I: antiallergic activity. Indian Drugs 1988;26(2):49–54.
[61] Saraf MN, Patwardhan B. Pharmacological studies on *S. brevistgma* part II: brochodilator activity. Indian Drugs 1988;26(2): 54–7.
[62] Patwardhan B, Kalbag D, Patki PS, Nagsampagi BA. Search of immunomodulatory agents: a review. Indian Drugs 1990;28(2): 56–63.
[63] Ayurveda and Future Drug Development, Patwardhan. Int J Altern Complementary Med 1992;10(12):9–11.
[64] Patwardhan B. Ethnopharmacology and drug discovery. J Ethnopharmacol 2005;100(1–2):50–2.
[65] Balasubramani SP, Venkatasubramanian P, Kukkupuni SK, Patwardhan B. Plant-based rasayana drugs from ayurveda. Chin J Integr Med 2011;17(2):88–94.
[66] Patil M, Patki P, Kamath HV, Patwardhan B. Antistress activity of *Tinospora cordifolia* (wild) miers. Indian Drugs 1997;34(4):211–5.
[67] Ziauddin M, Phansalkar N, Patki P, Diwanay S, Patwardhan B. Studies on the immunomodulatory effects of ashwagandha. J Ethnopharmacol 1996;50(2):69–76.
[68] Agarwal R, Diwanay S, Patki P, Patwardhan B. Studies on immunomodulatory activity of *Withania somnifera* (ashwagandha) extracts in experimental immune inflammation. J Ethnopharmacol 1999;67(1):27–35.
[69] Diwanay S, Chitre D, Patwardhan B. Immunoprotection by botanical drugs in cancer chemotherapy. J Ethnopharmacol 2004;90(1): 49–55.
[70] Grandhi A, Mujumdar AM, Patwardhan B. A comparative pharmacological investigation of ashwagandha and ginseng. J Ethnopharmacol 1994;44(3):131–5.
[71] Mitra SK, Datta HS, Paramesh R, Patwardhan B. Theories and management of aging: modern and ayurveda perspectives. Evidence-Based Complementary Altern Med 2001;2011.
[72] Gautam M, Saha S, Bani S, Kaul A, Mishra S, Patil D, et al. Immunomodulatory activity of asparagus racemosus on systemic Th1/Th2 immunity: implications for immunoadjuvant potential. J Ethnopharmacol 2009;121(2):241–7.
[73] Bani S, Gautam M, Sheikh FA, Khan B, Satti NK, Suri KA, et al. Selective Th1 up-regulating activity of *Withania somnifera* aqueous extract in an experimental system using flow cytometry. J Ethnopharmacol 2006;107(1):107–15.
[74] Patwardhan B, Gautam M. Botanical immunodrugs: scope and opportunities. Drug Discovery Today 2005;10(7):495–502.
[75] Gautam M, Diwanay S, Gairola S, Shinde Y, Patki P, Patwardhan B. Immunoadjuvant potential of asparagus racemosus aqueous extract in experimental system. J Ethnopharmacol 2004;91(2–3):251–5.
[76] Gautam M, Diwanay SS, Gairola S, Shinde YS, Jadhav SS, Patwardhan BK. Immune response modulation to DPT vaccine by aqueous extract of *Withania somnifera* in experimental system. Int Immunopharmacol 2004;4(6):841–9.
[77] Gautam M, Gairola S, Jadhav S, Patwardhan B. Ethnopharmacology in vaccine adjuvant discovery. Vaccine 2008;26(41): 5239–40.
[78] Patwardhan B, Gautam M. Process for making biologically active aqueous extracts of plant product. Indian Patent: 1246/Mum/2003, Filing Date: 2003-12-05, Publication Date: 2006-01-20, Applicant: Serum Institute of India Ltd., Pune University.
[79] Patwardhan B, Gautam M. Aqueous extracts of plant products. Indian Patent: 1247/Mum/2003, Filing Date: 2003-12-05, Publication Date: 2006-01-20. Applicant: Serum Institute of India Ltd., Pune.
[80] Patwardhan B, Gautam M. Process for making immunological adjuvants. Indian Patent: 1253/Mum/2003, Filing Date: 2003-12-08, Publication Date: 2006-01-20. Applicant: Serum Institute of India Ltd., Pune.
[81] Diwanay S, Gautam M, Patwardhan B. Cytoprotection and immunomodulation in cancer therapy. Curr Med Chem Anticancer Agents 2004;4(6):479–90.
[82] Patil D. Botanical drug interactions: case studies from traditional medicines. Germany: LAP Lambert Academic Publishing; 2013-12-06.
[83] Puranik AS, Halade G, Kumar S, Mogre R, Apte K, Vaidya ADB, *Cassia auriculata*: aspects of safety pharmacology and drug interaction. Evidence-Based Complementary and Alternative Medicine, vol. 2011, Article ID 915240, 8 pages, 2011. http://dx.doi.org/10.1093/ecam/nep237.

[84] Patil D, Gautam M, Gairola S, Jadhav S, Patwardhan B. Effect of botanical immunomodulators on human CYP3A4 Inhibition: implications for concurrent use as adjuvants in cancer therapy. Integr Cancer Ther 2014;13(2):167–75.

[85] Patil D, Gautam M, Mishra S, Kulkarni P, Suresh K, Gairola S, et al. Quantitative determination of protoberberine alkaloids in tinospora cordifolia by RP-LC-DAD. Chromatographia 2010; 71(3–4):341–5.

[86] Patil D, Gautam M, Jadhav U, Mishra S, Karupothula S, Gairola S, et al. Physicochemical stability and biological activity of *Withania somnifera* extract under real-time and accelerated storage conditions. Planta Med 2010;76(5):481–8.

[87] Chitlange SS, Kulkarni PS, Patil D, Patwardhan B, Nanda RK. High-performance liquid chromatographic fingerprint for quality control of *terminalia arjuna* containing ayurvedic churna formulation. J AOAC Int 2009;92(4):1016–20.

[88] Sahoo NK, Sarkar S, Patwardhan B. Stability study on *Boswellia serrata* (hydro-alcoholic) extract. Asian J Chem 2009;21(5):3529–34.

[89] Verpoorte R, Mukherjee P. GMP for botanicals: regulatory and quality issues on phytomedicines. New Delhi: Business Horizons; 2003.

[90] Patwardhan B. The quest for evidence-based Ayurveda: lessons learned. Curr Sci 2012;102(10).

CHAPTER

13

Evaluation of Bioactive Compounds as Acetylcholinesterase Inhibitors from Medicinal Plants

Santanu Bhadra, Manoj K. Dalai, Joydeb Chanda, Pulok K. Mukherjee

School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

OUTLINE

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00013-1

13.1 INTRODUCTION

From ancient times, plants have been widely used in traditional medicine for improving cognitive function and age-related memory loss. Until now, phytoconstituents such as huperzine (Chinese traditional medicine) and galantamine (origin from European herb) have been known for neuroprotective activity against cognitive disorder. It indicates that plants contain rich sources of active compounds and they have become a notable alternative to synthetic drugs in the treatment of cognitive and related disorders. A variety of plant-derived formulations have long been used for cholinesterase (ChE) inhibitory (ChI) activity in the Indian system of medicine [1]. Ethnopharmacological knowledge plays an important role in finding new lead compounds from plant sources. Simultaneously, traditional knowledge and modern scientific approach have provided templates of compounds from plants. These chemical compounds have emerged as promising candidates for the treatment of cognitive and related disorders, such as Alzheimer disease (AD), senile dementia, ataxia, myasthenia gravis, and Parkinson disease. The principal role of acetylcholinesterase (AChE) is to decrease the amount of acetylcholine (ACh) in the cholinergic neurons and to terminate the nerve impulse transmission. Inhibition of AChE and butyrylcholinesterase (BChE) in the cholinergic system are intimately linked with the treatment of cognitive-related disorders including AD [2]. Targeting these enzymes could mean success of the therapeutic approach to treat not only AD but also other forms of dementia linked with ACh deficiency. Many researchers are making considerable efforts to find quality AChE inhibitors derived from natural sources because several synthetic medicines, e.g., tacrine and donepezil, have been reported to have adverse effects. These medicines are also available for the symptomatic treatment of patients with mild to moderate AD. Certainly, synthetic AChE/BChE (tacrine and donepezil) inhibitors do not provide much benefit to the patient for their symptomatic relief and they also have adverse effects. Hence, naturally occurring AChE/BChE inhibitors from crude extracts of *Physostigma venenosa* (physostigmine), *Galanthus nivalis* galanthamine, and *Huperzia serrata* (huperzine) have been proven to be effective in cognitive-related disorders. Several scientific studies indicate that plant-derived chemical compounds have cognitive enhancing properties. The chemical compounds derived from sage (*Salvia cavandulaefolia/officinalis*), lemon balm (*Melissa officinalis*), and rosemary (*Rosmarinus officinalis)* are promising sources of AChE/BChE inhibitory activity [3]. Several plant secondary metabolites such as alkaloid, terpenoid, flavonoid, and polyphenol compounds have shown significant label of AChE/BChE inhibitory activity [4]. In this context, we have made every effort to demonstrate the importance of natural ChIs in learning and memory disorders. This chapter highlights several aspects of ChIs from medicinal plants, classes of bioactive compounds isolated from them, and their potential. Simultaneously, several comprehensive studies on plant's family and their potential phytoconstituents have been conducted for exploration of their potential anti-ChE activity.

13.2 CHOLINERGIC PATHWAY

ACh, the naturally occurring cholinergic neurotransmitter in the central nervous system, is considered to be involved in the learning process and responsible for intact memory. ACh is synthesized from choline and acetyl coenzyme A in the presence of choline acetyltransferase. The vesicular acetylcholine transporters (VAChTs) then transport ACh into synaptic vesicles. The VAChT essentially "exchanges" ACh for protons, where the pH gradient between the vesicle lumen and the cytoplasm provides the driving force for ACh transport. At the synaptic cleft, the released ACh binds with nicotinic and muscarinic receptors. Nicotinic receptors are ion channel receptors and muscarinic receptors are G-protein-coupled receptors, usually located on

postsynaptic cells. Normally, in the synaptic cleft, the excess unbound ACh, after activity, breaks down into acetate and choline by direct enzymatic hydrolysis. The enzyme that is found to be responsible for the hydrolysis of ACh is called AChE. Choline is then transported back into the presynaptic neuron by a high-affinity choline transporter for further ACh synthesis [5].

13.2.1 AChE and BChE

AChE is called a true cholinesterase, and BChE is a pseudocholinesterase; they share 65% amino acid sequence homology and have similar molecular forms and active center structures despite being products of different genes on human chromosomes 7(specifically 7q22) and 3(specifically 3q26), respectively. In addition, the AChE and BChE differ from each other by their substrate specificity, behavior in excess substrate, and susceptibility to inhibitors. AChE has more affinity towards ACh and hydrolyzes it faster than BChE, but it is less active on butyrylcholine (BCh). On the contrary, BChE preferably acts on BCh; it also hydrolyzes ACh. Excess of substrate inhibits AChE, whereas BChE exhibits substrate activation in excess substrate [6]. Tissue-specific distribution is also different for AChE and BChE; AChE is abundant in the brain, muscle, and erythrocyte membrane, whereas occurrence of BChE is higher in the liver, intestine, heart, kidney, and lungs. The two forms of ChEs differ significantly in substrate specificity, enzyme kinetics, expression, and activity in different brain regions, and complexity of gene regulation. BChE and AChE in the brain can cleave >10,000 molecules of ACh per second. AChE is one of the fastest enzymes and pivotal in hydrolyzing ACh, while BChE mainly works as a detoxification enzyme. Abnormality of BChE in the body does not cause any significant physiological abnormality; therefore, previously it did not draw much attention.

13.3 CHOLINESTARASE INHIBITORS FOR LEARNING AND MEMORY

That cognitive functions are highly dependent on central cholinergic neurotransmission goes back to the early 1960s, which was deduced from the experimentally proven fact that the effects of cholinergic antagonists and lesions of cholinergic nuclei are often related to cognitive deficits similar to those observed in aging and dementia (Figure 13.1). It was further confirmed by the use of cholinergic muscarinic antagonist scopolamine for the induction of amnesia in experimental subjects and the loss of cognitive abilities appeared to be comparable to untreated subjects. This scopolamine-induced cognitive deficits are directly related to a decrease in central cholinergic functions. Other cholinergic inhibitors like choline uptake inhibitor (hemicholinium), the specific muscarinic type 1 receptor antagonist (pirenzepine), and the nicotinic antagonist (mecamylamine) also have a negative effect on learning and memory performance. Furthermore, the AChE inhibitors increase the availability of ACh in the synaptic cleft, which is capable of reversing the scopolamine-induced cognitive deficit and impairment of learning and memory.

Recently, interest in the pharmacological and toxicological importance of BChE is growing, as some studies revealed that it hydrolyzes ester-containing drugs and scavenges ChIs including potent organophosphorus nerve agents before they reach their synaptic targets [7]. It was observed that selective BChE inhibition augmented long-term potentiation in the progression of AD. In AD, AChE is lost early by up to 85% in specific brain regions, whereas BChE levels rise with disease progression. Both AChE and BChE showed differentiated kinetic molecular properties within amyloid plaques and tangles than normal neuronal forms found in the brain. Histochemical studies showed that some cholinergic neurons contain BChE instead of AChE. Cytochemical studies have revealed that BChE in the brain of patients with AD regulates specific cholinergic pathways. BChE independently regulates 10−15% of ChE-positive cells in human amygdala and hippocampus. AChE-positive neurons project diffusely to the cortex, modulating cortical processing and responses to new and relevant stimuli. BChE-positive neurons project specifically to the frontal cortex, and may have roles in attention, executive function, emotional memory, and behavior. Furthermore, BChE activity progressively increases as the severity of dementia advances, while AChE activity declines. An important distinguishing feature of BChE from AChE is that BChE loses its efficiency at lower concentration of ACh, but the activity increases with the increase in ACh concentration. In contrast, at higher concentration of ACh, AChE becomes substrate inhibited. BChE has peptidase activity besides esterase activity [8]. Recent evidences also suggest that AChE and BChE may have roles beyond “classical” coregulatory esterase functions in terminating ACh-mediated neurotransmission. “Nonclassical” roles include modulating the activity of other proteins, regional cerebral blood flow, tau phosphorylation, and the amyloid cascade, which may affect rates of AD progression. AChE and BChE are efficient in cleaving amyloid precursor protein to β-amyloid protein and constitute β-amyloid plaques in AD. Thus, selective BChE inhibitors also prevent the formation of new β-amyloid plaques [9]. Researches showed that senile plaques contain

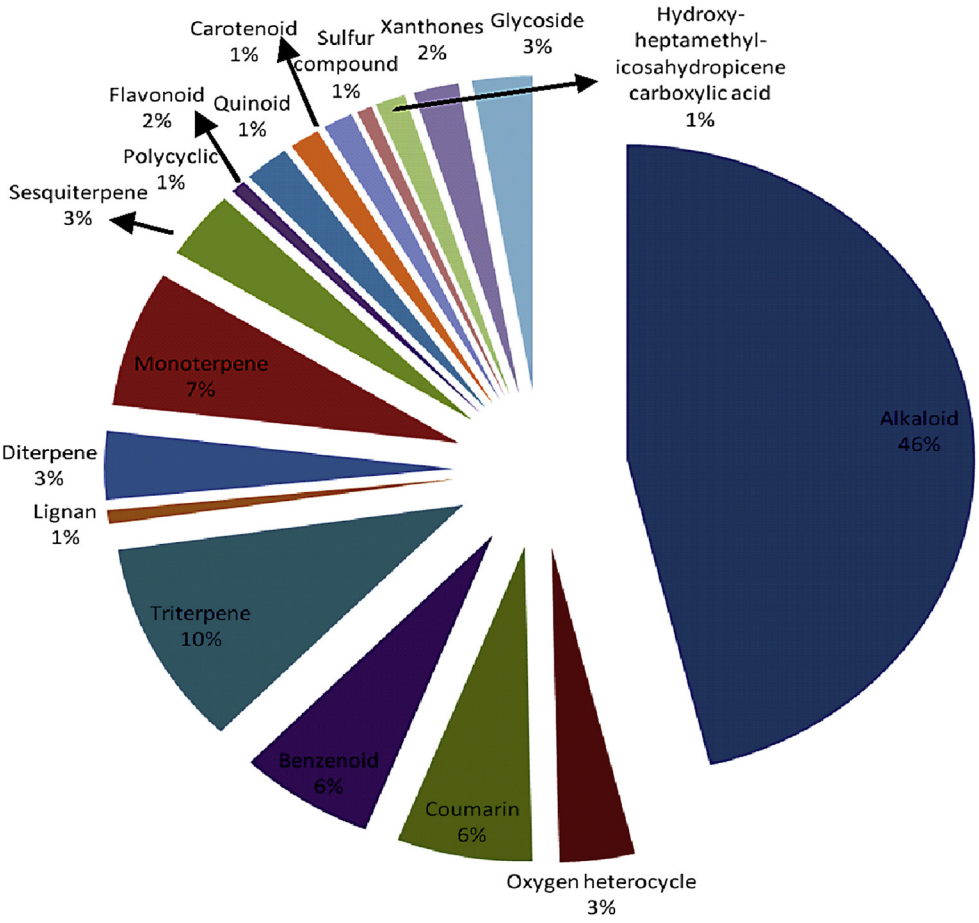


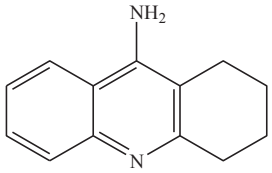
FIGURE 13.1 Role of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in the progression of Alzheimer disease (AD).

AChE and BChE and the incorporation of these enzymes result in the formation of a stable complex and may increase the neurotoxicity of amyloid beta fibrils in AD. A specific form of BChE is found in cerebrospinal fluid (CSF) of AD patients, and assay of this form in CSF can be used in sensitive and specific detection of AD; thus detection of BChE level is an essential for accurate and early diagnosis of AD.

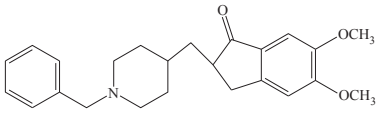
Recent studies showed that muscarinic neurotransmission plays an important role in attention, learning, memory, and cognition. It was observed that the muscarinic binding sites remain unchanged in different forms of dementia associated with cortical cholinergic deficits, including AD, when widespread decline in neuronal nicotinic receptors were reported in normal brain aging, which may be responsible in the cognitive process of the brain [10]. It was also found that the nicotinic receptors get severely affected in AD brain, which is related to the severity of dementia. A number of studies indicated that stimulation of the cholinergic nervous system might interfere with AD progression like nicotinic receptor agonists, which are reported to attenuate Aβ-induced toxicity and involved in neuroprotection. On the other hand, inhibition of muscarinic receptors are found to be beneficial in AD, as they regulate the posttranslational modification of two proteins PS1 and APP, which play a central role in the pathophysiology of AD [11]. On the contrary, the selective M1 agonists decrease tau phosphorylation and promote the nonamyloidogenic amyloid precursor protein processing pathways, which eventually improves neuroprotection. However, reports suggest that the specific M1 muscarinic or nicotinic agonists, M2 muscarinic antagonists, and AChE inhibitors are the novel leads for the management of cholinergic deficit and memory disorders [6].

Some of the US Food and Drug Administration (FDA) approved bioactive molecules should be named, as these molecules ignited the hope for the search of novel ChIs for the treatment of dementia. Tacrine **(I)** was the first drug approved by the US FDA for the treatment of AD in 1993. It reversibly inhibits both AChE and BChE. The most common side effects of tacrine are gastrointestinal symptoms such as nausea, vomiting, anorexia, and diarrhea. Hepatotoxicity is also a common side effect in a high percentage of

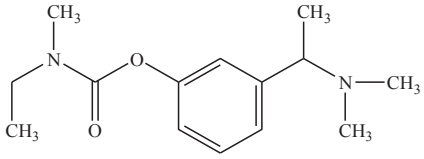
patients, necessitating frequent monitoring of liver transaminase levels. Drug interactions with theophylline are possible. Reversible ChI donepezil **(II)** was approved by the US FDA in 1997. The most common side effects of donepezil are diarrhea, anorexia, nausea, vomiting, muscle cramps, and fatigue [8]. Rivastigmine **(III)** (approved by US FDA in 2000) is a pseudo-irreversible inhibitor; it gets inactivated by enzymatic cleavage at the active site of the ChE rather than by metabolism. Unlike tacrine and donepezil, it does not go through the cytochrome P450 system. It inhibits both AChE and BChE. The main side effects of rivastigmine are nausea, vomiting, and diarrhea. Galantamine **(IV)** is the newest reversible ChI. It acquired USFDA approval in February 2001. It also inhibits both AChE and BChE. Galantamine also binds to nicotinic receptors in presynaptic neurons, stimulating greater release of ACh, which can simultaneously lead to improvement of memory, concentration, and attention. Nausea, vomiting, anorexia, diarrhea, and weight loss are the most common side effects noticed in galantamine therapy.



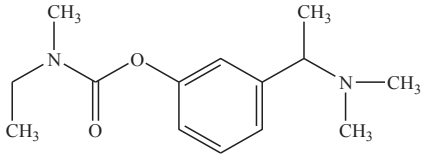
Tacrine (I)



Donepezil (II)



Rivastigmine (III)



Galantamine (IV)

13.4 MEDICINAL PLANTS FOR THE MANAGEMENT OF COGNITIVE DISORDER

Nature is a rich source of biological and chemical diversity. The unique and complex chemical leads found from natural sources are very difficult to obtain by chemical synthesis. There are many synthetic drugs originated from natural sources serving as plant-based complementary medicine. A variety of plants have been reported to exhibit AChE inhibitory activity and so may be relevant to the treatment of neurodegenerative disorders such as AD and other cognitive disorders. Recent findings reveal that over 350 plants belonging to more than 100 botanical families have been tested against AChE inhibition. In traditional practice, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases. These medicinal plants represent a great deal of untapped reservoir of drugs and the structural diversity of their component molecules makes a valuable source of novel lead compounds. According to ayurveda, AD-type of dementia is an imbalance of vata, pitta, and kapha. Medhya (intellectual promoting) herbs such as *Convolvulus microphyllus, Centella asiatica, Bacopa monnieri, Acorus calamus*, and *Celastrus paniculatus* are beneficial in cognitive disorders. Ethnopharmacological approach and bioassay-guided isolation have provided a lead in identifying potential AChE inhibitors from plant sources, including those for memory disorders.

Several food plants including fruits, vegetables, and spices (most of the spices contain essential oil) show potent AChE inhibitory activity that can improve cognition and memory in AD patients. Recently, the essential oils obtained from edible plants have become of great interest, due to their availability, fewer side effects or toxicity, as well as their biodegradability. Research on new bioactive compounds from medicinal plants has led to the isolation and structure elucidation of a number of new pharmacophores. Some of the important medicinal plants have been discussed in detail. Apart from that, a database of some of food plants, some essential oil containing plants, and some traditionally acclaimed plants that possess AChE inhibitory activity has been made (Table 13.1–13.3).

TABLE 13.1 List of Essential Oil Bearing Plants Having Anticholinesterase Activity

Name of the plant, family	Part(s) used	Biological activity (in % or μg/mL) AChE	BChE	References
Alpinia galangal, Zingiberaceae	Rhizome	83.9	–	[12]
Anethum graveolens, Apiaceae	Leaves	100.0	90.9	[13]
Artemisia dracunculus, Asteraceae	Leaves	0.14	–	[14]
Centella asiatica, Apiaceae	Whole	75.0	>10	[12]
Cinnamomum bejolghota, Lauraceae	Leaves	>25.0	>50.0	[12]
Cistus creticus, Cistaceae	Leaves	12.9	29.1	[15]
Cistus libanotis, Cistaceae	Leaves	71.2	23.7	[15]
Cistus monspeliensis, Cistaceae	Leaves	–	180.4	[15]
Cistus salvifolius, Cistaceae	Leaves	58.1	34.2	[15]
Cistus villosus, Cistaceae	Leaves	–	123.2	[15]
Citrus aurantifolia, Rutaceae	Fruit peel	85.8	82.9	[12]
	Fruit	139.3	235.5	[16]
Citrus aurantium, Rutaceae	Fruit	147.5	266.6	[16]
Citrus bergamia, Rutaceae	Fruit	161.6	243.6	[16]
Citrus hystrix, Rutaceae	Leaves	>20.0	>50.0	[12]
Citrus limon, Lamiaceae	Fruit peel	0.84	–	[17]
Citrus maxima, Rutaceae	Leaves	>50.0	>60.0	[12]
Citrus medica, Rutaceae	Fruit	171.3	154.6	[17]
Citrus reticulata, Rutaceae	Leaves	>25.0	>30.0	[12]
Cupressussem pervirens, Cupressaceae	Aerial	0.28	–	[17]
Cymbopogon citratus, Poaceae	Stem	>25.0	>50.0	[12]
Eucalyptus globulus, Myrtaceae	Aerial	0.12	–	[17]
Eupatorium odoratum, Asteraceae	Whole	>30.0	>25.0	[12]
Ferula lutea, Apiaceae	Flower	70.25	–	[18]
Foeniculum vulgare, Apiaceae	Aerial	1.18	–	[17]
Foeniculum vulgare, Umbelliferae	Leaves	80.8	65.4	[13]

TABLE 13.1 List of Essential Oil Bearing Plants Having Anticholinesterase Activity—cont'd

Name of the plant, family	Part(s) used	Biological activity (in % or μg/mL) AChE	BChE	References
Hypericum undulatum, Hypericaceae	Flowers	20.0	–	[19]
Inula graveolens, Asteraceae	Leaves	0.27	–	[14]
Laurus nobilis, Lauraceae	Leaves	51.3	–	[19]
Lavandula angustifolia, Lamiaceae	Flowers	33.7	–	[19]
Lavandula officinalis, Labiatae	Leaves	81.2	92.9	[13]
Lavandula pedunculata, Lamiaceae	Flowers	56.5	–	[19]
Malva silvestris, Malvaceae	Flowers	28.1	–	[19]
Melissa officinalis, Lamiaceae	Leaves	82.5	96.1	[12]
Mentha piperita, Lamiaceae	Leaves	81.5	93.5	[13]
Mentha suaveolens, Lamiaceae	Aerial	46.2	–	[19]
Ocimum basilicum, Lamiaceae	Leaves	10.7	74.1	[13]
Ocimum canum, Lamiaceae	Leaves	>20.0	>60.0	[12]
Ocimum gratissimum, Lamiaceae	Leaves	–	78.9	[12]
Ocimum sanctum, Lamiaceae	Leaves	1.6	–	[14]
Origa majorana, Lamiaceae	Leaves	94.6	94.4	[13]
Origa munitiflorum, Lamiaceae	Leaves	100.0	94.7	[13]
Origa numonites, Lamiaceae	Leaves	96.3	92.9	[13]
Origa vulgare, Lamiaceae	Leaves	97.9	88.4	[13]
Paronychia argentea, Caryophyllaceae	Aerial	44.6	–	[19]
Piper sarmentosum, Piperaceae	Leaves	>40.0	>50.0	[12]
Polygonum odoratum, Polygonaceae	Whole	>20.0	>40.0	[12]
Polyscias fruticosa, Araliaceae	Leaves	>55.0	>60.0	[12]
Salvia officinalis, Lamiaceae	Aerial	46.4	–	[19]

TABLE 13.1 List of Essential Oil Bearing Plants Having Anticholinesterase Activity—cont'd

Name of the plant, family	Part(s) used	Biological activity (in % or μg/mL)		References
		AChE	BChE	
Salvia sclarea, Lamiaceae	Leaves	11.6	45.1	[13]
Sanguisorba minor, Rosaceae	Aerial	38.8	–	[19]
Satureja montana, Lamiaceae	Aerial	53.0	34.0	[20]
Thymus lotocephalus, Lamiaceae	Aerial	0.90	0.50	[21]
Thymus praecox, Lamiaceae	Leaves	27.60	–	[22]
Thymus vulgaris, Lamiaceae	Aerial	0.21	–	[17]
Zingiber cassumunar, Zingiberaceae	Rhizome	>25.0	>50.0	[12]
Zingiber officinale, Zingiberaceae	Rhizome	>50.0	>53.0	[12]

13.4.1 Essential Oil-Bearing Plants Having Anticholinesterase Activity

Considering the plant-derived compounds in drug discovery, a large number of medicinal plants have been taken from various geographical sources with proper ethnopharmacological claims. Various research works have been executed on the essential oil composition and its effect on the AChE and BChE enzyme inhibition [12]. Some of the major plants containing essential oils are discussed below and has been cited in Table 13.1.

13.4.1.1 Acorus calamus *(Acoraceae)*

The ethanolic and hydroethanolic extracts of *Ac. calamus* have been used for a long time as neuroprotective agents as well as in the treatment of cognitive disorders. The major bioactive compound responsible for the pharmacological activity is α-asarone [28,29], which has been isolated from this plant.

13.4.1.2 Carom carvi *(Umbelliferae)*

The major phytoconstituents reported in *C. carvi* are (R)-carvone, D-limonene, α-pinene, *cis*-carveol, and myrcene, of which R-carvone and D-limonene were found to have potent inhibitory activity against AChE [30,31].

TABLE 13.2 List of Edible Plants Having Anticholinesterase Activity

Name of the plant, family	Part(s) used	Extract used	Biological activity (in % or μg/mL)		References
			AChE	BChE	
Anethum graveolens, Apiaceae	Leaf	Aqueous	6.10	8.57	[13]
Apium graveolens, Umbelliferae	Root	Dichloromethane	–	59.91	[23]
		Ethanol	–	40.39	
		Water	–	26.73	
Ap. graveolens, Apiaceae	Root	Aqueous	6.10	1.82	[13]
Brassica oleracea, Cruciferae	Leaves	Dichloromethane	44.92	52.75	[23]
		Ethanol	49.44	9.24	
		Water	44.06	6.55	
Petroselinum crispum, Apiaceae	Leaf	Aqueous	6.10	0.47	[13]
	Root		0.07	0.76	
Prunus domestica, Rosaceae	Fruit	Aqueous	3.09	2.22	[13]
Punica granatum, Lythraceae	Fruit	Aqueous	1.53	1.38	[13]
Spinacia oleracea, Chenopodiaceae	Aerial	Dichloromethane	–	30.60	[23]
		Ethanol	–	15.94	
		Water	–	19.05	

TABLE 13.3 List of Traditional Plants Having Anticholinesterase Activity

Name of the plant, family	Part(s) used	Extraction of the plant	Enzyme inhibition (in % or μg/mL)		References
			AChE	BChE	
Acacia nilotica, Mimosaceae	Leaves	Ethyl acetate	53.00	–	[24]
Acacia sieberiana, Mimosaceae	Root	Ethyl acetate	60.00	–	[24]
Acorus gramineus, Araceae	Rhizome	Methanol	7.2	–	[13]
Albizia adianthifolia, Fabaceae	Bark	Ethyl acetate	61.00	–	[24]
Arnica chamissonis, Asteraceae	Flower	Hexane	29.0	88.0	[19]
		Methanol	43.0	–	
Bacopa monniera, Scrophulariaceae	Whole	Ethanol	42.97	–	[25]
Bupleurum falcatum, Umbelliferae	Root	Methanol	24.8	–	[13]
Combretum kraussii, Combretaceae	Leaves	Ethyl acetate	96.00	–	[24]
Dioscorea batatas, Dioscoreaceae	Rhizome	Methanol	7.6	–	[13]
Epimedium koreanum, Berberidaceae	Whole	Methanol	47.5	–	[13]
Ginkgo biloba, Coniferae	Whole	Ethanol	>50	–	[25]
Hypericum perforatum, Hypericaceae	Whole	Methanol	178	–	[19]
Narcissus assoanus, Amaryllidaceous	Bulbs	Methanol	7.81	–	[26]
Narcissus baeticus, Amaryllidaceous	Bulbs	Methanol	44.61	–	[26]
Narcissus abcissus, Amaryllidaceous	Bulbs	Methanol	47.40	–	[26]
Ruta graveolens, Rutaceae	Whole	Hexane	34.00	61.0	[19]
Salix mucronata, Salicaceae	Bark	Ethyl acetate	82.00	–	[24]
Zizyphus jujuba, Rhamnaceae	Fruit	Methanol	2.4	–	[13]

Reproduced with permission from Elsevier Science, from Ref. [27].

13.4.1.3 Cinnamomum cassia (*Lauraceae*)

The bioactive compounds found in this plant are cinnamaldehyde and cinnamic acid, as well as coumarins, diterpenoids, and polyphenols. These compounds are responsible for its pharmacological activity [32]. The antioxidant property of this plant can be attributed to its cholinergic activity in vitro [33].

13.4.1.4 Illicium verum (*Illiciaceae*)

Bioactivity-guided fraction of *I. verum* fruit extract showed potent AChE/BChE inhibitory activity. The standardized extract of the plant has shown more affinity toward AChE (IC_{50}: 58.67 ± 0.16 μg/mL) than BChE (IC_{50}: 91.84 ± 1.29 μg/mL). Anethole, an important phytoconstituent found in this extract, has been reported for potent AChE/BChE inhibitory activity as compared to standard galanthamine [34].

13.4.1.5 Mentha spicata (*Lamiaceae*)

Decoction of *M. spicata* L leaves are used as folk medicine for the treatment of various neurological disorders [35]. Aqueous and methanol extracts of *M. spicata* leaves posses AChE inhibitory activity [36]. In another study, infusion of *M. spicata* leaves was examined for AChE inhibitory activity. Eriocitrin and eriodictyol are the main chemical constituents present in this extract that are responsible for the biological activities [37].

13.4.1.6 Myristica fragrans (*Myristicaceae*)

The n-hexane extract of *M. fragrans* seeds was evaluated for AChE inhibition activity in memory-deficient mice. Trimyristin and myristicin are the components of *M. fragrans* seed that were examined for the AChE inhibition activity by in vivo and in vitro studies [38]. The results of the studies showed that trimyristin and

myristicin significantly reduce the AChE activity in the nervous tissue of *Lymnaea acuminate* while enzyme kinetic study indicates components of *My. fragrans* have the mixed (competitive–noncompetitive) type of inhibition toward AChE [39].

13.4.2 Edible Plants Having Anticholinesterase Activity

During the last 50 years, many wonderful breakthroughs have improved our understanding of the role of food in our lives. As alternative therapy, food plants are of growing interest nowadays to address some major illnesses [40]. Several food plants including fruits and vegetables show potent AChE inhibitory activity that can improve cognition and memory in AD patients [41].A list of some major edible plants having ChE inhibitory activity is discussed below and has been shown in Table 13.2.

13.4.2.1 Centella asiatica *(Apiaceae)*

AChE inhibitory effect of *Ce. asiatica* aqueous extract was investigated in colchicine-induced memory-deficient and oxidative stress suffering rats. Treatment with 150 and 300 mg/kg p.o. of aqueous extract of *Ce. asiatica* for a period of 25 days shows significant AChE inhibitory activity in rats [42]. Asiatic acid, a triterpenoid component of *Ce. asiatica* was found to have inhibitory effect against AChE enzyme [43].

13.4.2.2 Eruca sativa *(Brassicaceae)*

It has been reported that *E. sativa* was found to possess potent antioxidant and anti-ChE activity. However, dichloromethane extract of *E. sativa* produces the highest inhibition against BChE [23].

13.4.2.3 Marsilea quadrifolia *(Marsileaceae)*

Ethnopharmacological evidence showed the uses of this plant as a nerve stimulant [44]. AChE inhibitory activity of the methanol extract of the species is observed at IC_{50}: 51.89 ± 0.24 μg/mL and found to have less affinity toward the BChE at IC_{50}: 109.43 ± 2.82 μg/mL. Anti-ChE activity of this species is reported due to the presence of alkaloids, polyphenols, steroids, and saponins [41].

13.4.2.4 Terminalia chebula *(Combretaceae)*

The hydroalcoholic extract of the plant showed potent antioxidant activity thus justifying its use in the management of oxidative stress [45]. *Terminalia chebula* aqueous extract shows significant anxiolytic effect [46] as well as cognition improvement in experimental rats [47].

13.4.3 Medicinal Plants Used in Traditional Medicine Having Anticholinesterase Activity

Traditional practices using medicinal plants are an important part of the primary health care system nowadays. Since ancient times, plants have been used as medicine, foods, agrochemicals, and pharmaceuticals by a large number of people. The ethnobotanical survey can bring out various leads for the drug development process to treat human diseases. A list of plants that have been traditionally used as AChE inhibitors as well as cognitive enhancers is shown below and summarized in Table 13.3.

13.4.3.1 Aloe vera *(Liliaceae)*

The major phytoconstituents found in this plant, sitosterol and stigmasterol, are found to possess potent AChE inhibitory activity in a dose-dependent manner (IC_{50}: 5.26 μg/mL) in vitro [48].

13.4.3.2 Alpinia galanga *(Zingiberaceae)*

Research on this plant exhibits potent cognitive improvement activity that promises its use in the treatment of AD. Alteration of behavioral parameter has been observed after treatment with *Alpinia* fractions, which indicates cognition improvement by enhancing cholinergic transmission [49].

13.4.3.3 Andrographis paniculata *(Acanthaceae)*

Andrographolide is a diterpene lactone obtained from this plant, which is responsible for various pharmacological activities. Psychopharmacological studies on *An. paniculata* extract reveal its effect on the nervous system that gives a significant alteration in behavior pattern [50].

13.4.3.4 Arnica montana *(Asteraceae)*

It contains sesquiterpene lactones, volatile oils, and thymol derivate, as well as phenolic compounds [51]. Wszelaki et al. (2010) described that a methanolic extract of the flower part of this plant showed prominent AChE and BChE inhibitory activity [52].

13.4.3.5 Bupleurum falcatum *(Umbelliferae)*

A strong AChE inhibitory activity of *Bu. falcatum* in an in vitro model was reported by Oh et al. (2004) [53]. *Bupleurum* root contains a series of triterpene saponins and sapogenins, which have been isolated with other minor compounds. The methanolic extract of its root shows significant inhibition that could play an important role in the treatment of AD [54].

13.4.3.6 Dioscorea bulbifera (*Dioscoreaceae*)

In 2013, Neha and her coworkers evaluated the AChE inhibition and antioxidant activity of an aqueous ethanol extract (50:50) of tubers of *D. bulbifera* in mice fed a high-fat diet and with streptozotocin-induced diabetes. A dose of 250, 500, or 1000 mg/kg for 90 days lowers the AChE activity in the brain of the mice and the oxidative stress also subsided [55].

13.4.3.7 Fumaria asepala (*Fumariaceae*)

The chloroform:methanol (1:1) extracts of various *Fumaria* species were investigated for their anti-ChE activity, which exhibit potent AChE (IC_{50}: 9.76 ± 0.89 μg/mL) and BChE (IC_{50}: 91.99 ± 0.70 μg/mL) inhibition [56]. Ten *Fumaria* species including *F. asepala* were screened for AChE/BChE inhibitory activity by spectrophotometric method, which confirms its pharmacological relevance in an acceptable range [57].

13.4.3.8 Hypericum perforatum (*Hypericaceae*)

Hypericum perforatum locally known as St. John's wort is one of the effective herbal medicine used in a number of neurological disorders including anxiety, neuralgia, and depression. Ethyl acetate, methanol, and water extracts of *Hy. perforatum* showed the maximum AChE inhibitory activity. The promising AChE inhibition activity of *Hy. perforatum* could be due to the content of flavonoid compounds like hypericin, hyperforin, and total flavonoids [58].

13.4.3.9 Lavandula angustifolia (*Lamiaceae*)

The extract of the *L. angustifolia* flowers were used as folk medicine for the treatment of nerve related disorders. The higher percentage of AChE inhibition activity was found in lavender oil at a dose label of 1 mg/mL (IC_{50}: 39.5 ± 8.6 μg/mL) [19]. It is also reported that linalool, a phytochemical component of the extract may be involved in AChE inhibitory activity [30].

13.4.3.10 Nardostachys jatamansi (*Valerianaceae*)

The plant extract inhibits oxidative stress by changing neurotransmitter level in different parts of brain in cold restraint stress model, which confirms the potential of *Na. jatamansi* as an effective anti-stress agent. Mukherjee et al. (2007) reported the AChE inhibitory potential of *Na. jatamansi* in an in vitro model based on Ellman's method [59]. The rhizome extract was also found to have potent anti-amnesic effect when given to sleep-deprived amnesic mice [60].

13.4.3.11 Nelumbo nucifera (*Nymphaceae*)

Flavonoids from *Ne. nucifera* have shown protective effects against exhaustive swimming exercise-induced oxidative stress, thus advocating its use as an anti-stress agent [61,62]. The plant's seedpod provides a feasible therapy in the treatment of AD and other forms of cognitive disorders. *N*-methylasimilobine, nuciferine, and nornuciferine isolated from this plant were found to have potent AChE inhibitory activity [63].

13.4.3.12 Origanum vulgare (*Lamiaceae*)

The strong AChE and BChE inhibitory activities of the essential oil of *O. vulgare* was reported in 2008 by Orhan and his coworkers [13]. Aqueous leaf extracts of this plant improved the learning parameters in rats [64]. It has been found that a novel phenolic glucoside, origanoside, isolated from *O. vulgare* helps in reducing oxidative damage [65] in the experimental model.

13.4.3.13 Pyrola japonica (*Pyrolaceae*)

Methanol extract of *Py. japonica* whole plant at a dose label of 5 mg/mL has been shown to possess AChE (IC_{50}: 37.00 ± 2.00 mg/mL) and BChE (IC_{50}: 36.00 ± 3.00 mg/mL) inhibitory activity [66].

13.4.3.14 Salix mucronata (*Salicaceae*)

Ethyl acetate extract of *S. mucronata* bark shows potential AChE inhibitory activity in vitro [67].

13.4.3.15 Tinospora crispa (*Menispermaceae*)

The major bioactive compound, columbamine, a quaternary alkaloid, isolated from *Ti. crispa* vine extract exerts strong inhibitory effect against AChE. Structure–activity relationship (SAR) analysis of the above compound suggests the quaternary nitrogen group could be involved in the inhibitory effect on AChE [68].

13.4.3.16 Withania somnifera (*Solanaceae*)

Withania somnifera is one of the most important medicinal plants whose aqueous extract improves psychomotor and cognitive performance when compared to placebo [69]. It gives a beneficial effect on cognitive deficit by decreasing oxidative damage caused by streptozotocin in a model of cognitive impairment [70]. It also reduces ibotenic acid-induced cognitive impairment and the loss of cholinergic markers after 2 weeks of treatment.

13.4.3.17 Ziziphus jujuba (*Rhamnaceae*)

The antioxidant activity of *Z. jujuba* fruit was reported, which improves spatial memory loss caused by ethanol. Study showed that the fruit extract contains an antiamnesic constituent that can reduce scopolamine- and β-amyloid peptide-induced amnesia [71]. Jujuboside A, extracted from the seed of *Z. jujube*, causes inhibitory effect on glutamate-mediated excitatory signal pathway in hippocampus [72,73].

Hemidesmus indicus is widely recognized in folk medicine against various diseases. The root of this plant has

been reported for its memory-enhancing potential [74]. Nerve tonic prepared from *Trapa bispinosa* has been used in folk medicine since ages, and it was scientifically supported by a recent study that demonstrated that it improves memory by reducing oxidative stress [11]. *Bacopa monniera Ginkgo biloba* is a well-known nootropic plant evidenced in many ancient literatures and it is one of the major ingredients found in several commercial nootropic products [75]. Standardized extracts of *B. monniera* and *Gingko biloba* both showed a dose-dependent inhibitory effect on AChE activity [25]. *Ocimum sanctum* was reported to improve scopolamine as well as aging-induced memory deficit, which suggested possible cholinergic modulation as the mechanism of its action and thereby indicated possible utility in the management of AD- and age-associated dementias. Vyawahare et al. (2008) reported that the alcoholic extract of the roots of *Clitoria ternatea* potentially repairs scopolamine-induced memory disruption using radial arm maze and condition avoidance response test and thereby validated its traditional claim. Animals treated with either ginseng extract or composite preparations containing ginseng is claimed to improve learning and memory. Aqueous extract of *Coriandrum sativum* has been reported to provide protection of pyramidal cells in cerebral cortex against neurodegenerative disorders and AD. Among plants that have been investigated for dementia therapy, *Salvia* is one of the most numerous genera within the family Lamiaceae; it causes inhibition of AChE as well as nicotinic activity. This type of ChIs is found in abundance in natural products, which encourages the researchers toward lead finding for a cure for AD from natural products. It has been found that plants belonging to the families Acanthaceae, Apocynaceae, Amaryllidaceae, Angelicae, Araceae, Asclepiadaceae, Berberidaceae, Buxaceae, Combretaceae, Compositae, Coniferae, Cyperaceae, Ebenaceae, Ericaceae, Euphorbiaceae, Fumariaceae, Gentianaceae, Guttiferae, Lamiaceae, Leguminosae, Lilliaceae, Lycopodiaceae, Malvaceae, Magnoliaceae, Menispermaceae, Molluginaceae, Moraceae, Musaceae, Nelumbonaceae, Papaveraceae, Piperaceae, Rubiaceae, Rutaceae, Sapotaceae, Solanaceae, and Tamaricaceae have promising AChE inhibitory potential [27,76].

13.5 PHYTOCONSTITUENTS FOR THE MANAGEMENT OF COGNITIVE DISORDER

The search for plant-derived inhibitors of AChE has accelerated in the treatment of AD and other forms of dementia, such as dementia with Lewy bodies, vascular dementia and Down syndrome. The bioactive molecules belonging to the classes of alkaloids, monoterpenes, coumarins, triterpenes, flavonoids, benzenoids, diterpenes, oxygen heterocycles, sesquiterpenes, stilbenes, lignans, sulfur compounds, proteids, polycyclic, quinoid, benzoxazinone, carotenoid, and alycyclic have been identified as AChE inhibitors from plant origin. Among the various classes of alkaloids, indole derivatives (such as physostigmine and related compounds), isoquinoline and related derivatives (such as galantamine and lycorine-type alkaloids), steroidal and terpenoid alkaloids, and many other derivatives possess inhibitory effects on AChE. Plant families that have been considered as potential sources of such alkaloids are Amaryllidaceae, Buxaceae, Apocynaceae, Papaveraceae, Lycopodiaceae, and Leguminosae. This great chemical diversity found in natural sources has been enormously studied to explore various chemical scaffolds for the development of potential new drugs to act on AChE enzymes for combating various cognitive disorders with different pharmacological profiles [77].

The bioactive molecules leading from plant origin are very much promising with high efficacy and lower side effects when compared to the drugs from synthetic origin. From Figure 13.1 it has been clearly understood that alkaloids are the promising group of compounds having potent AChE inhibitory properties. The plant family Lamiaceae promises maximum inhibitory potential followed by Buxaceae, Rutaceae, and others. This finding will help the researchers to screen the potent class of compounds on the basis of their origin and thus come up with some major bioactive pharmacophores with natural biodiversity. As the plant secondary metabolites readily depend on their species and genus so the content of bioactive compounds also depend on the plant taxonomy. Physostigmine, an indole alkaloid, isolated from *Physostigmina venenosum*, has been found to improve cognitive function in several in vivo studies through reversible AChE inhibition [78]. The chemical structure of physostigmine has provided a template for the development of rivastigmine, an AChE inhibitor that is licensed for use in the United Kingdom for the symptomatic treatment of mild-to-moderately severe AD. Galantamine is an amaryllidaceae alkaloid obtained from *Ga. nivalis*, which was used traditionally in Bulgaria and Turkey for neurological conditions through reversible competitive AChE inhibition that allosterically modulates nicotinic receptors [79]. Other Amaryllidaceae alkaloids such as assoanine, epinorgalantamine, oxoassoanine, sanguinine, and 11-hydroxygalantamine have also been reported to possess AChE inhibitory activity [80].The lycopodium alkaloid huperzine A is a natural compound first isolated from *Hu. serrata* (Thumb.) in 1986. It is a potent, reversible, and selective inhibitor of AChE with rapid absorption and penetration into the brain in animals, with a longer duration of action and higher therapeutic

index, and the peripheral cholinergic side effects are minimal at therapeutic doses in comparison to other available marketed ChIs [81]. It also attenuates memory deficits and neuronal damage that occur after ischemia, and therefore is beneficial in the treatment of cerebrovascular-type dementia [82]. The other major classes of compounds reported to have such activity are the terpenoids, glycosides, and coumarins. The hydrophobic active site of AChE is reported to be susceptible to hydrophobic interactions. SAR suggested that the nitrogen substituents at C-3 and/or C-20 of steroidal skeleton and the hydrophobic properties of the pregnane skeleton are the key structural features contributing to the inhibitory potency of pregnane-type steroidal alkaloids against AChE [83]. Terpenoids are relatively weak inhibitors of ChE. However, analogues of active terpenoid-like stilbene oligomer viniferin isolated from *Caragana chamlague* is a potential reversible and noncompetitive inhibitor of AChE [80]. ChIs have been chemically identified in several traditional Chinese medicinal plants, including *Angelica sinensis* and *Evodia rutaecarpa*. Another plant ChI, huperzine, derived from the moss *Huperzia*, traditionally used to treat inflammation and fever, is also being used in AD therapy in China [84]. It is a relatively selective inhibitor of cortical and hippocampal ChE and of AChE compared with BChE. In a placebo-controlled randomized trial, huperzine was found significantly better than placebo in improving memory, cognition, and behavioral function [85]. Systemic administration of *W. somnifera* led to differential inhibition of AChE and enhanced M1 -muscarinic receptor binding in rat brain [86]. Korean Ginseng (*Panax ginseng*) is considered to improve memory through enhancing cholinergic activity in similar animal models and also have neuroprotective activity in vitro [87].

13.5.1 Alkaloids

Alkaloids constitute a group of family of compounds that generally have in common the presence of nitrogen atom(s) in a cyclic ring. This is probably the largest group of metabolites with ChE inhibitory activity at lower concentrations. The first known AChE inhibitor was physostigmine, an alkaloid isolated for the first time in 1864 from *Ph. venenosum* Balf, which was used in therapy before the discovery of ACh as neurotransmitter. However, physostigmine is quite polar, being distributed throughout the body, and only a small amount reaches the central nervous system. Neostigmine, pyridostigmine, rivastigmine, cymserine, berberine, groenlandicine, tubocurarine, and galantamine are natural-derived alkaloids, having moderate AChE inhibitory activity [78,88].

Essential oils from natural sources contain complex mixtures of volatile compounds. Terpenoids and phenylpropanoid chemical class are the major chemical compounds found in essential oils. These chemicals have been investigated for AChE/BChE inhibitory activity and significant inhibitory effect on cognitive-related disorder like AD has been reported. Monoterpene constituents from *M. officinalis* and *R. officinalis* have been reported to inhibit erythrocyte AChE in in vitro studies. Geraniol, 3-carene, α-caryophyllene and limonene are other monoterpenes that are also reported to inhibit AChE. Monoterpenes may be cyclic (e.g., 1,8-cineole and α-pinene) or acyclic (e.g., geraniol and linalool) and consist of a hydrocarbon skeleton, which may be attributed to their anti-ChE activity. Some of the selected secondary metabolites and isolated compounds (Table 13.4) from natural sources have shown promising anti-ChE activity.

13.5.2 Terpenoids

Terpenes, which are built up from isoprene subunits, constitute the most numerous and structurally diverse group of secondary metabolites produced by plants. Several biological activities have been attributed to terpenes among which ChE inhibition predominates. Most of the terpenes inhibit ChE at higher concentrations than alkaloids. The essential oils from species such as *Thymus vulgaris* L. and *Eucalyptus globulus* Labill show ChE inhibitory activity. Monoterpenoids are the main constituents of these essential oils. In the monoterpene subgroup, 1, 8-cineole and α-pinene are the most effective compounds [118]. Arisugacin A, arisugacin B, arisugacin C, arisugacin D, arisugacin E, arisugacin F, arisugacin G, arisugacin H, α-nocerin, and territrem A [119–121] are also found to be potent AChE inhibitors. Other terpenes with potent AChE inhibition activity have been discussed in Table 13.4.

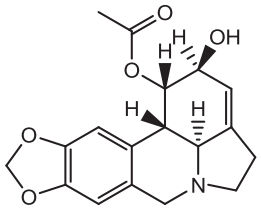
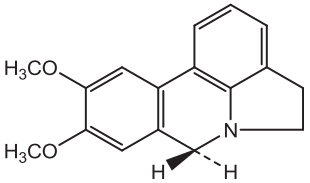
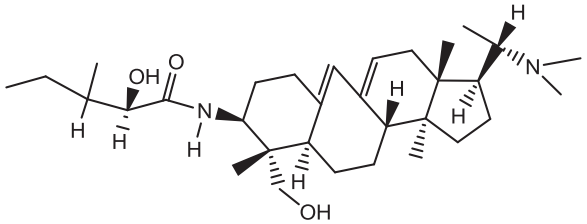
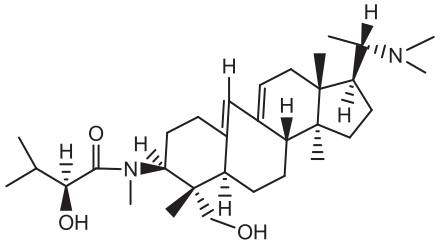
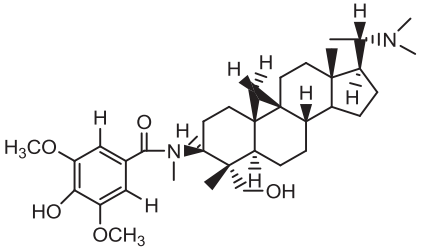
13.5.3 Phenyl Propanoids

Eugenol, *trans*-anethole, α-asarone, and β-asarone are found under this class of compounds. This type of compound is mainly identified in the essential oil of medicinal plants [14]. The important members of this class that show promising AChE inhibitory activity are shown in Table 13.4.

13.5.4 Coumarins

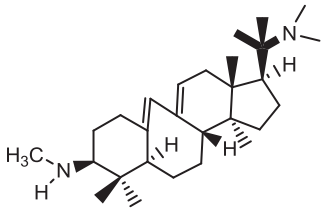
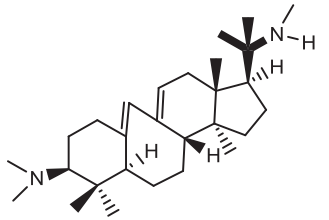
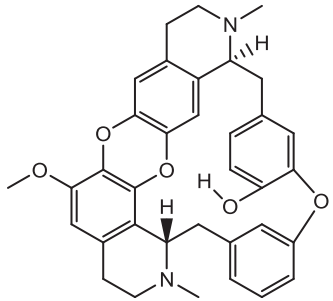
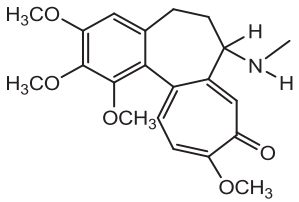
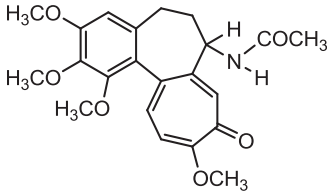
Coumarins are benzo-α-pyrones (a benzene ring joined to a pyrone ring) with important pharmacological properties. Decursin, decursinol, isoimperatorin, marmesin, nodakenin, xanthotoxin, xanthyletin, elagic

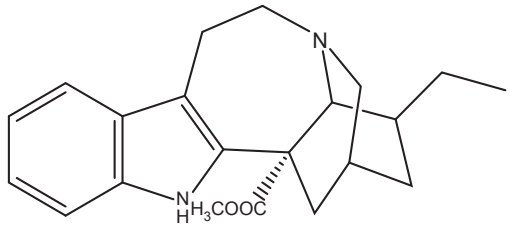
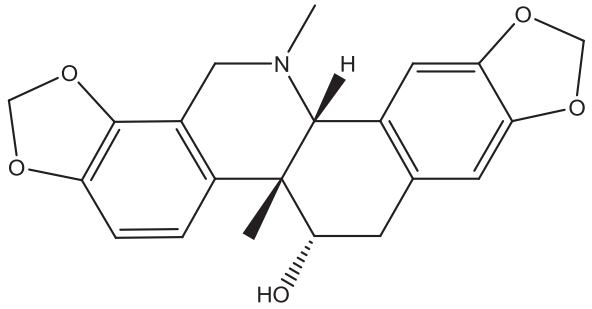
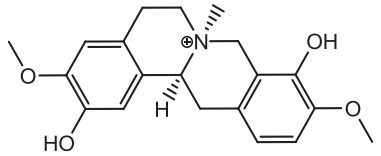
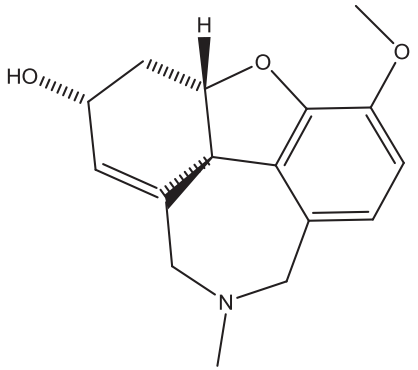
TABLE 13.4 Some Phytoconstituents Having Anticholinesterase Activity

Name of the chemical compound	Chemical structure	Plant name with family	Enzyme inhibition (in % or μg/mL) AChE	BChE	References
I. ALKALOIDS					
1-*O*-Acetyllycorine		*Crinum moorei*, Amaryllidaceae	0.96	–	[89]
Assoanine		*Narcissus assoanus*, *Amaryllidaceae*	3.87	–	[90]
Buxahejramine		*Buxus papillosa*, Buxaceae	162.0	–	[91]
Buxakarachiamine		*Buxus papillosa*, Buxaceae	143.0	–	[91]
Buxakashmiramine		*Buxus papillosa*, Buxaceae	25.4	0.74	[91]

Continued

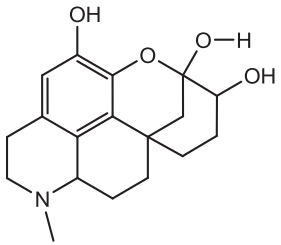
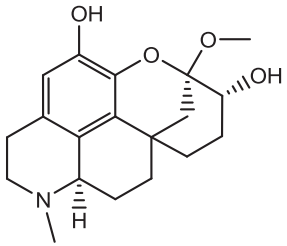
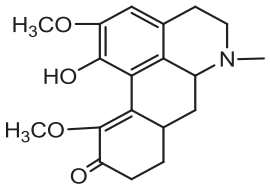
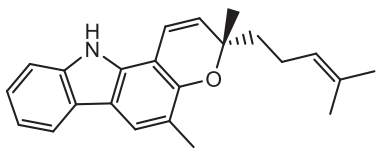
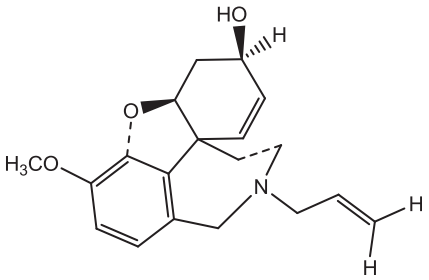
TABLE 13.4 Some Phytoconstituents Having Anticholinesterase Activity—cont'd

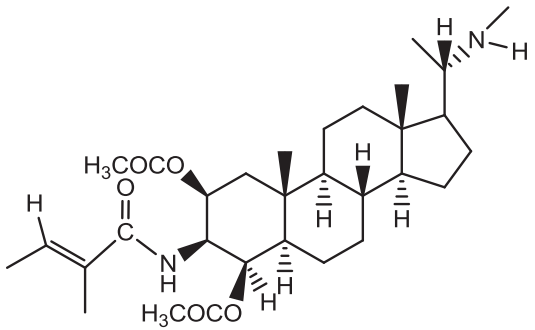
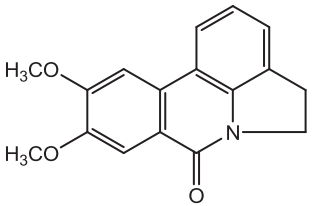
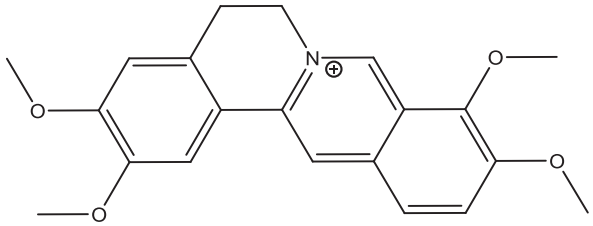
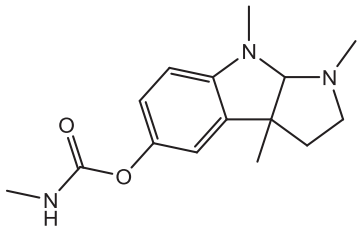
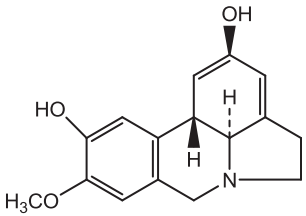
Name of the chemical compound	Chemical structure	Plant name with family	Enzyme inhibition (in % or μg/mL) AChE	BChE	References
Buxamine-B		*Buxus papillosa*, Buxaceae	74.0	–	[83]
Buxamine-C		*Buxus hyrcana*, Buxaceae	7.5	–	[83]
Chonemorphine	Refer the structure A	*Sarcococca hookeriana*, Buxaceae	28.0	0.5	[92]
Cocsuline		*Cocculus pendulus*, Menispermaceae	47.6	6.1	[93]
Colchamine		*Colchicum speciosum*, Colchicaceae	3.89	2.20	[92]
Colchicine		*Colchicum speciosum*, Colchicaceae	2.81	3.19	[92]

Coronaridine		*Tabernaemontana australis*, *Apocynaceae*	0.01	–	[94]
Corynoline		*Corydalis incisa*, Papaveraceae	30.6		[95]
Cyclanoline		*Stephania venosa*, Menispermaceae	9.23	–	[96]
Cyclomicrophylline-A	Refer the structure B	*Buxus papillosa*, Buxaceae	235.0	2.43	[91]
Cycloprotobuxine-C	Refer the structure B	*Buxus papillosa*, Buxaceae	38.8	2.73	[91]
Cyclovirobuxeine-A	Refer the structure B	*Buxus papillosa*, Buxaceae	105.7	2.05	[91]
Epipachysamine-*E*-5-en-4-one)	Refer the structure A	*Sarcococca hookeriana*, Buxaceae	9.9	0.6	[97]
Galanthamine		*Leucojum aestivum*, Amaryllidaceae	1.82	–	[11]
Hookerianamide J	Refer the structure A	*Sarcococca Hookeriana*, Buxaceae	48.5	0.8	[97]

Continued

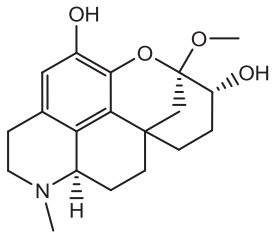
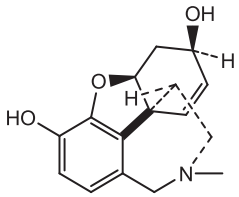
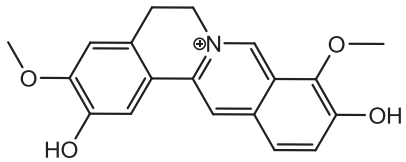
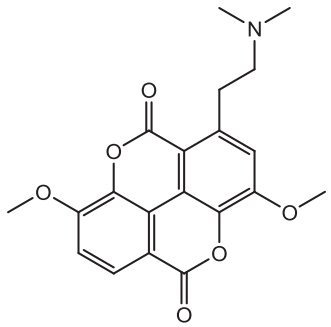
TABLE 13.4 Some Phytoconstituents Having Anticholinesterase Activity—cont'd

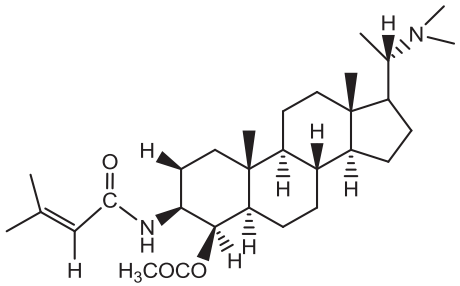
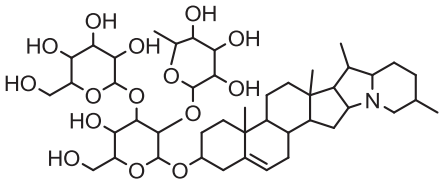
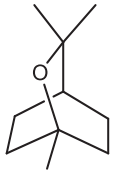
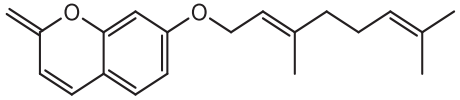
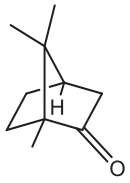
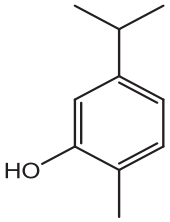
Name of the chemical compound	Chemical structure	Plant name with family	Enzyme inhibition (in % or μg/mL) AChE	BChE	References
Hookerianamide K	Refer the structure A	*Sarcococcahoo keriana*, Buxaceae	24.2	4.0	[97]
Kesselridine		*Colchicum speciosum*, Colchicaceae	3.02	3.38	[92]
Kesselringine		*Colchicum speciosum* Colchicaceae ,	3.47	4.26	[92]
Luteidine		*Colchicum speciosum*, Colchicaceae	4.21	3.85	[92]
Mahanimbine		*Murraya koenigii*, Rutaceae	0.03	–	[98]
N-allylnorgalanthamine		*Leucojum aestivum*, Amaryllidaceae	0.18	–	[11]

Nepapakist amine A		*Sarcococca coriacea*, Buxaceae	50.1	25.0	[99]
N-methylpachys amine A	Ref the structure A	*Sarcococcahoo hookeriana*, Buxaceae	22.1	1.6	[97]
Oxoassoanine		*Narcissus assoanus*, *Amaryllidaceae*	47.21	–	[90]
Palmitine		*Corydalis speciosa*, Papaveraceae	16.1	–	[100]
Physostigmine		*Physostigma venenosum*, Leguminosae	0.006	–	[101]
Pseudolycorine		*Narcissus assoanus*, Amaryllidaceae	152.3	–	[90]

Continued

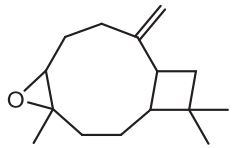
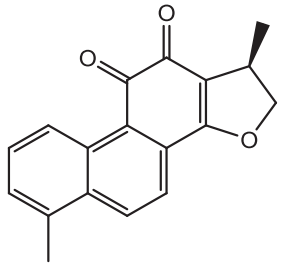
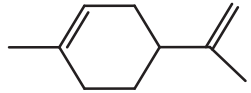
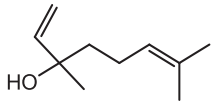
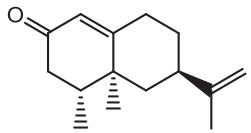
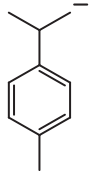
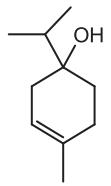
TABLE 13.4 Some Phytoconstituents Having Anticholinesterase Activity—cont'd

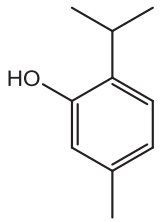
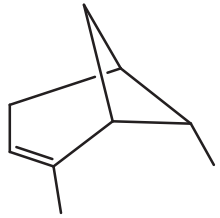
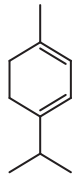
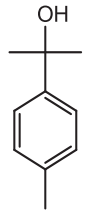
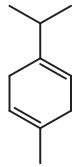
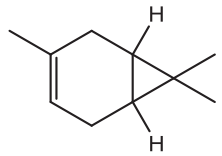
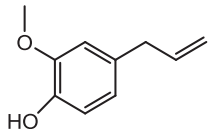
Name of the chemical compound	Chemical structure	Plant name with family	Enzyme inhibition (in % or μg/mL) AChE	BChE	References
Regeline		*Colchicum speciosum*, Colchicaceae	2.78	4.94	[92]
Sanguinine		*Eucharis grandiflora*, Amaryllidaceae	0.10	–	[90]
Sarcovagine C	Refer the structure A	*Sarcococca hookeriana*, Buxaceae	8.0	0.3	[102]
Stepharanine		*Stephania venosa*, Menispermaceae	14.1	–	[96]
Taspine		*Magnoliax soulangiana*, Magnoliaceae	0.33		[103]

Vaganine D		*Sarcococca coriacea*, Buxaceae	46.8	10.0	[99]
α-Solanine		*Solanum tuberosum*, Solanaceae	44.3	–	[104]
II. TERPENOIDS					
1,8-Cineole		*Melaleuca alternifolia*, Myrtaceae	49.0	–	[105]
Auraptene		*Citrus paradise*, Rutaceae	>17	–	[106]
Borneol		*Thymus vulgaris*, Lamiaceae	0.13	–	[107]
Camphor		*Salvia lavandulaefolia*, Lamiaceae	0.03	–	[3]
Carvacrol		*Th. vulgaris*, Lamiaceae	0.09	–	[107]

Continued

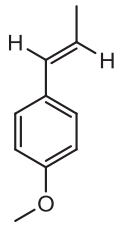
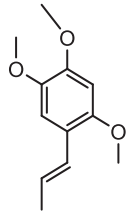
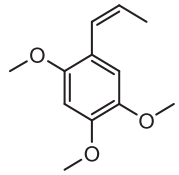
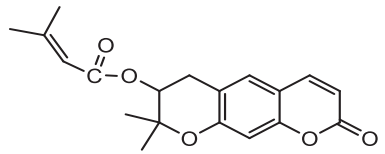
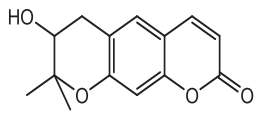
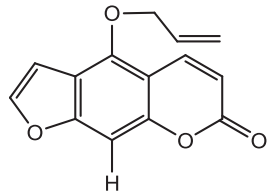
TABLE 13.4 Some Phytoconstituents Having Anticholinesterase Activity—cont'd

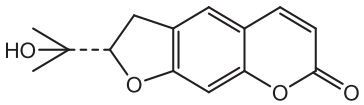
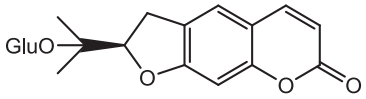
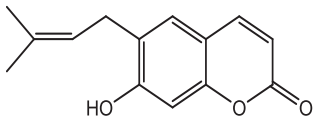
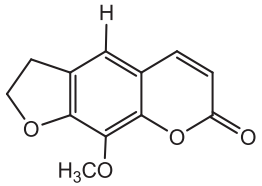
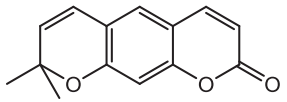
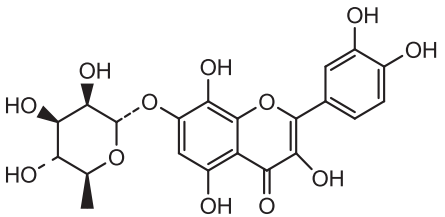
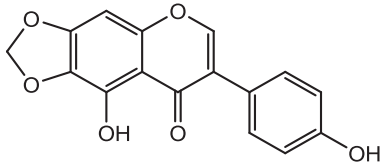
Name of the chemical compound	Chemical structure	Plant name with family	Enzyme inhibition (in % or μg/mL) AChE	BChE	References
Caryophyllene oxide		*Thymus lotocephalus*, Lamiaceae	4.32	16.57	[21]
Dihydrotanshinone		*Salvia miltiorhiza*, Lamiaceae	1.0	–	[108]
Limonene		*Citrus limon*, Rutaceae	0.58	–	[107]
Linalool		*Th. vulgaris*, Lamiaceae	2.21	5.78	[21]
Nootkatone		*Citrus paradise*, Rutaceae	>24	–	[105]
p-Cymene		*Melaleuca alternifolia*, Myrtaceae	38.3	–	[105]
Terpinen-4-ol		*Melaleuca alternifolia*, Myrtaceae	32.0	–	[105]

Thymol		*Th. vulgaris*, Lamiaceae	0.21	– [107]
α-pinene		*Salvia lavandulaefolia*, Lamiaceae	0.63	– [3]
α-Terpinene		*Melaleuca alternifolia*, Myrtaceae	31.7	– [105]
α-terpineol		*Inula graveolens*, Asteraceae	1.3	– [14]
γ-Terpinene		*Melaleuca alternifolia*, Myrtaceae	34.0	– [105]
δ-3-Carene		*Cupressus sempervirens*, Cupressaceae	0.03	– [107]
III. PHENYLPROPANOIDS				
Eugenol		*Ocimum sanctum*, Lamiaceae	0.48	– [14]

Continued

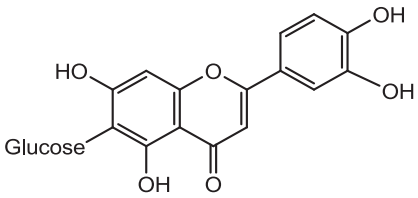
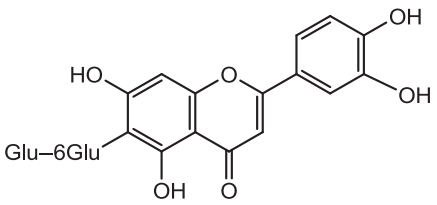
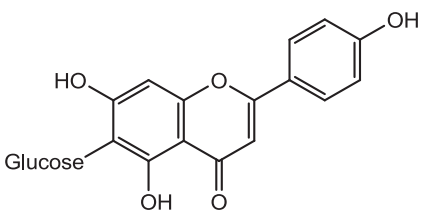
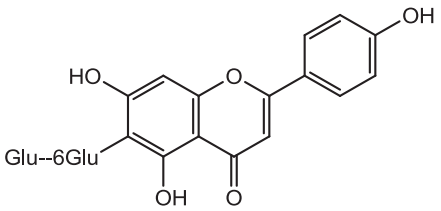
TABLE 13.4 Some Phytoconstituents Having Anticholinesterase Activity—cont'd

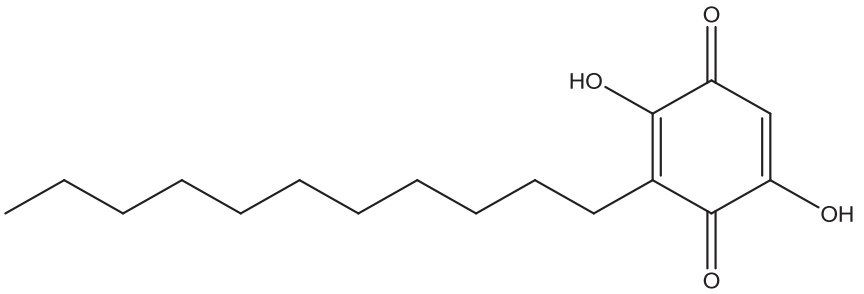
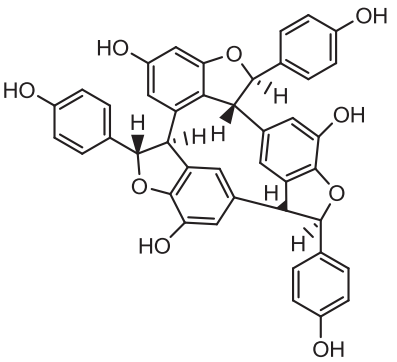
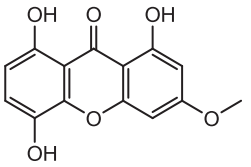
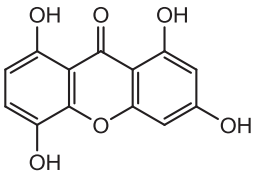
Name of the chemical compound	Chemical structure	Plant name with family	Enzyme inhibition (in % or μg/mL) AChE	BChE	References
trans-Anethole		*Foeniculum vulgare*, Apiaceae	1.32	–	[107]
α-asarone		*Acorus calamus*, Araceae	46.38	–	[109]
β-asarone		*Ac. calamus*, Araceae	3.33	–	[109]
IV. COUMARINS					
Decursin		*Angelica gigas*, Umbelliferae	3.9	–	[110]
Decursinol		*Angelica gigas*, Umbelliferae	2.8	–	[110]
Isoimperatorin		*Angelica gigas*, Umbelliferae	6.9	–	[110]

Marmesin		*Angelica gigas*, Umbelliferae	6.7	– [110]
Nodakenin		*Angelica gigas*, Umbelliferae	6.8	– [110]
Umbelliferone		*Angelica gigas*, Umbelliferae	2.9	– [110]
Xanthotoxin		*Angelica gigas*, Umbelliferae	5.4	– [110]
Xanthyletin		*Angelica gigas*, Umbelliferae	1.5	– [110]
V. FLAVONOIDS				
Gossypetin-7-*O*-L-rhamnopyranoside		*Rhodiola rosea*, Crassulaceae	58.0	– [111]
Irilone		*Iris pseudopumila*, Iridaceae	93.6	>100 [112]

Continued

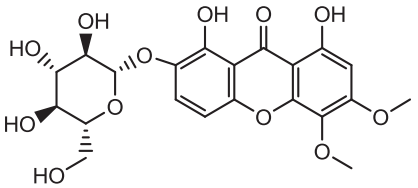
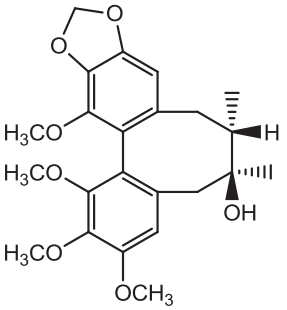
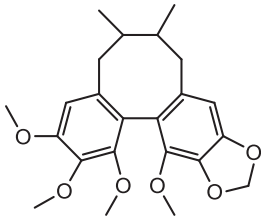
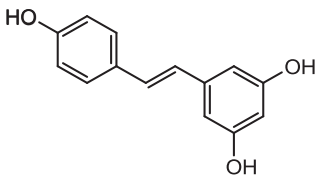
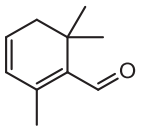
TABLE 13.4 Some Phytoconstituents Having Anticholinesterase Activity—cont'd

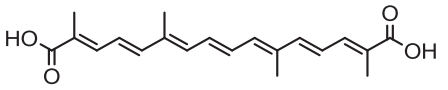
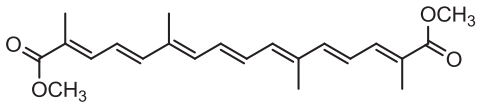
Name of the chemical compound	Chemical structure	Plant name with family	Enzyme inhibition (in % or μg/mL) AChE	BChE	References
Isoorientin		*Iris pseudopumila*, Iridaceae	26.8	31.5	[112]
Isoorientin-6-*O*″-β-D-glucopyranoside		*Iris pseudopumila*, Iridaceae	60.8	98.9	[112]
Isovitexin		*Iris pseudopumila*, Iridaceae	36.4	54.8	[112]
Isovitexin-6-*O*″-β-D-glucopyranoside		*Iris pseudopumila*, Iridaceae	85.9	>100	[112]

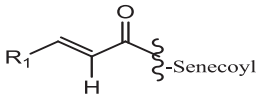
VI. QUINONES					
Embelin		*Embelia ribes*, Myrsinaceae	13.4	–	[113]
VII. STILBENES					
(+)-α-viniferin		*Caragana chamlague*, Leguminosae	96.4	16.9	[80]
VIII. GLYCOSIDES					
Acteoside	Refer the structure C	*Harpagophytum procumbens*, Pedaliaceae	19.9	35.0	[114]
Decaffeoyl verbascoside	Refer the structure C	*Harpagophytum procumbens*, Pedaliaceae	16.1	46.0	[114]
Isoacteoside	Refer the structure C	*Harpagophytum procumbens*, Pedaliaceae	21.9	29.7	[114]
IX. XANTHONES					
Bellidifolin		*Gentianella amarelle*, Gentianaceae	21.9	–	[115]
Bellidin		*Gentianella amarelle*, Gentianaceae	17.5	–	[115]

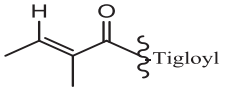
Continued

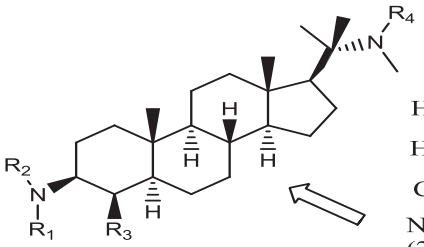
TABLE 13.4 Some Phytoconstituents Having Anticholinesterase Activity—cont'd

Name of the chemical compound	Chemical structure	Plant name with family	Enzyme inhibition (in % or μg/mL) AChE	BChE	References
Triptexanthoside C		*Gentianella amarelle*, Gentianaceae	43.7	–	[115]
X. LIGNANS					
Gomisin A		*Schizandra chinensis*, Schisandraceae	13.28	–	[116]
Schisandrol B		*Schizandra chinensis*, Schisandraceae	12.57	–	[116]
XI. BENZONOIDS					
Resveratrol		*Vitis vinifera*, Vitaceae	Not significant	Not significant	[13]
XII. CAROTENOID					
Safranal		*C. sativus*, Iridaceae	21.09	–	[117]

Crocetin		*C. sativus*, Iridaceae	96.33	– [117]
Dimethylcrocetin		*C. sativus*, Iridaceae	107.1	– [117]

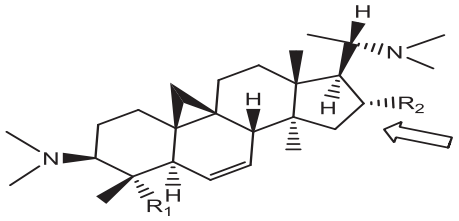






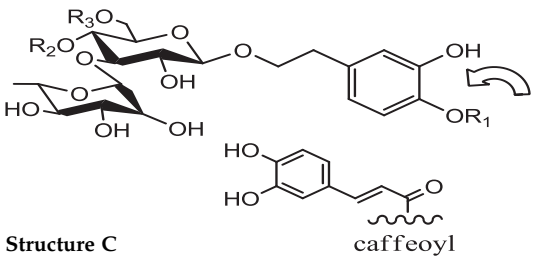
Structure A

Comp Name	Subsitiution of the structure R_1	R_2	R_3	R_4	Unstaturation
Hookerianamide J	H	Senecoyl	OH	Me	$C_{16\text{-}17}$
Hookerianamide K	Me	Me	H	Me	$C_{16\text{-}17}$ & $C_{4\text{-}5}$
Chonemorphine	H	H	H	Me	-
N-methy pachysamine A (26)	Me	Me	H	Me	-
Epipachysamine-*E*-5-en-4-one	H	Senecoyl	O	H	$C_{5\text{-}6}$
Sarcovagine C	H	Tigloyl	OAc	H	-



Structure B

Comp Name	Subsitiution of the structure R_1	R_2
Cycloprotobuxine-C	CH_3	H
Cyclovirobuxeine-A	CH_3	OH
Cyclomicrophylline-A	CH_2OH	OH



Structure C

Comp Name	Subsitiution of the structure R_1	R_2	R_3
Acteoside	H	caffeoyl	H
Isoacteoside	H	H	caffeoyl
Decaffeoyl verbascoside	H	H	H

Part of this table is reproduced with permission from Elsevier Science, from Ref. [27].

acid, and 3, 4-dimethyl herniarin [122] are some of the newer molecules under this class of compounds. Certain functionalized coumarin derivatives are considered as AChE inhibitors and also being proposed for the treatment of AD [123]. Studies showed that the benzylamine substructure was found in potent AChE inhibitory activity [124]. On the basis of these considerations, several coumarins and their derivatives were investigated as ChE inhibitors.

13.5.5 Flavonoids

Flavonoids constitute a class of polyphenols characterized by a diphenylpropane (C6-C3-C6) skeleton, which consists of two aromatic rings, each bearing at least one aromatic hydroxyl, connected by a carbon bridge, forming (or not) a third ring. Flavonoids are divided into subclasses, viz., flavanols, flavanones, anthocyanidins, flavones, flavonols, isoflavones, flavan-3-ols, flavanonols, aurones, and chalcones based on the connection of the two aromatic rings, degree of oxidation, and also the functional groups of the third ring. The majority of flavonoids naturally occur as glycosides or other conjugates, which explains the great variety of compounds, and is considered to be the most relevant class of phenolic compounds [125]. A series of chalcones were synthesized and investigated for their biological activity against AChE and BChE enzyme where the activity against AChE predominates [26].

13.5.6 Quinones

The quinonoid group seems to be important for AChE inhibition, since dopamine autoxidation can inactivate AChE, mainly by direct interaction of quinone or semiquinone oxidation products with the enzyme. Sargaquinoic acid is a natural benzoquinone extracted from the brown algae *Sargassum sagamianum* Yendo and exhibits AChE inhibitory activity [126]. *Thespesia populnea* (L.) Sol. ex Correa is a plant containing quinine group of compounds that have been reported to enhance memory and reduce brain ChE activity in mice [127]. Embelin is another major compound found in this class that potentially inhibits AChE [113].

13.5.7 Stilbenes

Stilbenes are a small family of secondary metabolites derived from the phenylpropanoid pathway. (+)-α-Viniferin (a trimer of resveratrol) is major stilbene compound that shows AChE inhibition in a dose-dependent manner [80].

13.5.8 Xanthones

Some xanthones like bellidin and bellidifolin were also reported to have potent AChE inhibition activity. Triptexanthoside C was the only xanthone isolated from *Gentianella amarella* (L.) with AChE inhibitory activity [115]. Xanthones isolated from *Gentiana campestris* (Gentianaceae) also promises AChE inhibitory activity [128]. Bruhlmann and coworkers have found some xanthones belonging to the families Gentianaceae, Clusiaceae, and Polygalaceae having AChE inhibitory activity [129].

13.5.9 Lignans

Several lignans have been isolated from *Vitex negundo* (Verbenaceae), showing AChE inhibitory activity [130]. Syringaresinol isolated from the aerial part of *Leptadenia arborea* (Asclepiadaceae) demonstrated inhibitory effect against AChE. Ligningomisin A, a component found in the fruits of *Schizandra chinesis* (Magnoliaceae), inhibited AChE activity with an IC_{50} value of 15.5 μM [131].

Gomisin C, gomisin G, gomisin D, and schisandrol B are other compounds isolated from the same species that entirely inhibits AChE in a dose-dependent manner [116].

13.5.10 Glycosides

Pregnaneglycosides were isolated from *Cynanchum atratum* (Asclepiadaceae) and screened for AChE inhibitory activity and it was found to be active due to the presence of cynatroside B and cynascyroside D [132].

13.5.11 Benzonoids

Plant-derived benzonoids such as curcumin, resveratrol, and green tea catechins have potential health-promoting properties as well as protective cellular effects [133]. It has been reported that curcumin (diferuloyl methane), resveratrol (*trans*-3, 5, 4′-trihydroxystilbene), and epigallocatechin-3-gallate exhibit antioxidant activity as well as AChE inhibition activity [103,134].

13.5.12 Quinoids

Embelin is a *p*-quinone class of compound that is found in fruits and seeds of *Embelia ribes*, reported to have AChE inhibitory activity [113].

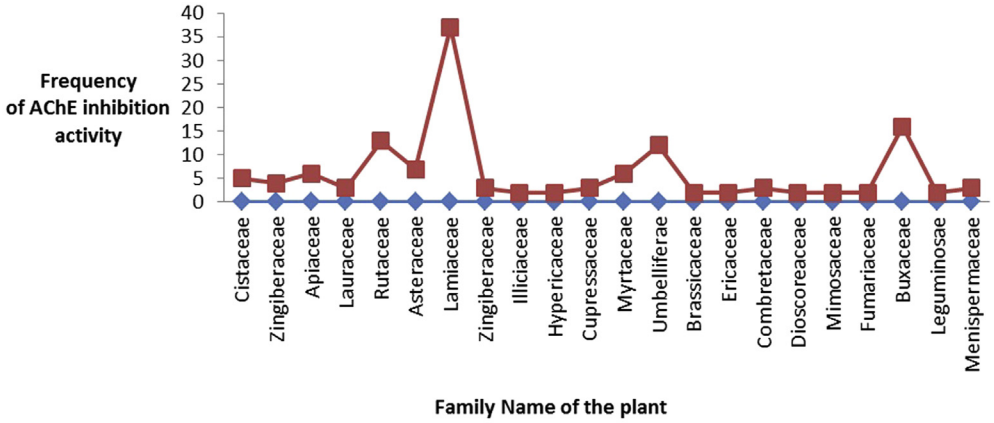


FIGURE 13.2 Different classes of phytoconstituents and their affinity toward cholinesterase inhibition. AChE, acetylcholinesterase.

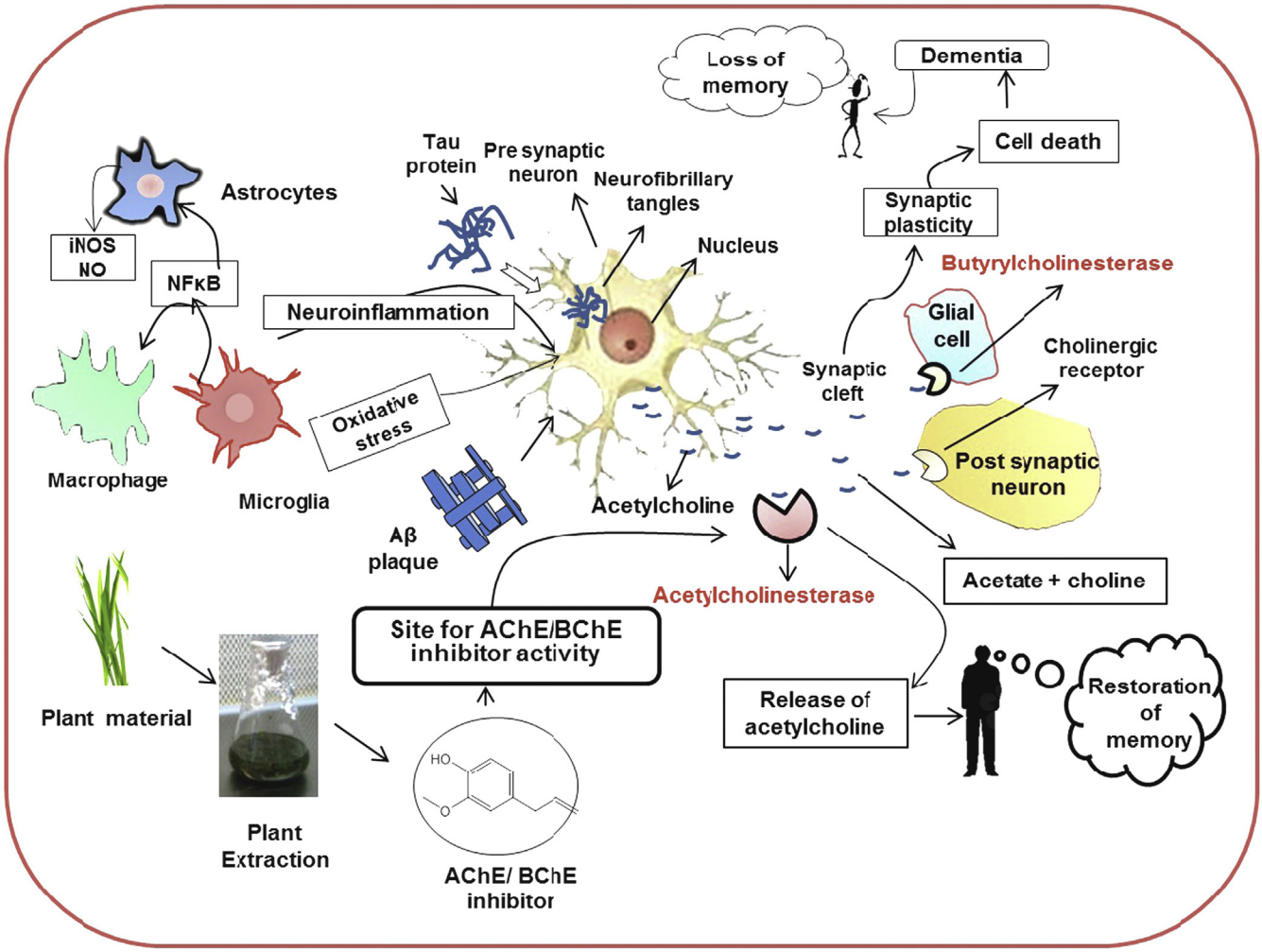


FIGURE 13.3 Different classes of plant family having potent cholinesterase inhibition. AChE, acetylcholinesterase; BChE, butyrylcholinesterase.

13.5.13 Benzoxazinone

2, 4-Dihydroxy-7-methoxy-1, 4-benzoxazin-3-one is a compound found in wheat extract that has been reported for its AChE inhibitory activity. This compound has shown potent inhibitory activity against AChE enzyme that is released from insects like *Epkestia kuehniella* (Zeller), *Musca domestica* L., *Sitophilus granarius* L., and *Rhopalosiphum padi* (L.) [89].

13.5.14 Carotenoids

Crocetin, dimethylcrocetin, and safranal are the main carotenoid constituents found in *C. sativus*. These compounds possess AChE inhibitory activity. In vitro study showed that safranal from *C. sativus* exhibits highest AChE inhibitory activity when compared to crocetin and dimethylcrocetin. Enzyme kinetics study of AChE exhibits mixed type of inhibition (competitive–noncompetitive) of carotenoid compounds. In silico study reveals that crocetin and and dimethylcrocetin simultaneously bind to the catalytic and peripheral anionic sites of AChE, while safranal fits with the catalytic site [117].

Inhibition of AChE, the enzyme principally involved in the catabolism of ACh, results in increased levels of ACh in the central nervous system, thus reversing the deficiency associated with AD. Bioassay-guided isolation of phytochemicals from medicinal plants of India and other parts of the globe have led to the discovery of several AChE inhibitors (Figure 13.2). They include furanocoumarins; isoimperatorin; imperatorin; oxypeucedanin (from *Angelica dahurica*); corynoxidine; protopine; palmatine; berberine (from *Corydalis speciosa*); buxamine B; *N*, *N*-dimethylbuxapapine; sarsalignone; vaganine; coronaridine; voacangine; voacangine hydroxyindolenine; rupicoline (from *Tabernaemontana australis*); and ursolic acid (from *Origanum majorana*) [95]. Some evidence exists for the relevant bioactivity that supports substantial scientific literature on relevant properties of the gingkolides, the chemical constituents of *Gi. biloba* considered to be responsible for the pharmacological activity of the plant.

In Figure 13.3, comparative data of different plant families have been presented where it can be seen that Lamiaceae (Labiatae) is one of the most diverse and widespread plant families in terms of AChE inhibition. This is one of the largest families among the dicotyledons. The plants belonging to the family secrete some major metabolites (mainly volatile oil) through their glandular trichomes present in leaves and flowers. The various medicinal properties of this family are mainly due to its essential oil content [135]. This study will further direct the researchers to explore newer plants having anti-ChE property. It can be concluded that medicinal plants from various families are the richest source of novel anti-ChIs, and there are several other plants remaining to be explored for safe and efficacious anti-ChE drugs for combating AD.

13.6 CONCLUSION

The discovery of AChE inhibitors has led to a new direction to AD therapy. It is associated with the deficiency of ACh in the brain, leading to cognitive impairment in old-aged patients. As the AChE inhibitors play a pivotal role in improving cognitive function, it remains a major research area in AD therapy for the past decades. Traditional medicine-inspired drug development could give some newer approach to the researchers to find out effective bioactive compounds targeted to treat AD [75]. Bioassay-guided isolation from botanicals is able to deliver a large number of chemical entities as AChE inhibitor. Among all the phytomolecules, alkaloids are ound to possess maximum inhibitory potential against AChE. Since most AChE inhibitors are known to contain nitrogen, the higher activity of this extract may be due to their rich alkaloid content. The content of various bioactive compounds present in the plant depends on its genera and species. Buxaceae, Amaryllidacea, Lycopodiaceae are the major families found to possess alkaloid and related compounds. Though the strongest AChE inhibitory activity is seen with alkaloid compounds, a large number of nonnitrogenous compounds like terpenoids, glycosides, and coumarins from plant sources have been discovered as AChE inhibitors [136]. It could be concluded that medicinal plants are a rich source of a newer chemical class of compounds for the management of cognitive disorders. Moreover, in vivo activity needs to be shown in animal and human models to confirm their therapeutic efficacy in case of AD and other related diseases. It is hoped that strong ethnobotanical evidences along with high-throughput screening will develop some novel compounds in drug development process, thus offering a newer discovery in the treatment of AD and other age-associated neurodegenerative diseases.

Acknowledgments

The authors express their gratitude to the Department of Science and Technology, Government of India, New Delhi, for providing financial assistance through project grant.

References

[1] Mukherjee PK, Houghton PJ. The worldwide phenomenon of increased use of herbal products: opportunities and threats. In: Mukherjee PK, Houghton PJ, editors. Evaluation of herbal medicinal products – perspectives of quality, safety and efficacy. London: Pharmaceutical Press; 2009. p. 3–12.

[2] Mukherjee PK. Evaluation of Indian traditional medicine. Drug Inf J 2001;35:620–3.

[3] Perry NSL, Houghton PJ, Theobald A, Jenner P, Perry EK. In-vitro inhibition of human erythrocyte acetylcholinesterase by *Salvia lavandulaefolia* essential oil and constituent terpenes. J Pharm Pharmacol 2000;52:895–902.
[4] Mukherjee PK, Satheeshkumar N, Venkatesh P, Venkatesh M. Lead finding for acetyl cholinesterase inhibitors from natural origin: structure activity relationship and scope. Mini Rev Med Chem 2011;11:247–62.
[5] Loewi O. About humoral transferability of the cardiac nerves effect. Eur J Physiol 1921;189:239–42.
[6] Francis PT, Palmer AM, Snape M, Wilcockc GK. The cholinergic hypothesis of Alzheimer's disease: a review of progress. J Neurol Neurosurg Psychiatry 1999;66:137–47.
[7] Greig NH, Utsuki T, Ingram DK, Wang Y, Pepeu G, Scali C, et al. Selective butyrylcholinesterase inhibition elevates brain acetylcholine, augments learning and lowers Alzheimer beta-amyloid peptide in rodent. Proc Natl Acad Sci USA 2005;102: 17213–8.
[8] Herholz KG. Acetylcholine esterase activity in mild cognitive impairment and Alzheimer's disease. Eur J Nucl Med Mol Imaging 2008;35:S25–9.
[9] Lacor PN. Aβ oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. J Neurosci 2007;27:796–807.
[10] Mesulam MM, Mash D, Hersh L, Bothwell M, Geula C. Cholinergic innervation of the human striatum, globus pallidus, subthalamic nucleus, substantia nigra, and red nucleus. J Comp Neurol 1992;323:252–68.
[11] Nordberg A, Svensson AL. Cholinesterase inhibitors in the treatment of Alzheimer's disease: a comparison of tolerability and pharmacology. Drug Saf 1998;19:465–80.
[12] Chaiyana W, Okonogi S. Inhibition of cholinesterase by essential oil from food plant. Phytomedicine 2012;19:836–9.
[13] Orhan I, Kartal M, Kan Y, Sener B. Activity of essential oils and individual components against acetyl- and butyrylcholinesterase. Z Naturforsch C 2008;63:547–53.
[14] Dohi S, Terasaki M, Makino M. Acetylcholinesterase inhibitory activity and chemical composition of commercial essential oils. J Agric Food Chem 2009;57:4313–8.
[15] Loizzo MR, Ben Jemia M, Senatore F, Bruno M, Menichini F, Tundis R. Chemistry and functional properties in prevention of neurodegenerative disorders of five *Cistus* species essential oils. Food Chem Toxicol 2013;59:586–94.
[16] Tundis R, Loizzo MR, Bonesi M, Menichini F, Mastellone V, Colica C, et al. Comparative study on the antioxidant capacity and cholinesterase inhibitory activity of *Citrus aurantifolia* Swingle, *C. aurantium* L., and *C. bergamia* Risso and Poit. Peel essential oils. J Food Sci 2012;77:H40–6.
[17] Menichini F, Loizzo MR, Bonesi M, Conforti F, De Luca D, Statti GA, et al. Phytochemical profile, antioxidant, anti-inflammatory and hypoglycemic potential of hydroalcoholic extracts from *Citrus medica* L. cv diamante flowers, leaves and fruits at two maturity stages. Food Chem Toxicol 2011;49: 1549–55.
[18] Loizzo MR, Bonesi M, Di Lecce G, Boselli E, Tundis R, Pugliese A, et al. Phenolics, aroma profile, and in vitro antioxidant activity of Italian dessert passito wine from Saracena (Italy). J Food Sci 2013;78:703–8.
[19] Ferreira A, Proenca C, Serralheiro ML, Araujo ME. The in vitro screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J Ethnopharmacol 2006; 108:31–7.
[20] Silva FVM, Martins A, Salta J, Neng NR, Nogueira JMF, Mira D, et al. Phytochemical profile and anticholinesterase and antimicrobial activities of supercritical versus conventional extracts of *Satureja montana*. J Agric Food Chem 2009;57:11557–63.
[21] Costa P, Goncalves S, Grosso C, Andrade PB, Valentao P, Bernardo-Gil MG, et al. Chemical profiling and biological screening of *Thymus lotocephalus* extracts obtained by supercritical fluid extraction and hydrodistillation. Ind Crops Prod 2012; 36:246–56.
[22] Orhan I, Senol FS, Gülpinar AR, Kartal M, Sekeroglu N, Deveci M, et al. Acetylcholinesterase inhibitory and antioxidant properties of *Cyclotrichium niveum*, *Thymus praecox* subsp. *caucasicus var. caucasicus*, *Echinacea purpurea* and *E. pallida*. Food Chem Toxicol 2009;47:1304–10.
[23] Boga M, Hacıbekiroğlu I, Kolak U. Antioxidant and anticholinesterase activities of eleven edible plants. Pharm Biol 2011;49: 290–5.
[24] Eldeen IMS, Elgorashi EE, Van Staden J. Antibacterial, anti-inflammatory, anti-cholinesterase and mutagenic effects of extracts obtained from some trees used in South African traditional medicine. J Ethnopharmacol 2005;102:457–64.
[25] Szwajgier D, Wydrych M, Wicaaw E, Targoeski Z. Anticholinesterase and antioxidant activities of commercial preparations from *Ginkgo biloba* leaves. Acta Sci Pol Hortorum Cultus 2013; 12:111–25.
[26] Hasan A, Khan KM, Sher M, Maharvi GM, Nawaz SA, Choudhary MI, et al. Synthesis and inhibitory potential towards acetylcholinesterase, butyrylcholinesterase and lipoxygenase of some variably substituted chalcones. Enzyme Inhib Med Chem 2005;20:41–7.
[27] Mukherjee PK, Kumar V, Mal M, Houghton PJ. Acetyl cholinesterase inhibitors from plants. Phytomedicine 2007 (4):289–300.
[28] Vohora SB, Shah SA, Dandiya PC. Central nervous system studies on an ethanol extract of *Acorus calamus* rhizome. J Ethnopharmacol 1990;28:53–62.
[29] Shukla PK, Khanna VK, Ali MM, Maurya RR, Handa SS, Srimal RC. Protective effect of *Acorus calamus* against acrylamide induced neurotoxicity. Phytother Res 2002;16:256–60.
[30] Adsersen A, Gauguin B, Gudiksen L, Jager KA. Screening of plants used in Danish folk medicine to treat memory dysfunction for acetylcholinesterase inhibitory activity. J Ethnopharmacol 2006;104:418–22.
[31] Yeom HJ, Kang JS, Kim GH, Park IK. Insecticidal and acetylcholine esterase inhibition activity of Apiaceae plant essential oils and their constituents against adults of German cockroach (*Blattella germanica*). J Agric Food Chem 2012;60:7194–203.
[32] Altuna ML, Yılmaza BS, Orhan IE, Citoglu GS. Assessment of cholinesterase and tyrosinase inhibitory and antioxidant effects of *Hypericum perforatum* L. (St. John's wort). Ind Crops Prod 2013;43:87–92.
[33] Lin CC, Wu SJ, Chang CH, Ng LT. Antioxidant activity of *Cinnamonum cassia*. Phytother Res 2003;17:726–30.
[34] Bhadra S, Mukherjee PK, Kumar NS, Bandyopadhyay A. Anticholinestesterase activity of standardized extract of *Illicium verum* Hook.f. fruits. Fitoterapia 2011;82:342–6.
[35] Bimakr M, Russly AR, Farah S, Taip AG, Liza Md S, Jinap S, et al. Comparison of different extraction methods for the extraction of major bioactive flavanoid compounds from spearmint (*Mentha spicata* L.) leaves. Food Bioprod Process 2011;89:67–72.
[36] Teixeira B, Marques A, Ramos C, Batista I, Serrano C, Matos O. European pennyroyal (*Mentha pulegium*) from Portugal: chemical composition of essential oil and antioxidant and antimicrobial properties of extracts and essential oil. Ind. Crop Prod 2012;36: 81–7.
[37] Dinis PC, Fale PL, Madeira PJA, Florencio MH, Serralheiro ML. Acetylcholinesterase inhibitory activity after *in vitro* gastrointestinal digestion of infusions of *Mentha* species. Eur J Med Plant 2013;3:381–93.
[38] Dhingra D, Parle M, Kulkarni SK. Comparative brain cholinesterase-inhibiting activity of *Glycyrrhiza glabra*, *Myristica*

fragrans, ascorbic acid, and metrifonate in mice. J Med Food 2006; 9:281–3.
[39] Jaiswal P, Kumar P, Singh VK, Singh DK. Enzyme inhibition by molluscicidal components of *Myristica fragrans* Houtt. in the nervous tissue of snail *Lymnaea acuminata*. Enzyme Res 2010; 2010:1–6.
[40] Hardy G. Nutraceutical and functional foods: introduction and meaning. Nutrition 2000;16:688–97.
[41] Bhadra S, Mukherjee PK, Bandyopadhyay A. Cholinesterase inhibition activity of *Marsilea quadrifolia* Linn. An edible leafy vegetable from West Bengal, India. Nat Prod Res 2012;26:1519–22.
[42] Kumar A, Prakash A, Dogra S. *Centella asiatica* attenuates D-galactose-induced cognitive impairment, oxidative and mitochondrial dysfunction in mice. Int J Alzheimers Dis 2011; 2011:1–9.
[43] Nasir MN, Abdullah J, Habsah M, Ghani RI, Rammes G. Inhibitory effect of asiatic acid on acetylcholinesterase, excitatory post synaptic potential and locomotor activity. Phytomedicine 2012; 19:311–6.
[44] Soni P, Singh L. *Marsilea quadrifolia* Linn. – a valuable culinary and remedial fern in jaduguda, Jharkhand, India. Int J Life Sci Pharm Res 2012;2:99–104.
[45] Mishra V, Agrawal M, Onasanwo SA, Madhur G, Rastogi P, Pandey HP, et al. Anti-secretory and cyto-protective effects of chebulinic acid isolated from the fruits of *Terminalia chebula* on gastric ulcers. Phytomedicine 2013;20:506–11.
[46] Chandrashekar R, Manohar VR, Rao SN. Chronic anxiolytic effect of aqueous extract of *Terminalia chebula* (AETC) in rats. Drug Invent Today 2012;42:697–706.
[47] Phachonpai W, Wattanathorn J, Tong-un T, Thipkaew C, Uabundit N, Thukhammee W, et al. Assessment of neuropharmacological activities of *Terminalia chebula* in rats. Am J Pharm and Toxicol 2012;7:41–8.
[48] Bawankar R, Deepti VC, Singh P, Subhashkumar R, Vivekanandan G, Subramaniam B. Evaluation of bioactive potential of an *Aloe vera* sterol extract. Phytother Res 2013;27: 864–8.
[49] Hanish, Singh JC, Alagarsamy V, Diwan PV, Sathesh, Kumar S, Nisha JC, Narsimha, Reddy Y. Neuroprotective effect of *Alpinia galanga* (L) fractions on Aβ (25–35) induced amnesia in mice. J Ethnopharmacol 2011;138:85–91.
[50] Maiti K, Gantait A, Mukherjee K, Saha BP, Mukherjee PK. Therapeutic potentials of andrographalide from *Andrographis paniculata*, a review. J Nat Rem 2006;6:1–13.
[51] Kiesel W. Sesquiterpene lactones with anti-inflammatory action in medicinal plants. Wiad Ziel 1995;7:24–5.
[52] Wszelaki N, Kuciun A, Kiss AK. Screening of traditional European herbal medicines for acetylcholinesterase and butyrylcholinesterase inhibitory activity. Acta Pharm 2010; 60:119–28.
[53] Oh MH, Houghton PJ, Whang WK, Cho JH. Screening of Korean herbal medicines used to improve cognitive function for anticholinesterase activity. Phytomedicine 2004;11:544–8.
[54] Tang W, Eisenbrand G. Chinese drugs of plant origin. Chemistry, pharmacology, and use in traditional and modern medicine. Berlin, Germany: Springer; 1992. 223–232, 491–498, 1017–1024.
[55] Neha B, Arun K, Preet K, Alka C. Evaluation of therapeutic potential of *Dioscorea bulbifera* tubers on learning and memory impairment in high fat diet (HFD) and IV streptozotocin (Stz) – induced experimental dementia in mice. Global J Res Med 2013;2:808–23.
[56] Orhan I, Sener B, Choudhary MI, Khalid A. Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants. J Ethnopharmacol 2004;91:57–60.
[57] Sener B, Orhan I. Molecular diversity in the bioactive compounds from Turkish plants – evaluation of acetylcholinesterase inhibitory activity of *Fumaria* species. J Chem Soc Pak 2004;26: 313–5.
[58] Bozin B, Kladar N, Grujic N, Anackov G, Samojlik I, Gavaric N, et al. Impact of origin and biological source on chemical composition, anticholinesterase and antioxidant properties of some st. John's wort species (*Hypericum* spp., hypericaceae) from the Central Balkans. Molecules 2013;18: 11733–50.
[59] Lyle N, Chakrabarti S, Sur T, Gomes A, Bhattacharyya D. *Nardostachys jatamansi* protects against cold restraint stress induced central monoaminergic and oxidative changes in rats. Neurochem Res 2012;37:2748–57.
[60] Rahman H, Muralidharan P. *Nardostacys jatamansi* DC protects from the loss of memory and cognition deficits in sleep deprived Alzheimer's disease (AD) mice model. Int J Pharm Sci Rev Res 2010;5:160–7.
[61] Jain R, Rajput S. Development of pharmacognostical parameters and estimation of quercetin using HPTLC in leaves of *Nelumbo nucifera* Gaertn. Pharmacogn J 2012;4:31–7.
[62] Xu HC, Wang MY. Effect of flavonoids from Lotus (*Nelumbo nuficera* Gaertn) leaf on biochemical parameters related to oxidative stress induced by exhaustive swimming exercise of mice. Biomedical Res 2014;25:1–5.
[63] Yang ZD, Zhang X, Du J, Li S, Yao XJ. An aporphine alkaloid from *Nelumbo nucifera* as an acetylcholinesterase inhibitor and the primary investigation for structure-activity correlations. Nat Prod Res 2012;26:387–92.
[64] Sheibani V, Afarinesh M, Hajializadeh Z, Arabnezhad R, Sepehri G, Abbasnejad M, et al. Evaluation of *Origanum vulgare* L. ssp. *viridis* leaves extract effect on discrimination learning and LTP induction in the CA1 region of the rat hippocampus. Iran J Basic Med Sci 2011;14:177–84.
[65] Chou TH, Ding HY, Chan LP, Liang JY, Liang CH. Novel phenolic glucoside, origanoside, protects against oxidative damage and modulates antioxidant enzyme activity. Food Res Int 2011;44: 1496–503.
[66] Kim JS, Shim SH, Xu YN, Kang SS, Son KH, Chang HW, et al. Phenolic glycosides from *Pyrola japonica*. Chem. Pharm Bull 2004;52:714–7.
[67] Adewusi EA, Moodley N, Steenkamp V. Medicinal plants with cholinesterase inhibitory activity: a review. Afr J Biotechnol 2010;9:8257–76.
[68] Pathak SK, Jain DC, Sharma RP. Chemistry and biological activities of the genera *Tinospora*, a review. Int J Pharm Pharm Sci 1995;33:277–87.
[69] Pingali U, Pilli R, Fatima N. Effect of standardized aqueous extract of *Withania somnifera* on tests of cognitive and psychomotor performance in healthy human participants. Pharmacogn Res 2014;6:12–8.
[70] Bhattacharya SK, Kumar A, Ghosal S. Effects of glycowithanolides from *Withania somnifera* on an animal model of Alzheimer's disease and perturbed central cholinergic markers of cognition in rats. Phytother Res 1995;9:110–3.
[71] Shanmugavasan A, Vaitheeswaran KSR, Ramachandran T. Design and development of pyrolyser to extract medicinal oil from the stem of *Ziziphus jujube*. J Anal Appl Pyrol 2011;92: 176–83.
[72] Zhang M, Ning G, Shou C, Lu Y, Hong D, Zheng X. Inhibitory effect of jujuboside A on glutamate-mediated excitatory signal pathway in hippocampus. Planta Med 2003;69:692–5.
[73] Peng WH, Hsieh MT, Lee YS, Lin YC, Liao J. Anxiolytic effect of seed of *Ziziphus jujuba* in mouse models of anxiety. J Ethnopharmacol 2000;72:435–41.
[74] Brahma SK, Debnath PK. Therapeutic importance of Rasayana drugs with special reference to their multi-dimensional actions. Aryavaidyan 2003;16:160–3.

[75] Das A, Shanker G, Nath C, Pal R, Singh S, Singh HK. A comparative study in rodents of standardized extracts of *Bacopa monniera* and *Ginkgo biloba* anticholinesterase and cognitive enhancing activities. Pharmacol Biochem Behav 2002;73:893–900.
[76] Mukherjee PK, Kumar V, Houghton PJ. Screening of Indian medicinal plants for acetylcholinesterase inhibitory activity. Phytother Res 2007b;21:1142–5.
[77] Loizzo MR, Tundis R, Menichini F, Menichini F. Natural products and their derivatives as cholinesterase inhibitors in the treatment of neurodegenerative disorders: an update. Curr Med Chem 2008;15:1209–28.
[78] Orhan IE, Orhan G, Gurkas E. An overview on natural cholinesterase inhibitors – a multi-targeted drug class and their mass production. Mini Rev Med Chem 2011;11:836–42.
[79] Grover JK, Khandkar S, Vats V, Dhunnoo Y, Das D. Pharmacological studies on *Myristica fragrans* – antidiarrheal, hypnotic, analgesic and hemodynamic (blood pressure) parameters. Methods Find Exp Clin Pharmacol 2002;24:675–80.
[80] Sung SH, Kang SY, Lee KY, Park MJ, Kim JH, Park JH, et al. (+)-α-Viniferin, a stilbene trimer from *Caragana chamlague* inhibits acetylcholinesterase. Biol Pharm Bull 2002;25:125–7.
[81] Wang T, Tang XC. Reversal of scopolamine-induced deficits in radial maze performance by (−)-huperzine A: comparison with E2020 and tacrine. Eur J Pharmacol 1998;349:137–42.
[82] Raves ML, Harel M, Pang YP, Silman I, Kozikowski AP, Sussman JL. Structure of acetylcholinesterase complexed with the nootropic alkaloid, (−)-huperzine A. Nat Struct Biol 1997;4: 57–63.
[83] Khalid A, Azim MK, Parveen S, Atta-ur-Rahman, Choudhary MI. Structural basis of acetylcholinesterase inhibition by triterpenoidal alkaloids. Biochem Biophys Res Commun 2005;331:1528–32.
[84] Cheng DH, Tang XC. Comparative studies of huperzine A, E2020 and tacrine on behavioural and cholinesterase activities. Pharmacol Biochem Behav 1998;60:377–86.
[85] Xu SS, Gao ZX, Weng Z, Du ZM, Xu WA, Yang JS, et al. Efficacy of tablet huperzine-A on memory, cognition and behaviour in Alzheimer's disease. Zhongguo Yao Li Xue Bao 1995; 16:391–5.
[86] Schliebs R, Liebmann A, Bhattacharya SK, Kumar A, Ghosal S, Bigi V. Systemic administration of defined extracts of *Withania somnifera* (Indian ginseng) and Shilajit differentially affects cholinergic but not glutamatergic and GABAergic markers in rat brain. Neurochem Int 1997;30:181–90.
[87] Salim KN, McEwan BS, Chao HM. Ginsenoside Rb1 regulates ChAT, NGF and trka mRNA expression in the rat brain. Mol Brain Res 1997;47:177–82.
[88] Houghton PJ, Ren Y, Howes MJ. Acetylcholinesterase inhibitors from plants and fungi. Nat Prod Rep 2006;23:181–99.
[89] Elgorashi EE, Stafford GI, Staden JV. Acetylcholinesterase enzyme inhibitory effects of amaryllidaceae alkaloids. Planta Med 2004;70:260–2.
[90] López S, Bastida J, Viladomat F, Codina C. Acetylcholinesterase inhibitory activity of some amaryllidaceae alkaloids and *Narcissus* extracts. Life Sci 2002;71:2521–9.
[91] Rahman AU, Parveen S, Khalid A, Farooq A, Choudhury MI. Acetyl and butyrylcholinestarase-inhibiting triterpenoid alkaloids from *Buxus papillosa*. Phytochemistry 2001;58:963–8.
[92] Devkota KP, Wansi JD, Lenta BN, Khan S, Choudhary MI, Sewald N. Bioactive steroidal alkaloids from *Sarcococca hookeriana*. Planta Med 2010;76:1022–5.
[93] Rahman AU, Wahab AT, Nawaz SA, Choudhary IM. New cholinesterase inhibiting bisbenzyl isoquinoline alkaloids from *Cocculus pendulus*. Chem Pharm Bull (Tokyo) 2004;52:802–6.
[94] Andrade MT, Lima JA, Pinto AC, Rezende CM, Carvalho MP, Epifanio RA. Indole alkaloids from *Tabernaemontana australis* (Muell. Arg) Miers that inhibit acetylcholinesterase enzyme. Bioorg Med Chem 2005;13:4092–5.
[95] Kim DK. Inhibitory effect of corynoline isolated from the aerial parts of *Corydalis incisa* on the acetylcholinesterase. Arch Pharm Res 2002;25:817–9.
[96] Ingkaninan K, Phengpa P, Yuenyongsawad S, Khorana N. Acetylcholinesterase inhibitors from *Stephania venosa* tuber. J Pharm Pharmacol 2006;58:695–700.
[97] Devkota KP, Lenta BN, Wansi JD, Choudhary MI, Kisangau DP, Naz Q, et al. Bioactive 5α-pregnane-type steroidal alkaloids from *Sarcococca hookeriana*. J Nat Prod 2008;71:1481–4.
[98] Kumar NS, Mukherjee PK, Bhadra S, Saha BP, Pal BC. Acetylcholinestarase inhibitory potential of a carbazole alkaloid, mahanimbine from *Murraya koenigii*. Phytother Res 2010b;24:629–31.
[99] Kalauni SK, Choudhari MY, Shaheen F, Manandhar MD, Rahman AU, Gewali MB, et al. Steroidal alkaloids from the leaves of *Sarcococca coriacea* of Nepalese origin. J Nat Prod 2001; 64:842–4.
[100] Kim DK, Lee KT, Baek NI, Kim SH, Park HW, Lim JP, et al. Acetylcholinesterase inhibitors from the aerial parts of *Corydalis speciosa*. Arch Pharm Res 2004;27:1127–31.
[101] Karczmar A. Invited review: anticholinesterases: dramatic aspects of their use and misuse. Neurochem Int 1998;32:401–11.
[102] Devkota KP, Lenta BN, Choudhary MI, Naz Q, Fekam FB, Rosenthal PJ, et al. Cholinesterase inhibiting and antiplasmodial steroidal alkaloids from *Sarcococca hookeriana*. Chem Pharm Bull (Tokyo) 2007;55:1397–401.
[103] Rollinger JM, Schuster D, Baier E, Ellmerer EP, Langer T, Stuppner H. Taspine: bioactivity-guided isolation and molecular ligand-target insight of a potent acetylcholinesterase inhibitor from *Magnolia x soulangiana*. J Nat Prod 2006;69:1341–6.
[104] Roddick JG. The acetylcholinesterase inhibitory activity of steroidal glycoalkaloids and their aglycones. Phytochemistry 1989;28: 2631–4.
[105] Miyazawa M, Yamafuji C. Inhibition of acetylcholinesterase activity by tea tree oil and constituent terpenoids. Flavour Fragrance J 2006;21:198–201.
[106] Ogawa K, Kawasaki A, Yoshida T, Nesumi H, Nakano M, Ikoma Y, et al. Evaluation of auraptene content in citrus fruits and their products. J Agric Food Chem 2000;48:1763–9.
[107] Aazza S, Lyoussi B, Miguel MG. Antioxidant and antiacetylcholinesterase activities of some commercial essential oils and their major compounds. Molecules 2011;16:7672–90.
[108] Ren Y, Houghton PJ, Hider RC, Howes MJ. Novel diterpenoid acetylcholinesterase inhibitors from *Salvia miltiorhiza*. Planta Med 2004;70:201–4.
[109] Mukherjee PK, Kumar V, Mal M, Houghton PJ. In vitro acetylcholinesterase inhibitory activity of the essential oil from *Acorus calamus* and its main constituents. Planta Med 2007;3:283–5.
[110] Kang SY, Lee KY, Sung SH, Park MJ, Kim YC. Coumarins isolated from *Angelica gigas* inhibit acetylcholinesterase: structure-activity relationships. J Nat Prod 2001;64:683–5.
[111] Hillhouse BJ, Ming DS, French CJ, Towers GHN. Acetylcholinesterase inhibitors in *Rhodiola rosea*. Pharm Biol 2004;42:68–72.
[112] Conforti F, Rigano D, Menichini F, Loizzo MR, Senatore F. Protection against neurodegenerative diseases of *Iris pseudopumila* extracts and their constituents. Fitoterapia 2009;80:62–7.
[113] Dhar SK, Johri RK, Zutshi U, Atal CK. Effect of potassium embelate, a novel analgesic compound on the neurotransmitter content of cerebrospinal fluid of the dog. Curr Sci 1986;55: 511–2.
[114] Bae YH, Cuong TD, Hung TM, Kim JA, Woo MH, Byeon JS, Choi JS, Min BS. Cholinesterase inhibitors from the roots of *Harpagophytum procumbens*. Arch Pharm Res 2013;37:1124–9.
[115] Urbain A, Marston A, Grilo LS, Bravo J, Purev O, Purevsuren B, et al. Xanthones from *Gentianella amarella* ssp. acuta with

acetylcholinesterase and monoamine oxidase inhibitory activities. J Nat Prod 2008;71:895–7.
[116] Kim DH, Hung TM, Bae KH, Jung JW, Lee S, Yoon BH, et al. Gomisin A improves scopolamine-induced memory impairment in mice. Eur J Pharm 2006;542:129–35.
[117] Geromichalos GD, Lamari FN, Papandreou MA, Trafalis DT, Margarity M, Papageorgiou A, et al. Saffron as a source of novel acetylcholinesterase inhibitors: molecular docking and in vitro enzymatic studies. J Agric Food Chem 2012;60:6131–8.
[118] Keane S, Ryan MF. Purification, characterisation, and inhibition by monoterpenes of acetylcholinesterase from the waxmoth, *Galleria mellonella* (L.). Insect Biochem Molec Biol 1999;29: 1097–104.
[119] Kuno F, Otoguro K, Shiomi K, Iwai Y, Omura S. Arisugacins A and B, novel and selective acetylcholinesterase inhibitors from *Penicillium* sp. J Antibiot 1996;49:742–7.
[120] Otoguro K, Shiomi K, Yamaguchi Y, Arai N, Sunazuka T, Masuma R, et al. Arisugacins C and D, novel acetylcholinesterase inhibitors and their related novel metabolites produced by *Penicillium* sp. J Antibiot 2000;53:50–7.
[121] Orhan I, Terzioglu S, Sener B. α- Onocerin: an acetylcolinesterase inhibitor from *Lycopodium clavatum*. Planta Med 2003;69: 265–7.
[122] Ho CC, Tasi HY, Lai YS, Chung JG. Effects of the ellagic acid on the *N*-acetyltransferase activity and acetylation of 2-aminofluorene in the rat. Toxicol Environ Chem 1999;71:319–29.
[123] Bruhlmann C, Ooms F, Carrupt PA, Testa B, Catto M, Leonetti F, et al. Coumarin derivatives as dual inhibitors of acetylcholinesterase and monoamine oxidase. J Med Chem 2001;44:3195–8.
[124] Sugimoto H, Yamanishi Y, Iimura Y, Kawakami Y. Donepezil hydrochloride (E2020) and other acetylcholinesterase inhibitors. Curr Med Chem 2000;7:303–39.
[125] Rice-Evans CA, Miller NJ, Paganga G. Structure antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biol Med 1996;20:933–56.
[126] Choi BW, Ryu G, Park SH, Kim ES, Shin J, Roh SS, et al. Anticholinesterase activity of plastoquinones from *Sargassum sagamianum*: lead compounds for Alzheimer's disease therapy. Phytother Res 2007;21:423–6.
[127] Vasudevan M, Parle M. Pharmacological actions of *Thespesia populnea* relevant to Alzheimer's disease. Phytomedicine 2006;13: 677–87.
[128] Urbain A, Marston A, Queiroz EF, Ndjoko K, Hostettmann K. Xanthones from *Gentiana campestris* as new acetylcholinesterase inhibitors. Planta Med 2004;70:1011–4.
[129] Bruhlmann C, Marston A, Hostettmann K, Carrupt PA, Testa B. Screening of non-alkaloidal natural compounds as acetylcholinesterase inhibitors. Chem Biodivers 2004;1:819–29.
[130] Haq AU, Malik A, Anis I, Khan SB, Ahmed E, Ahmed Z, et al. Enzyme inhibiting lignans from *Vitex negundo*. Chem Pharm Bull 2004;52:1269–72.
[131] Hung TM, Na M, Min BS, Ngoc TM, Lee I, Zhang X, et al. Acetylcholinesterase inhibitory effect of lignans isolated from *Schizandra chinensis*. Arch Pharm Res 2007;30:685–90.
[132] Bartoloni M, Cavrini V, Andrisano VJ. Choosing the right chromatographic support in making a new acetylcholinestarase-micro-immobilised enzyme reactor for drug discovery. Chromatogr A 2005;1065:135–44.
[133] Duthie SJ. Berry phytochemicals, genomic stability and cancer: evidence for chemoprotection at several stages in the carcinogenic process. Mol Nutr Food Res 2007;51:665–74.
[134] Marambaud P, Zhao H, Davies P. Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides. J Biol Chem 2005; 280:37377–82.
[135] Giuliani C, Bini LM. Insight into the structure and chemistry of glandular trichomes of Labiatae, with emphasis on subfamily Lamioideae. Plant Syst Evol 2008;276:199–208.
[136] Ahmad W, Ahmad B, Ahmad M, Iqbal Z, Nisar M, Ahmad M. In vitro inhibition of acetylcholinesterase, butyrylcholinesterase and lipoxygenase by crude extract of *Myricaria elegans* Royle. J Biol Sci 2003;3:1046–9.

LIST OF ABBREVIATIONS

ACh Acetylcholine
AChE Acetylcholinesterase
AD Alzheimer disease
APP Amyloid precursor protein
BChE Butyryl cholinesterase
ChAT Choline acetyltransferase
ChE Cholinesterase
CRS Cold restraint stress
CSF Cerebrospinal fluid
FDA Food and Drug administration
HAChT High-affinity choline transporter
MB-PQ Maneb and paraquat
TCM Traditional Chinese medicine
VAChT Vesicular acetylcholine transporters

CHAPTER

14

Drugs and Drug Leads Based on Natural Products for Treatment and Prophylaxis of Malaria

Søren Brøgger Christensen

Department of Drug Design and Pharmacology, University of Copenhagen, Copenhagen, Denmark

OUTLINE

14.1 MALARIA

Malaria is in many cases a fatal disease caused by infection with one of five protozoan parasites belonging to the genus *Plasmodium*: *P. vivax*, *P. malariae*, *P. falciparum*, *P. ovalis*, or *P. knowlesi* [1]. *Plasmodium knowlesi* has been known to be a parasite in long-tailed macaque monkeys, but the ability to infect humans was observed a decade ago by analyzing the DNA of parasites from patients in Malaysian Borneo [2]. The majority of deaths are caused by infections with *P. falciparum.*

14.1.1 The Burden of Malaria

World Malaria Report 2013 states that malaria is endemic in 104 countries, mainly in the tropical world, and that 3.4 billion people are at risk of malaria. It is estimated that 207 million malaria cases occurred globally and that the disease caused 627,000 deaths in 2012. About 80% of the cases and 90% of the deaths occurred in Africa. Most of the deaths (77%) were children younger than 5 years [3]. Even though this figure is alarming, earlier estimates suggested that between 1 and 2.5 million annual deaths were caused by malaria before 2003 [1,4], indicating that the World Health Organization (WHO) global malaria program has an effect. The goal of this program is to have reduced the global mortality rate of malaria by 56% in all ages and 63% in children younger than 5 years in 2015 [3]. Apparently, the burden of malaria has followed humans for at least 10,000 years. Studies of the polymorphism of the genome of the parasite principally responsible for the

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00014-3

death caused by malaria *P. falciparum* do not give a clear answer but indicate that a rapid increase in malaria might have occurred after the last glaciation and the spread of swidden agriculture [5].

The disease is transmitted by mosquitoes belonging to the genus *Anopheles*. A mathematical model for the transmission of the disease taking into account 31 parameters including weather conditions, rate of infection of humans, and the proliferation of mosquitoes has been suggested [6]. This model is continuously being refined [7–9].

14.1.2 The Cycle of the Disease

During feeding of the female mosquito on a human, on average 8–15 (but occasionally up to 100) plasmodial parasites are injected in the form of sporozoites, which either via the circulation or via lymph channels enter hepatocytes (Figure 14.1). In the liver, the parasites undergo asexual proliferation. After 5 days (in the case of *P. falciparum*) or 15 days (in the case of *P. malariae*), many thousand parasites are released from the liver as merozoites. In the cases of *P. vivax* or *P. ovale*, some parasites may remain in the liver in the form of hypnozoites, which may rupture several months later. Since only a few liver cells are infected, the patient does not experience any symptoms in the hepatic phase of the infection [1]. The merozoites liberated into circulation quickly enter erythrocytes. Attachment of the parasites to the erythrocytes is mediated via one or more erythrocyte receptors. For *P. vivax*, this receptor is Duffy blood group antigens, explaining why some persons in West Africa, who do not carry these antigens, are resistant toward infections with these parasites. In the erythrocytes, the parasites grow logarithmically and consume the proteins present, most of which is hemoglobin. After 12–14 h, cells infected with *P. falciparum* express a strain-specific antigen on the surface, enabling these erythrocytes to adhere to the walls of the venules or capillaries in vital organs and thereby disappear from circulation. During the time in the erythrocyte, the parasite goes through the stages of rings, trophozoites, and schizonts. Finally, the schizonts proliferate into merozoites, which rupture the cell after 48 h (for *P. falciparum*, *P. vivax*, and *P. ovales*) or after 72 h (for *P. malariae*) and liberate between 6 and 36 merozoites into the blood. If the infection is untreated, the characteristic fever spikes appear every 2 days (*P. vivax* and *P. ovales*, tertian malaria) or every 3 days with *P. malariae* (quartan malaria), when the erythrocytes rupture and release their content into the circulation. *P. falciparum* may never regulate to a tertian malaria [1].

After a series of asexual cycles of the parasites, a subpopulation develops into male and female gametocytes.

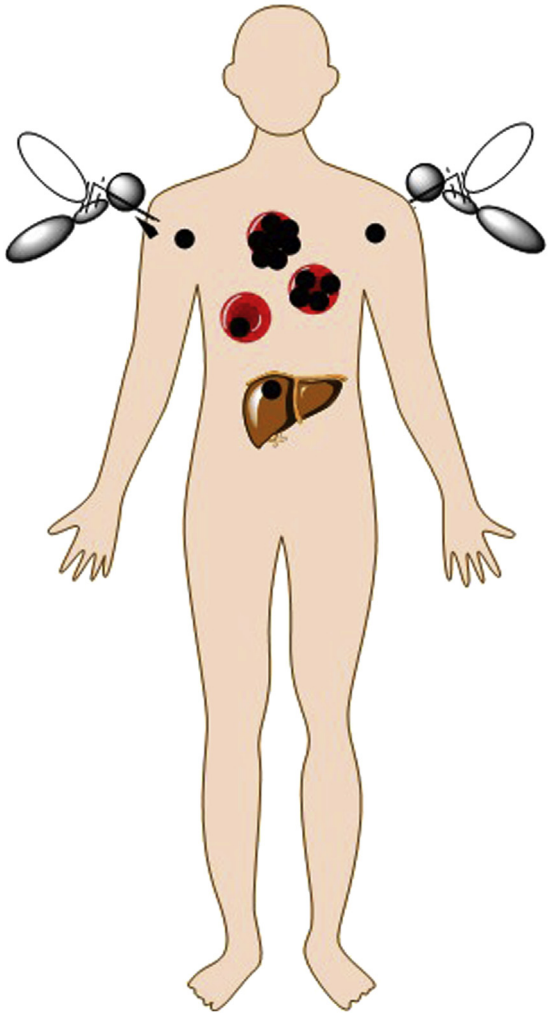


FIGURE 14.1 During feeding a mosquito injects approximately 10 parasites as sporozoites into the blood. The sporozoites infiltrate hepatocytes, where they proliferate asexually. After between 5 and 15 days, during which time the patient feels no symptoms, the parasites again are liberated into the blood where they enter the erythrocytes where they grow logarithmically. After 2 or 3 days, depending on the species of *Plasmodium*, the parasites rupture the cells, causing the characteristic fever strikes. Some parasites are converted into gametocytes, which can survive the digestive channel of the mosquito and perform a sexual proliferation.

When ingested by a mosquito, the gametocytes become activated and undergo a sexual proliferation. After having undergone several transformation cycles in the mosquito, the parasites finally penetrate into the salivary gland, from which they are injected into a human when the mosquito takes a blood meal. The development in the mosquito is known as sporogony and lasts between 8 and 35 days depending on the ambient temperature and the species of parasite. It is essential for the vectorial capacity that the mosquitoes survive for a period of time, which enables the parasites to undergo these cycles to give the infectious sporozoites [1].

14.2 DRUG FOR TREATMENT OF MALARIA

Development of a vaccine against malaria has proved difficult, meaning that at present, only drugs developed from small molecules can be used for the control of the disease. In 1991, a review was entitled "Can ethnopharmacology contribute to the development of antimalarial drugs?" [10]. Considering the fact that a surprisingly large fraction of drugs for treatment of malaria (about 50%) are either natural products or drugs derived from natural products [11–13], ethnopharmacology no doubt has contributed to the development of such drugs. It is interesting to note that the percentage of antimalarial drugs derived from natural products has not changed during the last 40 years, but the few drugs registered make it difficult to draw firm conclusions. No doubt the European use of the bark of *Cinchona* species as an antimalarial drug inspired Pelletier and Caventou to isolate the active principle, which was named quinine. It took more than a century before the configuration was established [14,15]. Similar Chinese investigations leading to artemisinin also were inspired by traditional use [16]. Two drugs for the treatment of malaria have been registered in the last 15 years as documented by inspection of the chapter To Market, To Market Annual Reports in *Medicinal Chemistry* volume 33 to 47 (1998–2013). One of the drugs is arteether dissolved in sesame oil for injection into children against severe infection, which actually only is a new formulation of a known drug. The trade name is Artemotil. The other drug is bulaquine, a derivative of primaquine, effective against relapse because it affects hypnozoites. The drug is registered as Aablaquin [17].

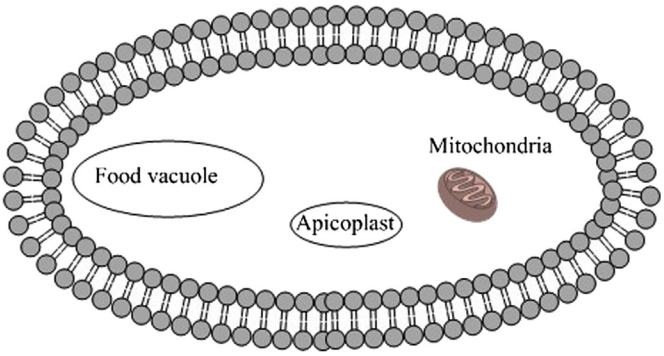


FIGURE 14.2 The organelles influenced by malaria drugs derived from natural products: food vacuole, mitochondria, apicoplasts.

14.2.1 Targets for Drugs Derived from Natural Products

Natural products or drugs derived from natural products primarily kill parasites by targeting processes in the food vacuole, the mitochondria, or the apicoplasts (Figure 14.2).

14.2.2 The Food Vacuole

The food vacuole is an organelle with a function similar to the digestive tract of higher organisms. Via a phagocytosis-like system, hemoglobin is transported into the food vacuole where it is digested to small peptides, which are released into the cytosol [18]. The remaining hem, which is an iron(II) complex, is oxidized into ferriprotoporphyrine, which spontaneously forms dimers. The dimers precipitate as submicrometer- to micrometer-sized crystals named hemozoin (Figure 14.3) [19]. By precipitation the potential toxicity of the ferriprotoporphyrine caused by the oxidative properties is avoided. The hemozoin formation is generally called polymerization. The heavy color of hemozoin is easily recognized by light microscopy of blood smears of malaria patients and is used for diagnostic purposes [1,20].

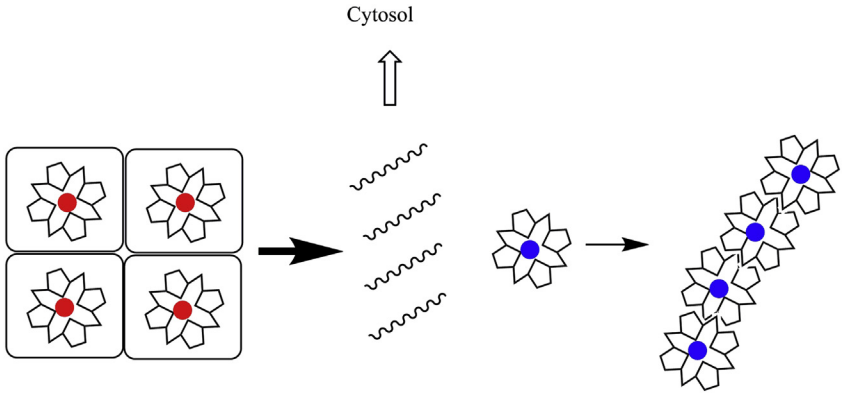


FIGURE 14.3 In the food vacuole, the tetrameric hemoglobin (four ovals with a porphyrin inside) is digested and the formed peptides (the helices) are transported out in the cytosol. The iron(II) ions (red circles) in the remaining heme will be oxidized into iron(III) form (blue circles), which is cytotoxic. Spontaneous dimerization affords an insoluble product, which precipitates as hemozoin (string of porphyrins) and thereby becomes nontoxic.

Alkaloids and other basic drugs will have an advantage in controlling *Plasmodium* parasites since the acidic pH of the food vacuole will make these compounds accumulate in the protonated form [21].

14.2.2.1 *Drugs Preventing Hemozoin Formation*

A number of drugs target the hemozoin precipitation by forming complexes with or covalently binding to the formed ferriprotoporphyrin. The more famous among these are quinine and artemisinin.

14.2.2.1.1 Quinine and 4-Aminoquinolines

Quinine, quinidine, cinchonine, and cinchonidine (Figure 14.4) are the major quinidine alkaloids isolated from the bark of trees belonging to the genera *Cinchona* and *Ramijia* (Rutaceae). It might be considered the irony of history that the first drug affecting malaria came from a continent (South America) in which malaria was no problem before the arrival of the Europeans. The Jesuits after arriving to the remains of the Inca Empire noticed that the Indians chewed the bark to prevent shivering of cold. Assuming that the bark also could cure the shivering caused by malaria attack they brought the bark back to Europe, a big part of which at that time was suffering under the burden of malaria. Indeed, correct dosing of the bark or extracts of the bark did cure several cases of malaria [22,23]. However, a serious drawback of this preparation was the problem of dosing correctly. The content of quininoline alkaloids in the bark of different species of *Cinchona* may vary between 4% and 7%, in some selected hybrids up to 17%. The small therapeutic window of quinine makes the variation in the content a severe problem for correct dosing of the drug. Too high doses might cause tinnitus, vomiting, and stimulatory action of the pancreatic β-cell causing hyperinsulinemic hypoglycemia [1]. Therefore, it was a major step in the fight against malaria when Pelletier and Caventou in 1820 isolated quinine sulfate in a decent quality. A major illustration of the importance of the possibility of administration of quinine in reproducible doses was illustrated by the four expeditions on the river Niger in the nineteenth century. During the first two expeditions (1805 and 1833), 39 of 44 and 32 of 40, respectively, Europeans died from malaria. In the third expedition, the crew consisted of 62 Europeans. Of these, 55 were infected with malaria but 39 were successfully treated with quinine. Finally, during the fourth expedition (1854), all the Europeans were prophylactically treated with quinine. No European died from malaria during this trip [14]. Also, the use of quinine drastically reduced the military mortality in areas in the tropics [22].

Quinine prevents the hemozoin crystals from growing by intercalating the quinolone rings between the aromatic groups of the ferriprotoporphyrine molecules [19]. A similar mechanism of action explains the antimalarial effect of a series of drugs derived using quinine as scaffold such as mefloquine, amodiaquine, and mepacrine (Figure 14.5) [19]. Other mechanisms of actions, however, have been suggested for mefloquine, such as inhibition of vacuole−vesicle fusion [24] or binding to essential proteins in the parasite [25]. The ability of mefloquine to affect hypnozoites is difficult to understand should the mechanism of action solely be prevention of hem formation.

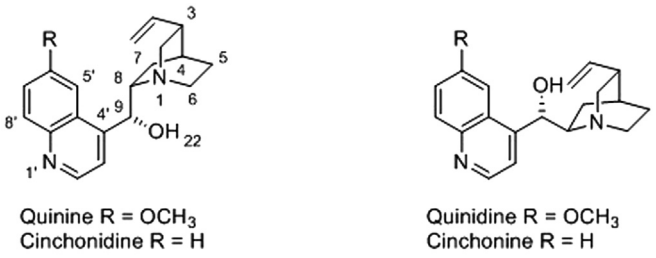


FIGURE 14.4 The four major quinolone alkaloids in bark of trees belonging to the genera *Cinchona* and *Remijia*.

14.2.2.1.2 Chloroquine

The most successful drug and without comparison, chloroquine [20], was not developed using quinine as a scaffold but methylene blue (Figure 14.6). Ehrlich concluded that the ability of the *Plasmodium* parasite to take up this dye so efficiently had to cause a toxic effect on the parasite. He succeeded in curing two patients with malaria, but the drug was not sufficient

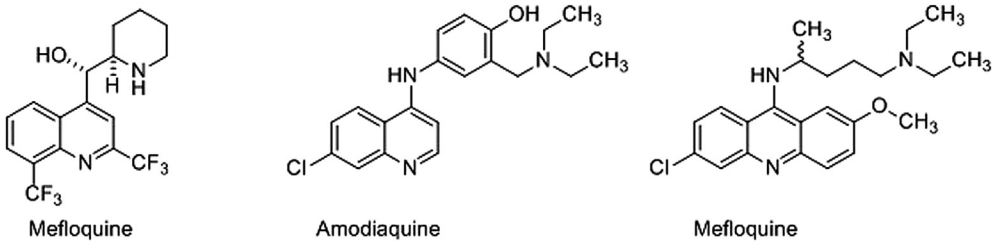


FIGURE 14.5 Drugs containing a four-substituted quinolone nucleus. The mechanism of action of all of these is assumed to depend on their ability to intercalate with heme.

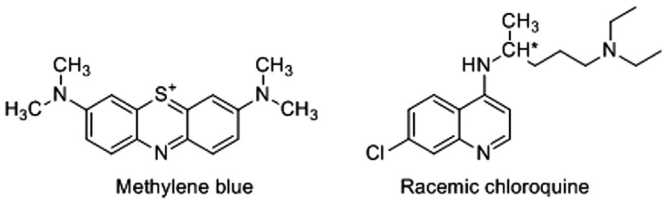


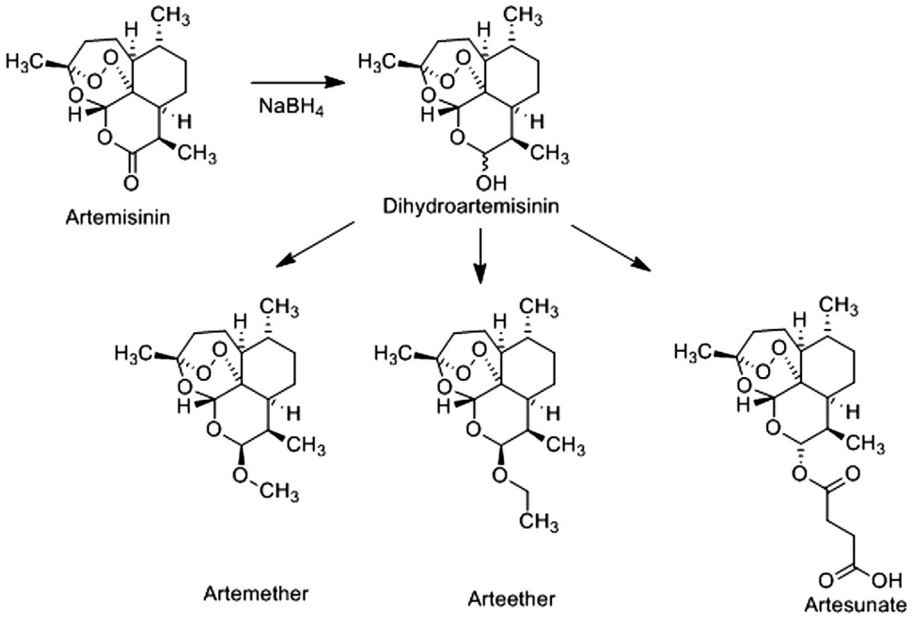
FIGURE 14.6 Methylene blue and chloroquine. Chloroquine is used as a racemic mixture. The missing chirality of the target molecule (the precipitating hem) must mean that the two enantiomers have the same affinity for the target, but they may be differently metabolized or distributed in the body.

efficient for general use [22]. Attempts to optimize the molecule led to chloroquine, the potential of which, however, was first realized after the Second World War [22]. Chloroquine became the drug of first choice in malaria therapy for more than two decades until resistance limited the use of the drug. The resistance is correlated to point mutations in the gene *pfcrt* [26]. The gene codes for a transporter PfCRT. Mutations in the gene like *K76T* has been assumed to remove a positively charged lysine from the transporter thereby enabling it to remove the positively charged chloroquine from the food vacuole [20]. Other *PfCRT* mutations, however, also induce resistance, suggesting a more complex situation. Like quinine, chloroquine prevents hemozoin formation [19]. An interesting feature of chloroquine is that the racemic form of this drug is used. The achirality of the hem molecule leads to the expectation that the two isomers have the same affinity toward the biological target, but obviously different distribution or metabolism of the two enantiomers cannot be excluded.

14.2.2.1.3 Artemisinin

Artemisinin (Scheme 14.17) is a sesquiterpene lactone isolated from *Artemisia annua* L. (Asteraceae) [16]. The structure is unusual by possessing a endocyclic peroxy bridge. Even more surprisingly the peroxy bridge resists reduction with borohydride, affording only a reduction of the lactone to give the corresponding semiacetal [27,28]. This compound is used for making ethers (artemether, arteether), which are soluble in oil, enabling injection of the drug or rectal administration [29], or a semi succinate ester (artesunate), which is soluble in water and therefore may be injected intravenously. Rectal administration might be advantageous for patients in whom vomiting prevents oral administration. Artemether, arteether, and artesunate are prodrugs of dihydroartemisinin, which is the active drug [30]. The mechanism of action has been debated, but it is now generally believed that artemisinin like quinine prevents hemozoin formation. In contrast to quinine, however, artemisinin is assumed to form a covalent binding to the hem skeleton at either Cα or $C\beta^{19}$ (Figure 14.7). A study using Mn(II) as radical inducer has revealed how a reactive intermediate of artemisinin may be formed (Scheme 14.18) [31]. Studies of mass spectra of the reaction product also have confirmed suggested structure [32].

Fast metabolism and excretion necessitates treatment with artemisinin derivatives for at least 5 days, which is inconvenient in large parts of the world. The drugs are therefore preferentially used in combination with other drugs with large half-life times like mefloquine, lumefantrine, amodiaquine, sulfadoxine, pyrimethamine, piperaquine, chlorproguanil, and dapsone [18].



SCHEME 14.17 Conversion of artemisinin into oil-soluble (arteether and artemether) or water-soluble drugs (artesunate).

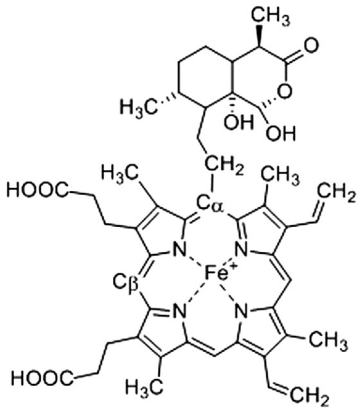


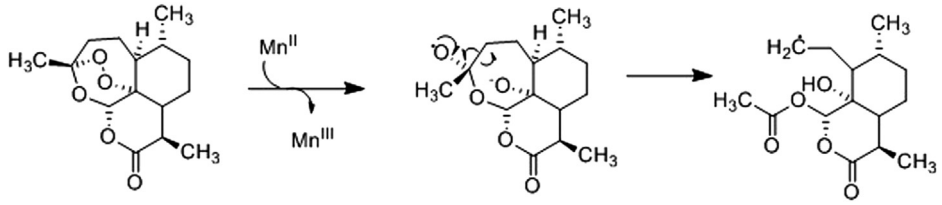
FIGURE 14.7 Reaction product of artemisinin and hem.

14.2.3 The Apicoplast

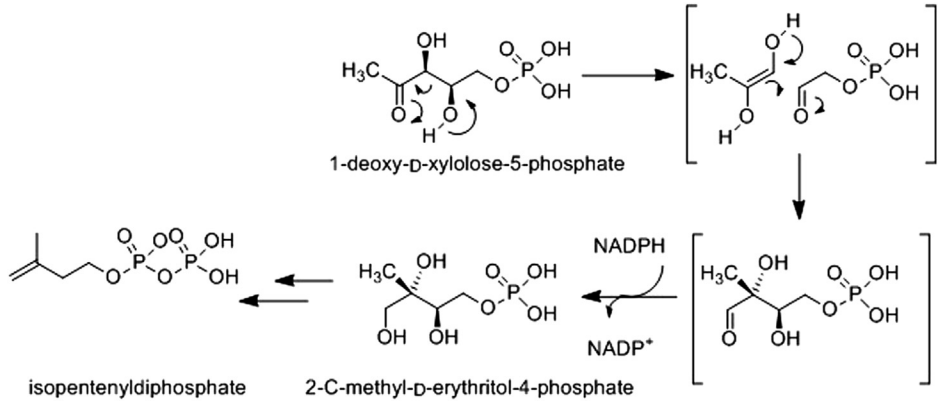
The apicoplast of the *Plasmodium* parasites is assumed to be a reminiscence of cyanobacteria domesticated by the eukaryotic cells. The organelle is an analog to the chloroplasts performing the photosynthesis in plants and algae. The ability to perform photosynthesis is lost in the parasites [33,34]. However, the metabolic functions including biosynthesis of fatty acids and terpenoids are essential for the survival of the organism [35]. In plants and microorganisms, terpenoids are synthesized by two pathways, the mevalonate pathway and the deoxyxylulose pathway [36]. In both pathways the final product is isopentenyl diphosphate. In the first pathway, the final product is obtained from mevalonic acid, whereas the later pathway includes a rearrangement of 1-D-deoxyxylulose-5-phosphate into 2-*C*-methyl-D-erythriol-4-phosphate followed by a reduction of the aldehyde to the corresponding alcohol (Scheme 14.19) [36,37]. Absence of the deoxyxylulose pathway in humans and animals makes it an obvious target for treatment of infections with organisms like *P. falciparum*, for which it is essential [34]. The apicoplasts possess a 35-kilobase circular genome that encodes for a prokaryote transcription and translation system, which makes the organelle sensitive toward antibiotics targeting such a system [30].

14.2.3.1 *Fosmidomycins*

A search for antibiotics in *Streptomyces rubellomurinus* lead to the isolation of a phosphonic acid FR900098 (Figure 14.8) [38]. Extension of the studies to *Streptomyces lavendulae* and other strains of *S. rubellomurinus* afforded three additional compounds [39] (Figure 14.8). Comparison of the structures of FR31564, which has been named fosmidomycin, and the substrate for 1-deoxy-D-xylulose-reductoisomerase reveals a very good overlay (Figure 14.9), indicating that it is likely that fosmidomycin inhibits the enzyme by binding to the active site. The amide function cannot be reduced by the enzyme in contrast to the aldehyde group of the natural substrate. Unfortunately, several clinical trials with fosmidomycin or combination of fosmidomycin with artemisinin or clindamycin have been performed but the results were not convincing [40–43].



SCHEME 14.18 Manganese (II) provoked conversion of artemisinin into a reactive radical.



SCHEME 14.19 Formation of isopentenyldiphosphate by the deoxyxylose pathway. The compounds in the brackets are intermediates, which are bound to the enzyme 1-deoxy-D-xylulose-reductoisomerase.

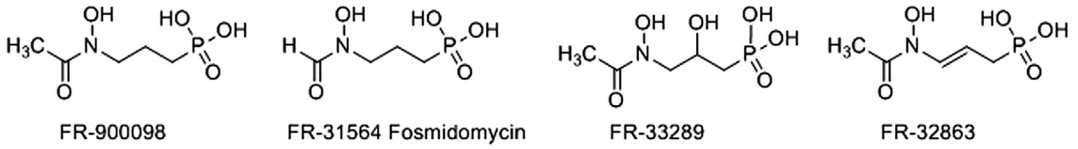


FIGURE 14.8 Structure of the four phosphonic acids isolated from species of *Streptomyces* (FR900098, FR31564, FR32863, FR33289) inhibiting 1-deoxy-D-xylulose-reductoisomerase.

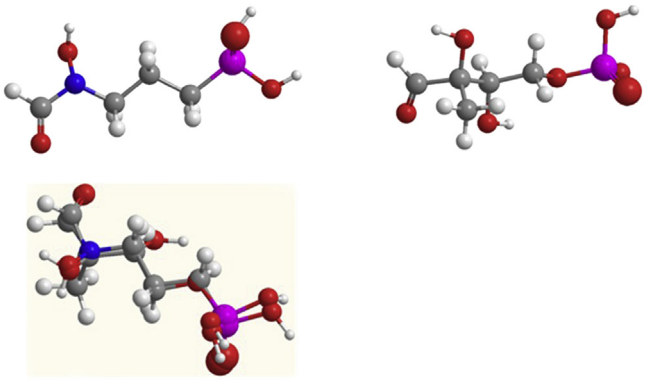


FIGURE 14.9 Overlay of the substrate for 1-deoxy-D-xylulose-reductoisomerase (Scheme 14.19) and FR31564 (fosmidomycin). The two molecules were energy minimized in Chem3D and overlaid. White spheres are hydrogen, red spheres are oxygen, gray spheres are carbon, blue spheres are nitrogen, and magenta spheres are phosphorous atoms.

14.2.3.2 *Tetracyclines*

The tetracyclines (Figure 14.10) are a group of orally active broad-spectrum antibiotic originally isolated from cultures of species of *Streptomyces* [44]. The mechanism of action is believed to be an inhibition of prokaryotic translation of proteins. This is achieved by interfering with the binding of aminoacyl-tRNA to the 30S subunit of the prokaryotic ribosome. The tetracyclines show a much smaller affinity for eukaryotic ribosomes [44]. From this point of view it might be surprising that the tetracyclines also effect protein expression in *Plasmodium* parasites. Incubation of *P. falciparum* parasites with tetracyclines reveals that no apparent changes of the mitochondria or apicoplasts are visible after the first cell cycle. However, in daughter cells, the incubation affords nonfunctional apicoplasts and subsequent blockage of parasite development.

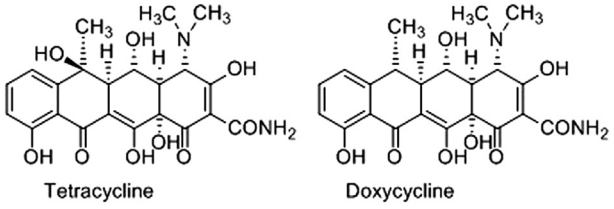


FIGURE 14.10 Structure of tetracycline and doxycycline.

Based on these observations it is concluded that the mechanism of action is a prevention of protein synthesis in the apicoplasts [45–47]. Slow onset of the effects of tetracyclines explains why WHO on the list with essential medicine only suggests doxycline to be used in combination with quinine for treatment of malaria, whereas it might be used alone for prophylaxis [48].

14.2.3.3 *Lincosamide*

Lincomycine (Figure 14.11) is an antibiotic isolated from cultures of *Streptomyces lincolnensis* var. *lincolnensis* [44]. The semisynthetic derivative clindamycin (Figure 14.11) is used in combination with quinine or artesunate for treatment of malaria [45]. Like the tetracyclines the lincosamides are assumed to affect the protein synthesis.

14.2.4 The Mitochondria

In higher organisms, reduction of oxygen to generate energy occurs in the mitochondria. In the erythrocyte stage of *P. falciparum*, however, the mitochondria seem to be of major importance for generating oxidized complex III (cytochrome bc_1). The parasite appears to miss the enzymes necessary for generating ATP [49]. Besides feeding the electron transport chain and thereby maintaining the electron gradient over the membrane, the oxidized plasmodium complex III enables reduction of dihydroorotate dehydrogenase, which reduces dihydroorotate to orotate, a precursor for the pyrimidines (Figure 14.12). Since parasites are unable to salvage pyrimidines formed by catabolism of DNA, blockage of the orotate synthesis will be fatal [50].

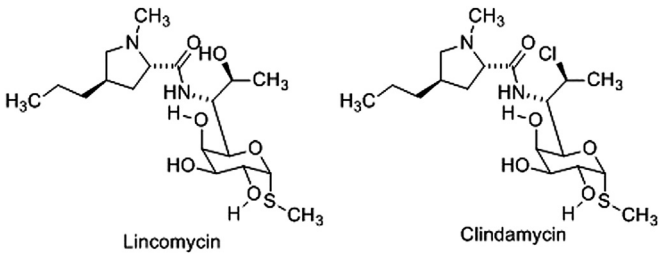


FIGURE 14.11 Structure of the lincosamides lincomycin and clindamycin.

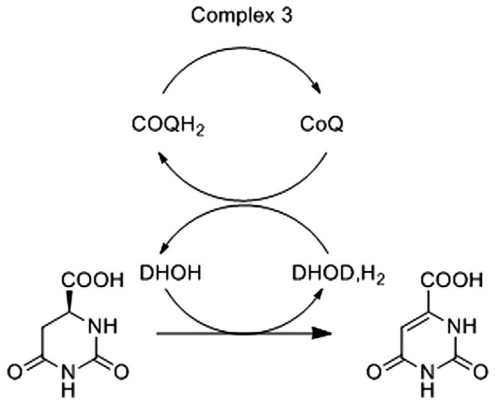


FIGURE 14.12 The electron path from complex III to dihydroorotate.

14.2.4.1 Naphthoquinones

Lapachol (Figure 14.13) and other naphthoquinones are widely distributed in plants belonging to the family Bignoniaceae but they have also been found in other families from the order Lamiales [51]. In an ethnopharmacological approach to find potential antimalarial drugs, a screening program was performed and lapachol was found to possess some activity against *Plasmodium lophurae* in ducks [52]. Based on medicinal chemical optimization of the drugability of the naphthoquinones lapinone (Figure 14.13) was developed and clinical trials revealed positive results by intravenous injections of high doses (2 g per day for 4 days). Of nine patients two had recrudescence 2 or 3 weeks after treatment, one was free of recrudescence after 10 months and the remaining six had no recrudescence after 13 months [53]. The rather high doses, however, suggested that the drug might be optimized. One problem was a low half-life, which could be prolonged by changing the side chain. Based on these studies, atovaquone was designed and registered (Figure 14.13). The mechanism of action of the naphthoquinone was understood when studies revealed that the hydroxyquinones had three orders of magnitude higher affinity for the complex III of the parasites than for that of rats [54]. Atovaquone intercalates in cytochrome bc_1 by forming hydrogen bonds with His181, interacting with Thr122, and forming water bridge with Glu272 (Figure 14.14) [55]. The complex formation prevents the electron transfer in the mitochondria, which leads to depolarization of the mitochondria membrane potential. A further consequence is a disruption of the pyrimidine synthesis [49].

To avoid resistance it is recommended to use ataovaquone in combination with other drugs in particular proguanil (Malarone). Proguanil, which is a prodrug of cycloguanil (Scheme 14.20), is an inhibitor of dihydrofolate reductase [1]. Atovaquone and proguanil synergistically enforce the effect of each other.

14.3 MALARIA PREVENTION THROUGH VECTOR CONTROL

In addition to treatment of infected patients WHO also recommend prevention of malaria through vector control such as indoor spraying and the use of insecticide-treated mosquito nets [56]. The use of bed nets reduces the contact time between humans and mosquitoes and thereby minimizes the risk of transmission. Some of the recommended pesticides like cypermethrin and deltamethrin are derived from the pyrethrins isolated from *Chrysanthemum cinerarifolium* Vis. (Asteraceae) [44].

14.4 EVIDENCE-BASED USE OF PHYTOMEDICINES

In spite of the advantages of the use of the active principle isolated from plants and in particular the use of active principle improved by optimization of the natural products (e.g., lapachol into atovaquone, artemisinin into artesunate), arguments for the use of phytomedicines still are put forward. Synergistic interaction between the secondary metabolites in, e.g., *A. annua* and quinine, quinidine, and cinchonine (Quinimax), have been used as an argument for the use of the juice obtained by wringing wet *A. annua* herb or to use a mixture of the three alkaloids instead of quinine, respectively [57]. Other constituents also might increase the effect by increasing the bioavailability of the active principles [57]. On the other hand, if different metabolites can increase the pharmacological effects, it might also be possible that secondary metabolites might counteract each other.

In addition to possible positive interactions, advantage of the use of locally produced phytomedicines could be that this would empower poor communities to become more self-reliant [58]. Furthermore, it is argued that traditional use in several generations without observation of side effects must ensure that the traditional use is safe and efficient. Obviously this argument must be supported with experiments including clinical trials proving the effects of the drug [59]. The performance of such trials, however, should be done under strict ethical guidelines [60]. Even after a successful outcome of such a trial, this gives no evidence for chronic safety of the drug. In Africa, children and pregnant women may be infected with malaria several times a year meaning that continuous dosing could cause unacceptable chronic toxicity [59]. An additional problem with locally produced phytomedicines is proper dosing. A reproducible content of the active principle in the medicine must be ensured to avoid either too

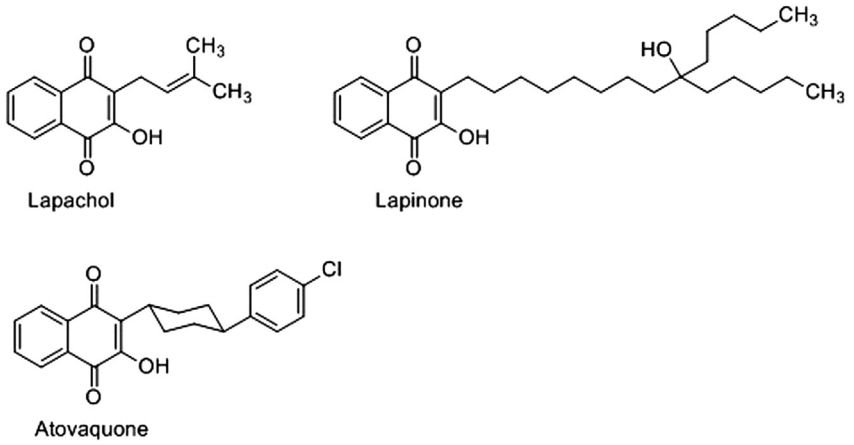


FIGURE 14.13 Antimalarial hydroxynaphthoquinones.

low dosing, which prevents curing the patent and facilitates development of resistance, or too high dosing causing toxic side effects. The latter problem might be overcome by production of standardized phytomedicines, e.g., at a national level [58]. The control systems needed for making standardized products after good manufacturing practices standards, however, would add considerable costs to the production, meaning that the final product not necessarily needs to be cheaper than conventional medicines produced in large scale in the pharmaceutical industry [58], especially when produced after expiration of patent rights and in countries with low production costs.

Complex mixtures of botanicals are typically used in traditional Chinese and Indian medicine. Such mixtures have also been recommended for treatment of malaria. Examples are Ayush-64 [a mixture of *Caesalpinia bonducella* L. (Fabaceae), *Swetia chirata* Buch (Gentianaceae), *Alstonia scholaris* L. (Apocyanaceae), and *Picrorhiza kurrooa* Royle ex Benth (Plantaginaceae)], and Malarial-5 [a mixture of *Cassia occidentalis* L. (Caesalpinaceae), *Lippia chevalieri* Mold (Verbenaceae), and *Spilanthes oleraceae* L. (Asteraceae)]. A clinical trial has revealed that Ayush-64 clears the infection after treatment for 3 days, although the risk of recrudescence was not investigated; Malarial-5 appeared to have no effect [58].

A number of phytomedicines, some standardized for treatment of malaria, are listed in two reviews [58,59]. Below are some of these listed in alphabetical order.

14.4.1 Argemone mexicana

Two studies on the effects of decoction of *Argemone mexicana* L. (Papaveraceae) have revealed significant reduction in parasitemia [58,59,61,62], although not meeting the demands of WHO, which expects that >95% of the patients should obtain an adequate clinical and parsitological response at day 28. A preparation named after the inventor Soumafoura Tiemoko Bengali

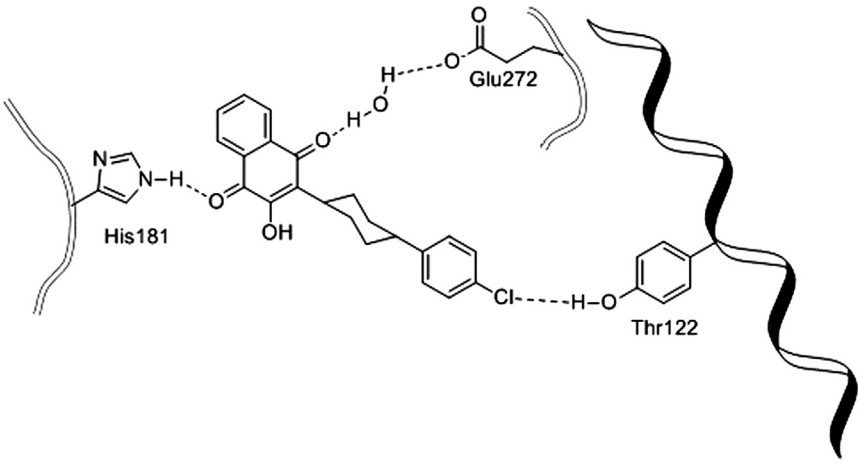
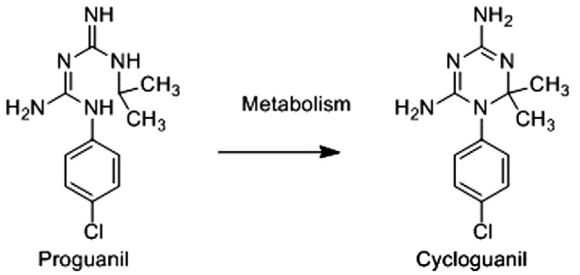


FIGURE 14.14 Binding of atovaquone in complex III (cytochrome bc_1). Notice the postulated interaction between the phenolic group of Thr122 and the chlorine atom of atovaquone.



SCHEME 14.20 Metabolic conversion of proguanil into cycloguanil.

has been prepared by the Department of Traditional Medicine in Mali [58]. A problem with these studies, however, is that standardized preparations have not been used, making interpretation of the results difficult. Protopine and berberine (Figure 14.15) have been isolated from *A. mexicana*. Berberine very potently inhibits proliferation of malaria parasites in vitro but has no effect in vivo [63], whereas protopine has been shown to control malaria infections in mice [64].

14.4.2 Artemisia annua

Ethanolic extracts and decoction of *A. annua* L. (Asteraceae) and are recommended for treatment of malaria [58]. Clinical trials have been performed on an ethanolic extract. A 100% clearance was observed; however, as is feared with the use of single drug therapy with artemisinin (Scheme 14.17), recrudescence was seen in 33% of the patients. Synergistic effects of other flavonoids and terpenoids in the extract are suggested to improve the effect of the crude extract compared to the effect of the purified artemisinin [57,58]. The flavone casticin is reported to enhance the in vitro activity of artemisinin by three- to five-fold [57].

14.4.3 Artobotrys uncinatus

Artobotrys uncinatus C. Agardh (Annonaceae) has been used in traditional Chinese medicine for treatment of malaria [59]. The active principle yingzhaosu A (Figure 14.16) was isolated. The presence of the peroxide yingzhaosu A led to the suggestion that the mechanism of action might be similar to that of artemisinin. Drug development based on yingzhaosu A was discontinued because the compound showed no convincing advantages over artemisinin [59].

14.4.4 *Cinchona* Bark

In the 1920s and 1930s, some clinical trials for the effects of crude preparations of *Cinchona* bark were performed [58]. The drug was named totaquina. In spite of some attempt to perform standardization, a wide variation of the doses administered must be expected and the exact species of *Cinchona* was not given. A total clearance of parasites was reported but the possibility of recrudescence was not investigated. Synergistic interaction of the different alkaloids in the drug is suggested to improve the effects of the drug [58]. A mixture of quinine, quinidine, and cinchonine (Quinimax, Figure 14.4) has been marketed as an antimalarial drug, which might

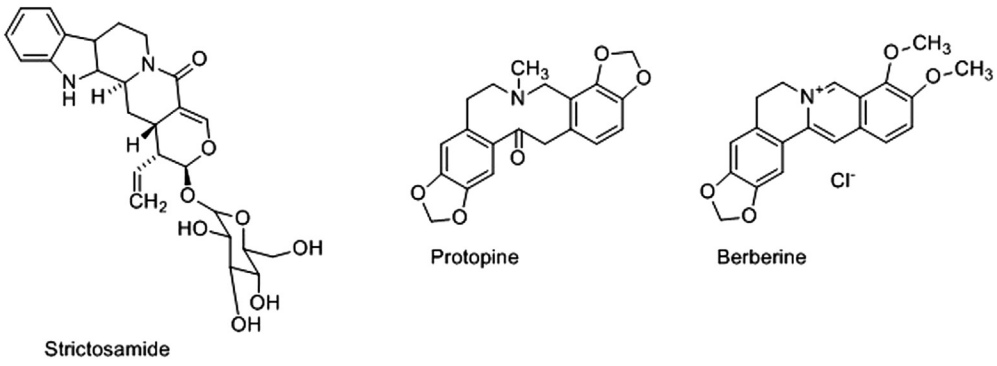


FIGURE 14.15 Structure of strictosamide, berberine, and protopine.

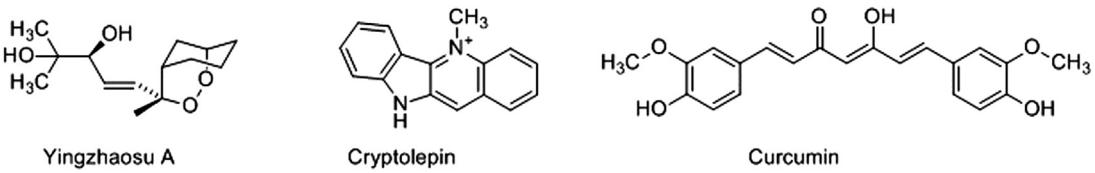


FIGURE 14.16 Structure of yinzhaosu, cryptolepine, and curcumin.

be efficient toward infections with quinine-resistant *P. falciparum* [57].

14.4.5 Cryptolepis sanguinolenta

An extract of *Cryptolepis sanguinolenta* Lindl. (Apocynaceae) has cured 12 patients with parasitemia between 10^3 and $10^4/\mu l$ [59]. The assumed active ingredient cryptolepine (Figure 14.16), however, could not cure mice infected with *Plasmodium berghei* [65]. A standardized decoction of the plant has been marketed as PHYTOLARIA by the Faculty of Pharmacy, Kwame Nkrumah University of Science and Technology, Ghana.

14.4.6 Curcuma longa

A nanomilled preparation of *Curcuma longa* L. (Zingiberaceae) has been used for treatment of patients infected with *P. vivax* or *P. falciparum*. The active principle is assumed to be curcumin (Figure 14.16). Unfortunately, no clinical data are available for the use of extracts of *C. longa* against malaria [59].

14.4.7 Nauclea pobeguinii

Stembark of *Nauclea pobeguinii* (Hua ex Pobég) Merr. (Rubiaceae) was extracted with 80% ethanol and the extract concentrated to give a residue standardized to contain 5.6 % of strictosamide Figure 14.15. The residue was put into capsules (500 mg in each). Patients with a proven *P. falciparum* infection were treated three times daily with 2 capsules followed by one capsule three times daily for four days. This treatment was almost as efficient as an artesunate-amodiaquine treatment and showed fewer side effects [66,67]. The suspected active principle strictosamide showed no activity in vivo, indicating a metabolic conversion into an active compound [68].

14.5 CONCLUSION

Unfortunately, there is no simple answer to the previously asked question "Can ethnopharmacology contribute to the development of antimalarial agents?" [10]. None of the promising compounds mentioned in this contribution [10] has made their way into the market and no new natural product or derivative of a natural product has been marketed since then. However, it is still evident that natural products have been the major source for antimalarial drugs in the past [11–13]. In addition, many lessons have been learned from natural products. Thus, investigation of the target of quinine taught us that the hemozoin formation might be a pharmacological target and that inhibition of protein formation and inhibition of the deoxyxylulose pathway could be fatal for the parasite. No doubt natural products still will be an important source of inspiration in the future.

A new strategy for discovery of lead compounds has been suggested. Not only should the secondary metabolites in the traditionally used medicine be investigated but also interesting structures might be found by including investigation of metabolites formed in the body [59]. This suggestion is based on the observation that clinical trials of extracts of *N. pobeguinii* have shown antimalarial effects but no active principle could be found in the extract [59]. In addition, valuable clues in particular for developing combination therapies might be found by reinvestigating the profile of secondary metabolites in crude traditional medicines, which in clinical trials have shown an effect far better than expected from the known secondary metabolites [57,58].

New technologies have made marine organisms, microorganisms, and fungi more accessible. These sources often offer very potent natural products [69,70]. Studies of these organisms should also be included in searches for new antimalarial drugs, even though ethnopharmacological clues only will be available for these sources in very few cases [59].

The effect of fosmidomycin was realized by mining the plasmodial genome and realizing the presence of the deoxyxylulose pathway [71]. No doubt the knowledge of this genome enables realization of new targets for drugs.

Development of high-throughput screenings also has facilitated fast screenings of big chemical libraries. A screening of 309,474 compounds led to the identification of 172 representative drug candidates. A reverse chemical genetic study identified 19 new inhibitors of four validated drug targets and 15 possible new targets [72]. Similarly, a screening of almost 2 million compounds has led to 13,533 compounds with an IC_{80} (the concetration in which the growth of the parasites is inhibited 80%) below 2 μM. Analysis of the data suggested that the compounds might target protein kinases or host–parasite interactions [73]. At present, no registered drug interferes with these targets. All of the found lead structures are publicly available.

In conclusion, many new methods for developing new antimalarial drugs have been suggested, which is fortunate since it is feared that resistance against the presently used drug might develop. However, a major problem is the poor possibility for a sufficient profit from such drugs, which limits the interest of the pharmaceutical industry. Hopefully, the academic society encouraged by substantial funding, which might be obtained from foundations like the Linda and Bill Gate Foundation, can compensate. Even though the WHO malaria program has achieved noticeable results, there still is some way to go before the malaria situation is acceptable.

References

[1] White NJ. Malaria. In: Cook GC, Zumla AI, editors. Manson's tropical diseases. 21 ed. China: Elsevier Science Limited; 2003. p. 1205–93.

[2] Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SSG, Cox-Singh J, et al. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet 2004;363:1017–24.

[3] WHO. World malaria report. 2013.

[4] Bloland PB. Drug resistance in malaria. WHO; 2001.

[5] Hartl DL. The origin of malaria: mixed messages from genetic diversity. Nat Rev Microbiol 2004;2:15–22.

[6] Hoshen MB, Morse AP. A weather-driven model of malaria transmission. Malar J 2004;3:32.

[7] Ermert V, Fink AH, Jones AE, Morse AP. Development of a new version of the Liverpool Malaria Model. I. Refining the parameter settings and mathematical formulation of basic processes based on a literature review. Malar J. 2011;10:35.

[8] Ermert V, Fink AH, Jones AE, Morse AP. Development of a new version of the Liverpool Malaria Model. II. Calibration and validation for West Africa. Malar J 2011;10:62.

[9] Gething PW, Patil AP, Smith DL, Guerra CA, Elyazar Iqbal RF, Johnston GL, et al. A new world malaria map: *Plasmodium falciparum* endemicity in 2010. Malar J 2011;10:378.

[10] Phillipson JD, Wright CW. Can ethnopharmacology contribute to the development of antimalarial agents? J Ethnopharmacol 1991; 32:155–65.

[11] Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. J Nat Prod 1997;60:52–60.

[12] Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981–2002. J Nat Prod 2003;66: 1022–37.

[13] Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. J Nat Prod 2007;70:461–77.

[14] Rocco F. The miraculous fever-tree. Malaria, medicine and the cure that changed the world. London: HarperCollins Publisher; 2003.

[15] Eiden F. Ausflug in die Vergangenheit. Chinin und andere Chinaalkaloide. 2. Teil: die Aufklärung des räumlichen Baus der Chinolin-Chinaalkaloide. Pharm Unserer Zeit 1999;28:11–20.

[16] Qinhaosu Antimalaria Coordinating Research Group. Antimalaria studies on qinhaosu. Chin Med J 1979;92:811–6.

[17] Dotherty AM. To market, to market. Ann Rep Med Chem 2001;36: 293–318.

[18] Wiesner J, Ortmann R, Jomaa H, Schlitzer M. New antimalarial drugs. Angew Chem Int Ed 2003;42:5274–93.

[19] Weissbuch I, Leiserowitz L. Interplay between malaria, crystalline hemozoin formation, and antimalarial drug action and design. Chem Rev 2008;108:4899–914.

[20] Ecker A, Lehane AM, Clain J, Fidock DA. PfCRT and its role in antimalarial drug resistance. Trends Parasitol 2012;28:504–14.

[21] Dzekunov SM, Ursos LM, Roepe PD. Digestive vacuolar pH of intact intraerythrocytic *P. falciparum* either sensitive or resistant to chloroquine. Mol Biochem Parasitol 2000;110:107–24.

[22] Meshnich SR, Dobson MJ. The history of antimalarial drugs. In: Rosenthal PJ, editor. Antimalarial chemotherapy. Totowa, New Jersey: Humana Press; 2001. p. 15–25.

[23] Greenwood D. The quinine connection. J Antimicrob Chemother 1992;30:417–27.

[24] Hoppe HC, van Schalkwyk DA, Wiehart UI, Meredith SA, Egan J, Weber BW. Antimalarial quinolines and artemisinin inhibit endocytosis in *Plasmodium falciparum*. Antimicrob Agents Chemother 2004;48:2370–8.

[25] Foley M, Tilley L. Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. Pharmacol Ther 1998;79:55–87.

[26] Sidhu AbS, Verdier-Pinard D, Fidock DA. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations. Science 2002;298:210–3.

[27] Casteel DA. Antimalarial agents. In: Wolff ME, editor. Burger's medicinal chemistry and drug discovery. 5th ed. New York: John Wiley & Sons Inc; 1997. p. 3–91.

[28] Klayman DL. Qinhaosu (Artemisinin): an antimalarial drug from China. Science 1985;228:1045–55.

[29] Barnes KI, Mwenechanya J, Tembo M, Mcilleron H, Folb PI, Ribeiro I, et al. Efficacy of rectal artesunate compared with parenteral quinine in initial treatment of moderately severe malaria in African children and adults: a randomised study. Lancet 2004; 363:1598–605.

[30] Ridley RG. Medical need, scientific opportunity and the drive for antimalarial drugs. Nature 2002;415:686–93.

[31] Robert A, Meunier B. Alkylating properties of antimalarial artemisinin derivatives and synthetic trioxanes when activated by a reduced heme model. Chem Eur J 1998;4:1287–96.

[32] Christensen SB, Bygbjerg IC. Drugs for the neglected disease malaria based on natural products. In: Tringali C, editor. Bioactive compounds from natural sources. Boca Ranton: CRC Press; 2012. p. 525–50.

[33] Köhler S, Delwiche CF, Denny PW, Tilney LG, Webster P, Wilson RJ, et al. A plastid of probable green algal origin in apicomplexan parasites. Science 1997;275:1485–9.

[34] Lichtenthaler HK, Zeidler J, Schwender J, Muller C. The non-mevalonate isoprenoid biosynthesis of plants as a test system for new herbicides and drugs against pathogenic bacteria and the malaria parasite. Z Naturforsch C 2000;55:305–13.

[35] Choi SR, Mukherjee P, Avery MA. The fight against drug-resistant malaria: novel plasmodial targets and antimalarial drugs. Curr Med Chem 2008;15:161–71.

[36] Dewick PM. The mevalonate and methylerythritol phosphate pathways: terpenoids and steroids. In: Medicinal natural products. 3rd ed. Chicester, UK: John Wiley and Sons Ltd; 2009. p. 187–310.

[37] Hunter WN. The non-mevalonate pathway of isoprenoid precursor biosynthesis. J Biol Chem 2007;282:21573–7.

[38] Okuhara M, Kuroda Y, Goto T, Okamoto M, Terano H, Kohsaka M, et al. Studies on new phosphonic acid antibiotics.1. Fr-900098, isolation and characterization. J Antibiot 1980;33:13–7.

[39] Kuroda Y, Okuhara M, Goto T, Okamoto M, Terano H, Kohsaka M, et al. Studies on new phosphonic acid antibiotics.4. Structure determination of Fr-33289, Fr-31564 and Fr-32863. J Antibiot 1980;33:29–35.

[40] Borrmann S, Adegnika AA, Matsiegui PB, Issifou S, Schindler A, Mawili-Mboumba DP, et al. Fosmidomycin-clindamycin for *Plasmodium falciparum* infections in African children. J Infect Dis 2004; 189:901–8.

[41] Borrmann S, Issifou S, Esser G, Adegnika AA, Ramharter M, Matsiegui P, et al. Fosmidomycin-clindamycin for the treatment of *Plasmodium falciparum* malaria. J Infect Dis 2004;190:1534–40.

[42] Borrmann S, Adegnika AA, Moussavou F, Oyakhirome S, Esser G, Matsiegui P, et al. Short-course regimens of artesunate-fosmidomycin in treatment of uncomplicated *Plasmodium falciparum* malaria. Antimicrob Agents Chemother 2005;49:3749–54.

[43] Borrmann S, Lundgren I, Oyakhirome S, Impouma B, Matsiegui P, Adegnika AA, et al. Fosmidomycin plus clindamycin for treatment of pediatric patients aged 1 to 14 years with *Plasmodium falciparum* malaria. Antimicrob Agents Chemother 2006;50:2713–8.

[44] Dewick PM. Medicinal natural products. Chicester, UK: John Wiley and Sons Ltd; 2009. p 1–539.

[45] Schlitzer M. Medizinische chemie der wirkstoffer gegen malaria. Pharm Unserer Zeit 2009;38:512–20.

[46] Dahl EL, Shock JL, Shenai BR, Gut J, DeRisi JL, Rosenthal PJ. Tetracyclines specifically target the apicoplast of the malaria parasite

Plasmodium falciparum. Antimicrob Agents Chemother 2006;50: 3124–31.
[47] Briolant S, Almeras L, Belghazi M, Boucomont-Chapeaublanc E, Wurtz N, Fontaine A, et al. *Plasmodium falciparum* proteome changes in response to doxycycline treatment. Malar J 2010;9.
[48] WHO. Essential medicines WHO model list. 2013.
[49] Mather MW, Henry KW, Vaidya AB. Mitochondrial drug targets in apicomplexan parasites. Curr Drug Targets 2007;8:49–60.
[50] Painter HJ, Morrisey JM, Mather MW, Vaidya AB. Specific role of mitochondrial electron transport in blood-stage *Plasmodium falciparum*. Nature 2007;446:88–91.
[51] Hussain H, Krohn K, Ahmad VU, Miana GA, Green IR. Lapachol: an overview. Arkivoc 2007;(ii):145–71.
[52] Fieser LF, Berlinger E, Bondhus FJ, Chang FC, Dauben WG, Ettlinger MG, et al. Naphthoquinone antimalarials. I. General survey. J Am Chem Soc 1948;70:3151–5.
[53] Fawaz G, Fieser LF. Naphthoquinone antimalarials. XXIV. A new synthesis of lapinone. J Am Chem Soc 1950;72:996–1000.
[54] Fry M, Pudney M. Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4′-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). Biochem Pharmacol 1992;43: 1545–53.
[55] Nayak SK, Mallik SB, Kanaujia SP, Sekar K, Ranganathan KR, Ananthalakshmi V, et al. Crystal structures and binding studies of atovaquone and its derivatives with cytochrome bc1: a molecular basis for drug design. CrystEngComm 2013;15:4871–84.
[56] WHO. WHO recommended insecticides for indoor residual spraying against malaria vectors. 2013.
[57] Rasoanaivo P, Wright CW, Willcox ML, Gilbert B. Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions. Malar J 2011;10.
[58] Willcox M. Improved traditional phytomedicines in current use for the clinical treatment of malaria. Planta Med 2011;77:662–71.
[59] Wells TNC. Natural products as starting points for future antimalarial therapies: going back to our roots? Malar J 2011;10:S3.
[60] WHO. General guidelines for methodologies on research and evaluation of traditional medicine. 2000.
[61] Willcox ML, Graz B, Falquet J, Sidibe O, Forster M, Diallo D. *Argemone mexicana* decoction for the treatment of uncomplicated falciparum malaria. Trans R Soc Trop Med Hyg 2007;101:1190–8.
[62] Graz B, Willcox ML, Diakite C, Falquet J, Dackuo F, Sidibe O, et al. *Argemone mexicana* decoction versus artesunate-amodiaquine for the management of malaria in Mali: policy and public-health implications. Trans R Soc Trop Med Hyg 2010;104:33–41.
[63] Christensen SB, Kharazmi A, Antimalarial. Natural products. In: Tringali C, editor. Bioactive compounds from natural sources. London: Taylor and Francis; 2001. p. 381–431.
[64] Zhao Y, Zheng J, Huang S, Li X, Lin Q, Zhang J. Experimental studies on the antimalarial effect of protopine derivatives. Yao Hsueh T'ung Pao 1981;16:7–10.
[65] Wright CW, Phillipson JD, Awe SO, Kirby GC, Warhurst DC. Quetinleclercq, J.; Angenot, L. Antimalarial activity of cryptolepine and some other anhydronium bases. Phytother Res 1996;10:361–3.
[66] Mesia K, Cimanga K, Tona L, Mampunza MM, Ntamabyaliro N, Muanda T, et al. Assessment of the short-term safety and tolerability of a quantified 80% ethanol extract from the stem bark of *Nauclea pobeguinii* (PR 259 CT1) in healthy volunteers: a clinical phase I study. Planta Med 2011;77:111–6.
[67] Pieters L, Mesia K, Tona L, Mampunza M, Ntamabyaliro N, Muanda T, et al. A Phase IIA and IIB clinical trial of a quantified extract of *Nauclea pobeguinii* stem bark against uncomplicated *falciparum* malaria. Planta Med 2011;77:SL65.
[68] Mesia K, Cimanga RK, Dhooghe L, Cos P, Apers S, Totte J, et al. Antimalarial activity and toxicity evaluation of a quantified *Nauclea pobeguinii* extract. J Ethnopharmacol 2010;131:10–6.
[69] Cragg GM, Newman DJ. Biodiversity: a continuing source of novel drug leads. Pure Appl Chem antim 2005;77:7–24.
[70] Bhatnagar I, Thomas NV, Kim SK. Natural flora and anticancer regime: milestones and roadmap. Anticancer Agents in Med Chem 2013;13:910–22.
[71] Kissinger JC, Brunk BP, Crabtree J, Fraunholz MJ, Gajria B, Milgram AJ, et al. The *Plasmodium* genome database—Designing and mining a eukaryotic genomics resource. Nature 2002;419: 490–2.
[72] Guiguemde WA, Shelat AA, Bouck D, Duffy S, Crowther GJ, Davis PH, et al. Chemical genetics of *Plasmodium falciparum*. Nature 2010;465:311–5.
[73] Gamo F-J, Sanz LM, Vidal J, de CC, Alvarez E, Lavandera JL, et al. Thousands of chemical starting points for antimalarial lead identification. Nature 2010;465:305–10.

This page intentionally left blank

CHAPTER

15

Evaluation of Natural Products against Biofilm-Mediated Bacterial Resistance

Tuhinadri Sen, Sanmoy Karmakar, Ratul Sarkar

Department of Pharmaceutical Technology, School of Natural Product Studies, Jadavpur University, Kolkata, West Bengal, India

OUTLINE

15.1 INTRODUCTION

Since the Golden Era of antibiotics, when the discovery of sulfonamides and penicillins brought a revolution in the world of medical science by bringing about a drastic reduction in the mortality rates, the world has gradually witnessed a slowdown in the antibiotic pipeline. The problem arising out of such paucity of new molecules is further compounded by the rapid spread of antibiotic resistance, a fact that has now triggered the alarm bells, probably warning us of a "Post Antibiotic Era."

From ancient days, microbial infections have always contributed to various diseases in plants, animals and humans. The advent of antibiotics brought hope and it was felt that mankind would be able to overcome the burden of infectious diseases. However, bacteria have been able to evolve successfully to become resistant to antibiotics. Bacteria are one of the earliest life forms to appear on earth and are found in almost every habitat on the planet. Today, the problem of antibiotic resistance is a clinical reality and it poses a major threat to mankind. Currently, much effort is being made for a better understanding of antibiotic resistance and smarter ways to overcome it [1].

Over the last 60 years, approximately several million tons of antibiotics have been released into the biosphere [2]. Moreover, the nonjudicious utilization of antibiotics has been further compounded by their irrational application in both veterinary medicine and agriculture, particularly in economically advanced countries [3,4]. Such indiscriminate use of antibiotics often leads to an increase

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00015-5

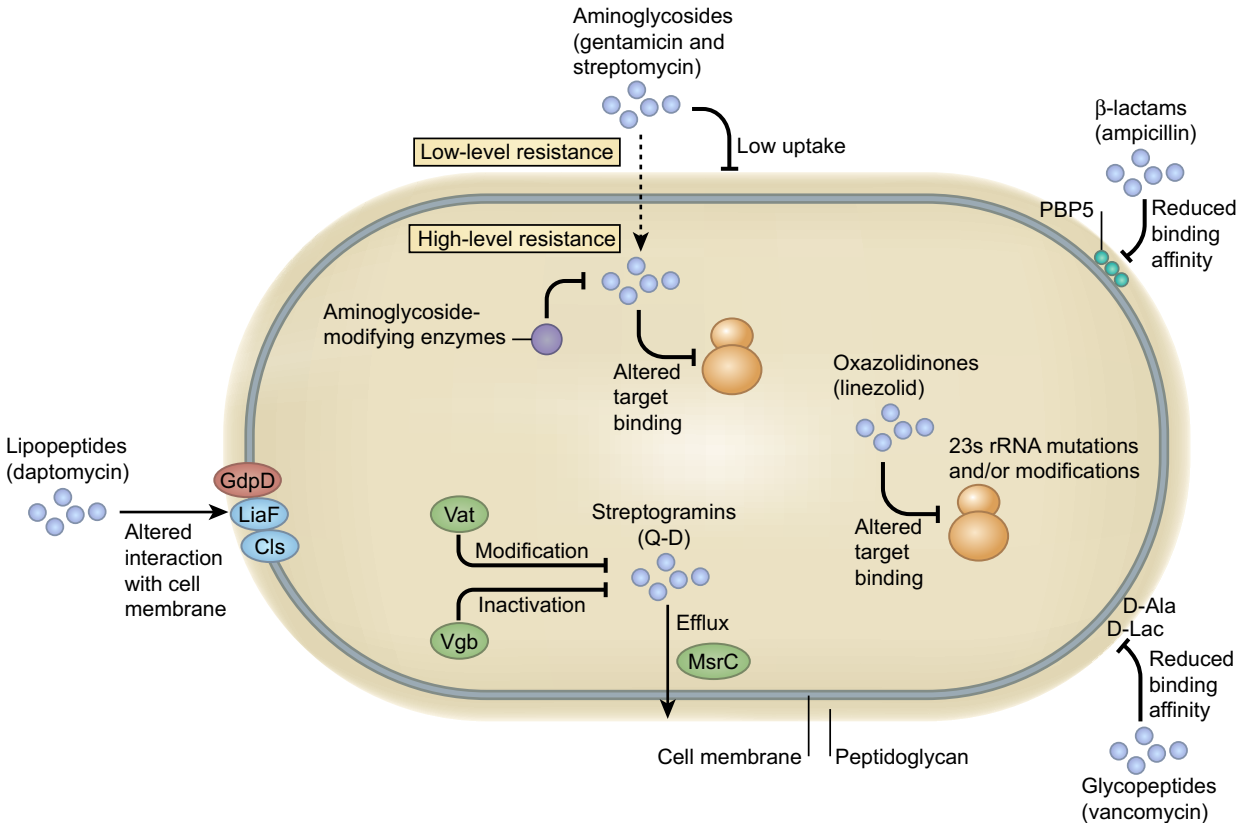


FIGURE 15.1 The main mechanisms of antibiotic resistance are shown. In *Enterococcus faecium*, resistance to ampicillin occurs through the production of penicillin-binding protein 5 (PBP5), which has low affinity for β-lactams. Enterococci exhibit intrinsic low-level resistance to aminoglycosides such as streptomycin or gentamicin owing to low uptake of these highly polar molecules. High-level resistance results from the acquisition of aminoglycoside-modifying enzymes or, for streptomycin, can result from ribosomal mutations that result in altered target binding. Resistance to the glycopeptide vancomycin occurs through a well-characterized mechanism of reduced vancomycin-binding affinity, involving alterations in the peptidoglycan synthesis pathway. Resistance of *Enterococcus* spp. to streptogramin quinupristin–dalfopristin (Q–D) involves several pathways, including drug modification (by virginiamycin acetyltransferase (Vat)), drug inactivation (through virginiamycin B lysase (Vgb)), and drug efflux (via the adenosine triphosphate-binding cassette protein macrolide–streptogramin resistance protein (MsrC)). Resistance to the oxazolidinone linezolid is rare, but the most common pathway involves mutation in the 23S ribosomal RNA ribosome-binding site. Resistance of *E. faecalis* to the lipopetide daptomycin has been shown to involve altered interactions with the cell membrane and requires the membrane protein LiaF and enzymes involved in phospholipid metabolism, such as a member of the glycerophosphoryl diester phosphodiesterase family (GdpD) and cardiolipin synthase (Cls). *Permission obtained from "Nature Publishing Group"© [13].*

in the selective pressure on the organism, compelling the physician to write expensive broad-spectrum antibiotics, thus increasing the treatment costs, duration of infection [5,6], morbidity and mortality [7–10].

According to available reports, around 25,000 lives are lost each year in Europe and such mortality has been linked to infections caused by antibiotic-resistant bacteria [11]. Similar trends have been recorded in the United States, where around 19,000 deaths (during 2005) were linked to methicillin-resistant *Staphylococcus aureus* infections [11]. As per the estimates of the US Center for Disease Control and Prevention, antibiotic resistance has been held responsible for about 2 million illnesses and 23,000 deaths per year in the United States [12]. According to another survey, it was found that treatment cost for ear infection has gone up by 20% between 1997 and 1998 [12], indicating a steep rise in antimicrobial therapy.

Several mechanisms are held responsible for the development of drug resistance, and these may be summarized as follows: (i) mutations of the antibiotic target, (ii) changes in cell permeability and multiple efflux pumps and (iii) horizontal transfer of resistance genes Figure 15.1 [14–17]. In hostile environments (stress conditions), the organisms are known to form multicellular, surface adherent communities, which are known to form a protective environment, thereby providing a safe house to the organism [18]. More precisely, bacteria entrench themselves in a self-produced hydrated matrix of polysaccharides and protein (a slimy layer), known as a biofilm [19]. The biofilm matrix acts as an environmental barrier, thereby diminishing the effectiveness of

the various antibiotics [20]. Around 70–80% cases of drug resistance has been linked to the biofilm entrapped bacteria and is a major cause of concern, particularly with reference to chronic infections [20].

The traditional medicinal systems (often containing plant-derived products) have been popularly used by different civilizations for healing purposes. Even today, some of such products are still being used for a variety of therapeutic purposes. Age old documents from India, China, Egypt, Arab and Greek literature also provide ample evidences of plant-based remedies. Despite tremendous advances in the field of chemistry, biology and medicine, natural products of plant origin still continue to play a significant role in the management of different diseases, even in this century. However, only a fraction (around 6% of 300,000 species) of the terrestrial plants (higher plants) of our globe have been evaluated (systematic studies) in true scientific terms [21].

On survey of the literature, it is evident that a major portion of bioactive compounds, with therapeutic potential, are secondary plant metabolites, which are truly not essential for either growth or development. However, the vast array of these secondary metabolites, with complex chemical structures (chemical diversity), has been found to display a diverse range of pharmacological properties. Today, scientists have started a reevaluation of these products, which are not only utilized for self defense by the plants (i.e., protection from microbes and the herbivores) but also assist in pollination and seed dispersal.

15.2 BIOFILM FORMATION

At the initial stage of biofilm formation, planktonic cells are known to swim across the surface and scan for the appropriate surface (inert or living, tissues or artificial devices) for attachment [23]. Once bacteria attach to the surface, the cells are known to shift to the resting state. The cells then use twitching motility instead of swimming motility for their movement, which are otherwise 100-fold slower [24]. At the same time, some bacterial genes related to the synthesis of extracellular polymeric substances (EPS) and alginate (*algC*) are found to be upregulated (three to fivefold increase may occur) [25,26]. Micro colonies then differentiate and form mature biofilm displaying tower or mushroom shapes [27]. During maturation, surface-attached bacteria create a protective environment around themselves by secreting EPS, which prevents the entry of the antibiotics into the biofilm. Water channels, found to be present in the biofilms, are known to facilitate the exchange of nutrients and waste products [27,28]. It has been observed that there is variable oxygen concentration (high in the periphery and decrease toward the core, thereby creating anaerobic condition) inside the biofilm. Due to the gradual decrease in oxygen and nutrition, the cells in the periphery grow at a faster rate as compared to the cells present in the core of the biofilm [27]. Thus, cells residing inside the biofilms become tolerant and start expressing a range of virulence factors during such entrapped conditions [29]. Ultimately, on return of favorable conditions, cells may detach from the biofilms (through enzymatic disruption; alginate lyase), a phenomenon that has been documented in *Pseudomonas aeruginosa* [29] Figure 15.2.

Presence of nutrients, moisture and solid surface (inert or living) are some of the most important factors that are known to influence biofilm formation. Interestingly, different species of biofilm-forming bacteria have been found to be associated with a number of diseases involving lungs, teeth, tissue, blood vessels, middle ear, or artificial medical devices (catheters, prosthesis, cardiac valves, contact lenses) [20]. Biofilms of polymicrobial nature have also been observed in chronic wounds

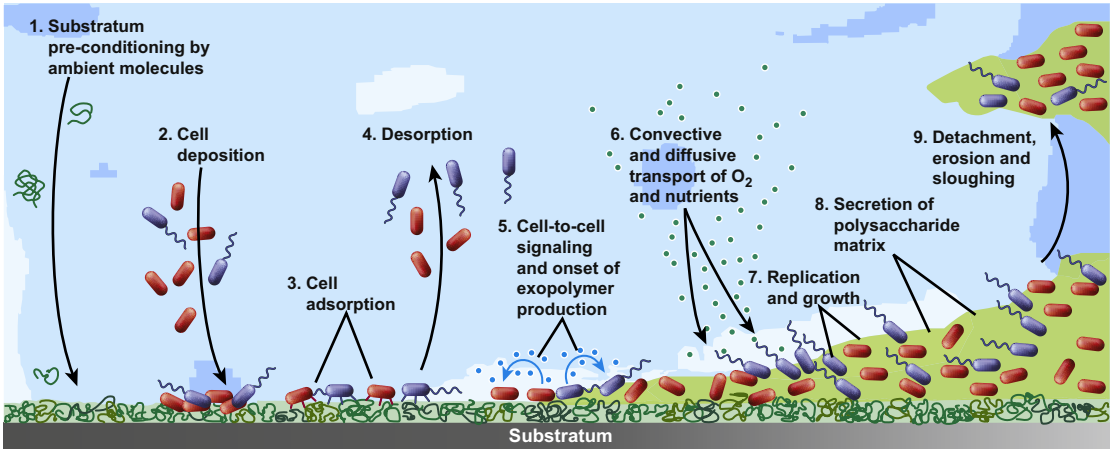


FIGURE 15.2 Processes governing biofilm formation. *Permission obtained from Elsevier Ltd © [22].*

such as foot ulcer, pressure ulcers and venous leg ulcers [30]. Microbes associated with the formation of dental plaque are also known to form biofilm, which provide a protective barrier to the cells. The slimy EPS protects the bacteria from destruction and organic acids produced by the biofilm-forming bacteria, may in turn cause teeth erosion and decay. *P. aeruginosa*, a common environmental bacterium and a major cause of persistent lung infections associated with cystic fibrosis (CF) [31], may trigger the excess secretion of thick mucus, which eventually blocks the passage of air, thereby rendering clinical management more critical [32].

Besides infecting living tissues, many bacterial species are known to colonize indwelling catheters and form biofilms, thereby inducing unnecessary complications in patient care Figure 15.3. This phenomenon is considered as one of the most common causes of health care-associated bloodstream infections (BSIs) [34]. Among the various catheter-borne infections, the complication arising out of the crystalline biofilms of *Proteus mirabilis* are considered to be the most problematic, as they are known to initiate pyelonephritis and septicemia [35]. According to recent estimates, there may be around 8.5–19.8 (per 1000 catheter day) cases of BSI (related to biofilm) that may be associated with various catheter implants [33–35] Table 15.1.

15.3 MECHANISM FOR RESISTANCE DUE TO BIOFILM

Bacteria residing inside the biofilm, display resistance against a range of antimicrobial drugs [19]. Modifications of enzymes, target site mutation and multiple efflux pumps are some of the important factors contributing to antibiotic resistance in biofilm-forming bacteria [36]. However, only a few hypotheses are currently available regarding the mechanism of antibiotic resistance through biofilms [37].

The presence of EPS, which accounts for a majority of the dry mass of the biofilm, is known to act as a mechanical barrier, thereby diminishing the effectiveness of various antibiotics [38]. Moreover, the negatively charged polymers present on the surface of the biofilm matrix are known to interact with positively charged antibiotics (e.g., aminoglycoside), thereby limiting/slowing the penetration of these drugs [39]. Additionally, some antibiotics are known to be enzymatically inactivated inside the biofilms or in other cases, may also be removed via efflux pumps [40–44].

Altered environmental conditions may trigger differential oxygen concentration, i.e., the surface of the biofilms may show higher oxygen levels, whereas a

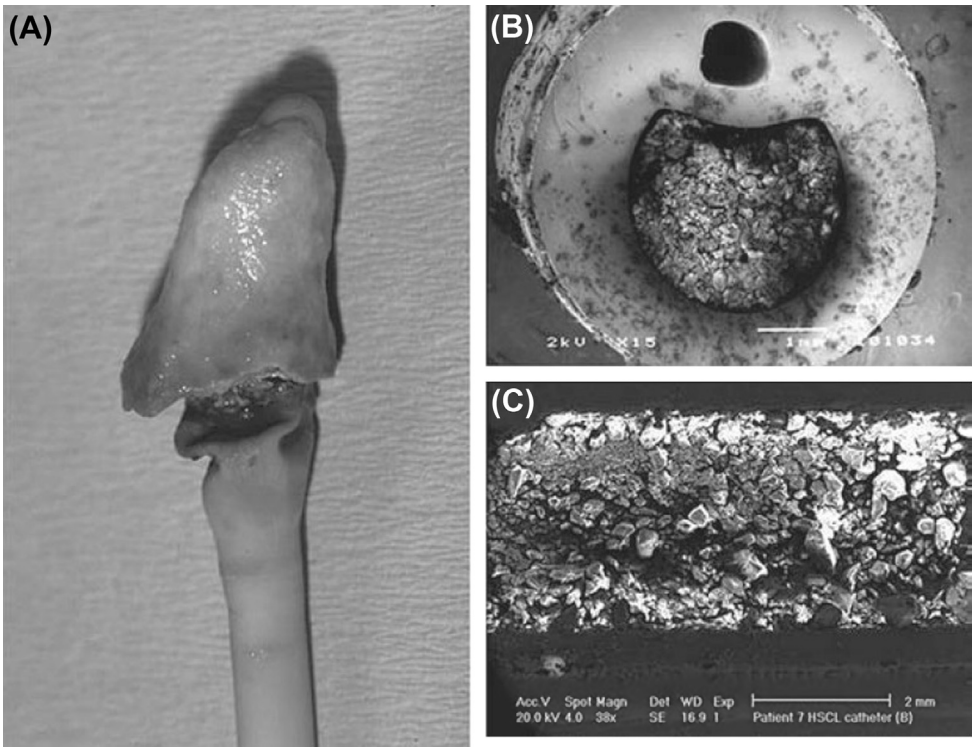


FIGURE 15.3 Images of crystalline biofilms on blocked catheters taken from patients. (A) Catheter that had been indwelling suprapubically for 6 months. It was removed surgically. Crystalline material completely covered the eyehole and balloon of the hydrogel-coated latex catheter. (B) A cross-section of a silicone catheter that had been indwelling for 8 weeks. The image shows that the central lumen is occluded by crystalline biofilm. (C) A longitudinal section of a silver–hydrogel-coated latex catheter that blocked after 11 days in situ. *Permission obtained from "Nature Publishing Group"© [33].*

TABLE 15.1 The Incidence of Bacterial Species Isolated from 106 Catheter Biofilms [33]

	Number (%) of catheters colonized by each species		
	All catheter	Mixed-species	Single-species
Species	Biofilms	Biofilms (76 catheters)	biofilms (30 catheters)
Pseudomonas aeruginosa[a]	38 (35.9)	31 (40.8)	7 (23.3)
Enterococcus faecalis	36 (34.0)	34 (44.7)	2 (6.7)
Escherichia coli	33 (31.1)	31 (40.8)	2 (6.7)
Proteus mirabilis[a]	32 (30.2)	26 (34.2)	6 (20.0)
Klebsiella pneumoniae[a]	19 (17.9)	18 (23.7)	1 (3.3)
Morganella morganii[a]	14 (13.2)	11 (14.5)	3 (10.0)
Providencia stuartii	11 (10.4)	9 (11.8)	2 (6.7)
Staphylococcus aureus[a]	11 (10.4)	10 (13.2)	1 (3.3)
Enterobacter cloacae	9 (8.5)	7 (9.2)	2 (6.7)
Klebsiella oxytoca[a]	9 (8.5)	8 (10.5)	1 (3.3)
Providencia rettgeri[a]	5 (4.7)	4 (5.3)	1 (3.3)
Coagulase-negative staphylococci[a]	5 (4.7)	4 (5.3)	1 (3.3)
Citrobacter species	4 (3.8)	4 (5.3)	0 (0.0)
Proteus vulgaris[a]	3 (2.8)	2 (2.6)	1 (3.3)

[a]*Indicates species capable of producing urease.*
Permission obtained from "Nature Publishing Group"© Stickler (2008).

scarcity of oxygen may be observed toward the core of the biofilms, thereby making the core less accessible to the antibiotics, namely, aminoglycosides [45–49]. In chronic lung infections (*P. aeruginosa*) associated with CF, the organism residing in the core of the biofilm may become nondividing due to scarcity of nutrients and oxygen and such low metabolic state of nondividing cell often acts as a barrier to the antibiotic activity [20,50,51]. The altered osmotic environment within the biofilms may trigger a stress response altering the relative proportion of porins (channels) and this may lead to reduced permeability of antimicrobials Figure 15.4 [52]. In certain conditions, bacteria may differentiate into a protected phenotype state, thereby enhancing the tolerance of the cells [43].

15.4 BIOFILM AND QUORUM SENSING

Following an infection, the cells start displaying an ability to express certain chemicals that are necessary for development of virulence, which is normally achieved in a well-regulated fashion. A well-coordinated expression of virulence factors has been found to be necessary for survival of the bacterium as well as for the purpose of invasion [53]. When the bacterial population reaches a quorum, cell-to-cell communication is established through certain chemical signaling molecules (autoinducers), which at a minimum threshold concentration, may influence the expression of certain genes. As bacteria grow, they communicate (both intra and interspecies) with each other by utilizing the chemical signals (mentioned above) and the process is known as quorum sensing (QS) [53–55]. Different microorganisms are known to utilize QS for regulating antibiotic production, virulence, bioluminescence, motility, symbiosis and biofilm formation [54,55]. In other words, as the QS signaling molecules reach an optimum concentration, these chemicals diffuse back into the cell and regulate the expression of certain genes and also coordinate their behavior in a group-based manner, assisting in bacterial colonization [56–58]. Thus, QS is perceived to provide evolutionary advantages to the bacterium, which include improved access to environmental niches and an enhanced ability to combat with its competitors [59,60].

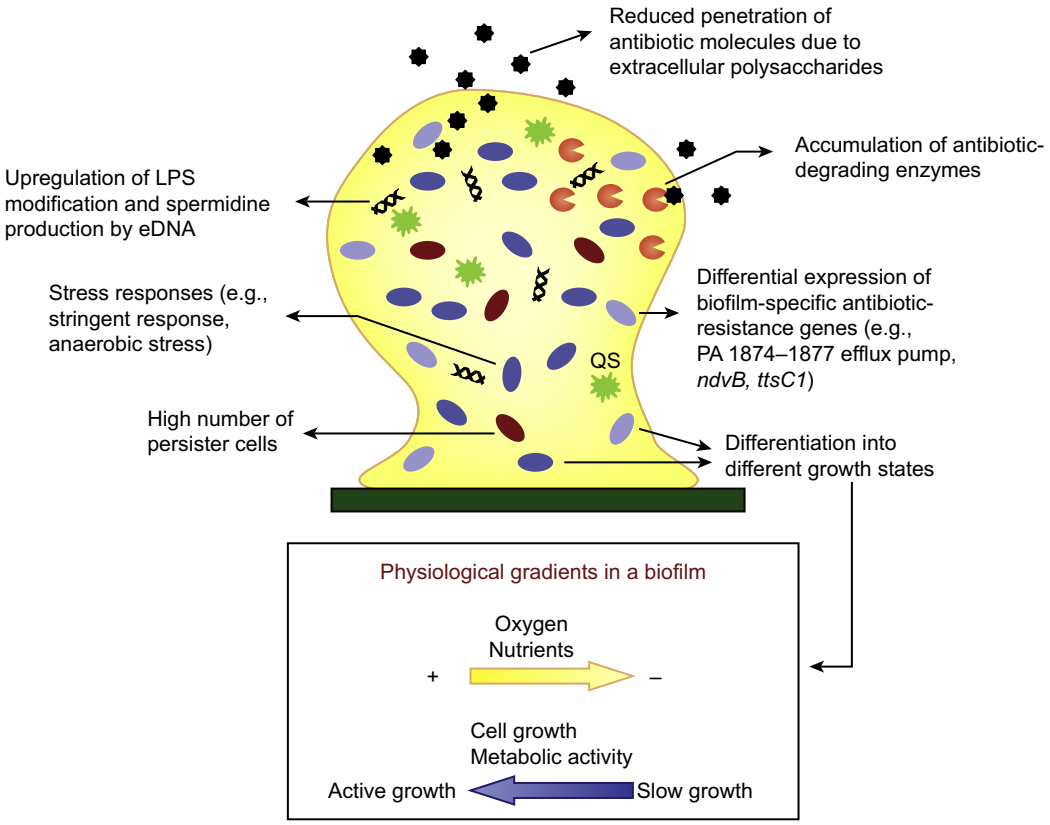


FIGURE 15.4 Schematic representation of a *Pseudomonas aeruginosa* biofilm indicating various examples of adaptive resistance mechanisms exhibited during the multicellular growth state. The box below shows the gradients of oxygen and nutrients formed within the biofilm structure and how they relate to cell differentiation into different growth states. Abbreviations: QS, quorum sensing signal; eDNA, extracellular DNA. *Permission obtained from Elsevier Ltd © [18].*

The QS was first described in *Vibrio fischeri*, a bioluminescent marine bacterium, known to colonize the light organ of the *Euprymna scolopes* [61]. In this microorganism, the bioluminescent luciferase production is known to be under the regulatory control of the QS mechanism (LuxI is the autoinducer synthase responsible for the production of *N*-acylhomoserine lactones (AHLs); LuxR, the receptor for AHLs, can interact with DNA, thus acting as a transcriptional activator), which in turn modulate the expression of the *luxICDABE* (the luciferase operon) [62].

AHL is one of the main QS signaling molecules found in gram-negative bacteria, whereas oligopeptide-based signaling is observed in gram-positive bacteria. More than 70 species of gram-negative bacteria are known to use AHL as a signaling molecule [63,64]. Apart from the lactones, both gram-positive and gram-negative bacteria also utilize a common signaling molecule (furanosyl borate), known as autoinducer-2 (AI-2) [65–67]. Besides these, an autoinducer AI-3 has also been reported to be present in enterohemorrhagic *Escherichia coli* (EHEC; O157:H7) and in several species of intestinal bacteria [68].

15.4.1 AHL-Dependent Bacterial QS Systems

In case of *P. aeruginosa*, the LasR-I and RhlR-I systems are involved in QS [28]. The lactone synthase gene (such as luxI) is responsible for the synthesis of the AHL molecule, which is secreted in the media [28]. As the cell density increases (reach the quorum), the AHLs present in the media attain a critical concentration and during this phase, the AHLs diffuse back into the cell and subsequently interact with the transcriptional regulators. *P. aeruginosa* produces two AHLs molecules, 3OC12--HSL and C4-HSL, which bind to their respective transcriptional regulators, LasR and RhlR [28,69,70]. The AHL-regulator complex thus formed, may then activate the transcription of a number of genes, including *lasB*, *toxA*, *rhlR* and *lasI* [70,71]. It is also interesting to note that different concentrations of AHLs may be necessary for regulating a diverse range of target genes [72] Figure 15.5.

15.4.2 Peptide-Based QS System

Gram-positive bacteria such as *S. aureus* utilize modified autoinducing oligopeptides (AIP) as signaling

molecules, that are known to recognize the membrane-bound histidine kinases as receptors [62,73]. This AIP peptide (I–IV) is encoded by *agrD* [62]. The membrane-bound protein AgrB is known to modify AIP (as peptide signals are not diffusible through membrane) through the addition of a thiolactone ring and its subsequent export to the extracellular media [74]. At a critical concentration, extracellular AIP (signify high population density) binds to AgrC (membrane-bound sensor kinase), ultimately leading to autophosphorylation of AgrA [75,76]. The phosphorylated AgrA is responsible for the expression of *agrBDCA* along with the other secreted factors. It has been observed that the increase in cell density leads to activation of the *agr* system, which ultimately switches the bacterium from an adhesive, colonizing commensal to an invasive as well as aggressive pathogen by increasing the secretion of toxins and proteases Figure 15.6 [77].

Every gram-positive bacterium uses a unique signal for communication [62]. In *S. aureus*, *the agr* system is known to exist in four polymorphic forms, categorized as I–IV [76,78]. Interestingly, each group has a distinct AIP, different from the other, with an ability to activate its AgrC but inhibit the activation of the other receptor from the same group [76,79]. It may also be important to note that coinfection with two different groups of *S. aureus* often lead to a competition and the group of cells demonstrating faster expression of QS signals may cause suppression of the others [80].

15.4.3 AI-2 System

AI-2, a furanosyl borate diester is another signaling molecule, which is found in both gram-negative and gram-positive bacteria. It can serve as an inter- and intra-species signal molecule [54,62,81]. This signaling system was first reported in *Vibrio harveyi*, but later on it has been detected in a wide range of bacteria including *E. coli*, *Salmonella typhimurium*, *Porphyromonous gingivalis*, and *Streptococcus gordonii* [54,62]. It has also been observed that some bacterial species are unable to produce their own AI-2 synthase but are capable of AI-2 signal transduction, and by this manner they can sense as well as monitor a variety of bacteria in their immediate surroundings. In *E. coli* and *S. typhimurium*, the AI-2 system is regulated by the luxS-regulated operon (*lsr*) [82]. AI-2 is synthesized by luxS (metalloenzyme) from the precursor molecule *S*-adenosyl homocysteine (SAH).

Apart from the diffusion, AI-2 is known to be exported by YdgG, a membrane-bound extracellular spanning protein [83]. As observed with other signaling molecules, AI-2 at a critical extracellular concentration traverses into the cell using the Lsr transporter (constituted by LsrB, LsrC, and LsrA), where it undergoes phosphorylation by Lsrk and this phosphorylated AI-2 is known to regulate the *Lsr* operon [84,85] Figure 15.7.

15.4.4 Other QS Systems

A natural variation in the QS system can also be observed in bacteria that do not express the signaling molecule but respond to those produced by others: (i) *E. coli*, which possess SdiA, a homolog of LuxR [87] and (ii) in CF, *Burkholderia cepacia* respond to QS signals produced by *P. aeruginosa* [88]. The QS system in *Candida albicans*, (fungal pathogen) is known to produce farnesol, which has been found to be necessary for its virulence [89,90].

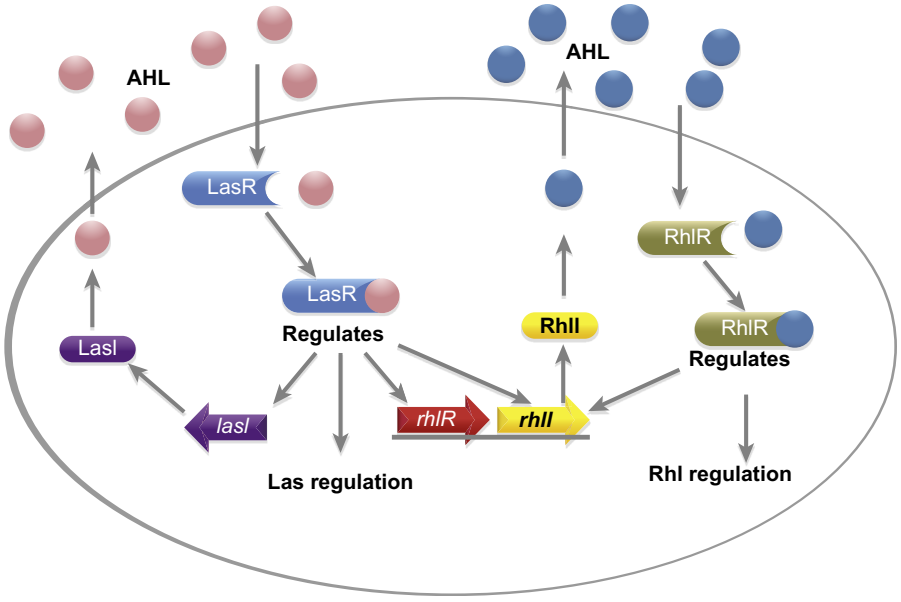


FIGURE 15.5 QS pathway showing the LasR-I and RhlR-I systems of *Pseudomonas aeruginosa*. The LasI autoinducer is represented by pink circle and the RhlI autoinducer is shown as blue circle. When the cells attain the quorum, AHLs secreted into the media, attend a critical concentration, and during this phase, the AHLs once again diffuse into the cell and subsequently interact with the transcriptional regulators (LasR – blue; RhlR- green) and regulate the expression of *lasB, toxA, rhlR,* and *lasI*.

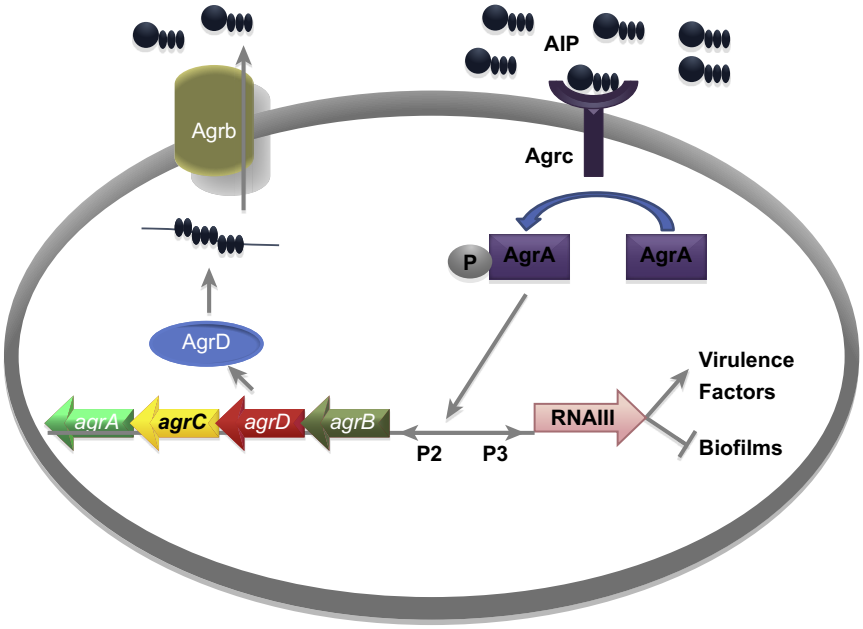


FIGURE 15.6 The quorum sensing pathway in gram positive *Staphylococcus aureus*. Auto inducer peptide (AIP - small dark blue circles; encoded by AgrD) is modified by the membrane bound protein AgrB (through addition of a thiolactone ring) and then it is exported to the external media. As the extracellular AIP reaches a critical concentration, it binds to AgrC, a membrane-bound sensor kinase (trans membrane receptor histidine kinase) of *S. aureus*. This causes autophosphorylation of AgrA and this phosphorylated form of AgrA is responsible for the expression of RNAIII (repress cell adhesion factors) along with *agrBDCA* (P2 – promoter for *agrBDCA* and P3 – for RNAIII) which in turn increases the AIP level.

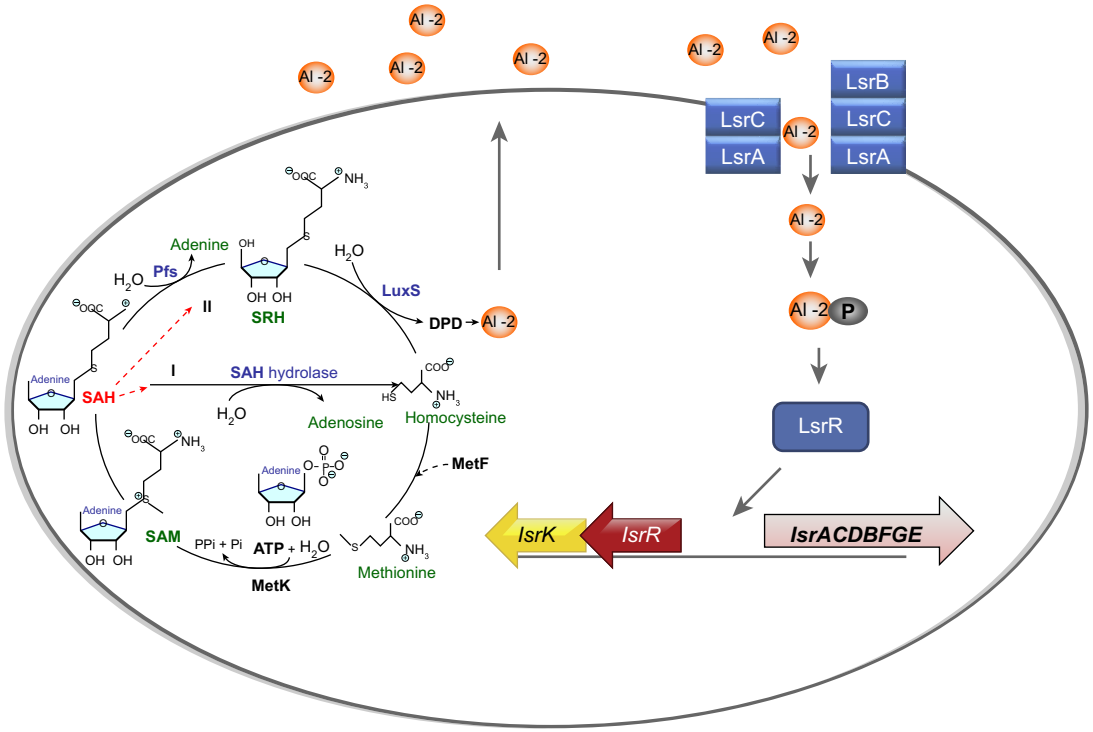


FIGURE 15.7 The analogous *lsr* QS signaling circuits in *Escherichia coli* and *Salmonella typhimurium*. Synthesis of autoinducer AI-2 by LuxS. During AI-2 biosynthesis, the methyl transfer from *S*-adenosylmethionine (SAM) to its various substrates leads to the production of the *S*-adenosyl homocysteine (SAH). SAH is toxic and is removed in two ways, either using one-step conversion (I) through SAH-hydrolase or using a two-step conversion (II) by Pfs and LuxS enzymes. The Pfs nucleosidase enzyme hydrolyzes adenine from SAH to form *S*-ribosylhomocysteine (SRH). LuxS acts on SRH to produce 4,5-dihydroxy-2,3-pentanedione (DPD) and homocysteine. DPD undergoes further rearrangements to produce the active AI-2 molecule. *Figure modified, with permission, obtained from Elsevier Ltd © [86].*

15.5 QUORUM SENSING AS A TARGET FOR ANTIMICROBIAL THERAPY

The emergence of antibiotic resistance is an insurmountable problem, which necessitates the development of novel therapeutic approaches to combat this global issue [92]. According to researchers, targeting the QS citadel, without necessarily inducing lethal effects may turn out to be a useful approach against the emerging threat of escalating antibiotic resistance [93]. The new-generation QS-inhibitory compounds might serve as a viable alternative to overcome the massive challenge posed by the drug-resistant pathogens, at least in part. Several strategies have been considered for interrupting/disrupting the bacterial QS system, namely, (a) inhibition of signal generation, (b) inhibition of signal dissemination and (c) inhibition of signal reception Figure 15.8.

15.5.1 Inhibition of Signal Generation

Bacteria communicate through small signaling molecules, which enable them to express genes responsible for their survival in the hostile environment. Thus, these signaling molecules and the process involved in it might prove to be sensitive clinical targets for overcoming the problem of bacterial resistance.

According to reports, various analogs of *S*-adenosylmethionine (SAM; *S*-adenosylhomocysteine, butyryl-SAM, *S*-adenosylcysteine, including holo-ACP and sinefungin) can potentially inhibit the synthesis of AHLs by the *P. aeruginosa* RhlI protein [93–95].

The precursor molecule SAM gets converted to methylthioadenosine (MTA) or SAH by transfer of methyl moiety [85]. Thereafter, it is further catalyzed by 5′-MTA/*S*-adenosylhomocysteine nucleosidase (MTAN/Pfs) to produce AHLs and AI-2 [85]. Inhibiting this process could potentially alter both AI-1 and AI-2 signaling and this can be achieved by MTAN inhibitors [96]. Analogs of MTA, with alkyl or aryl substitution, may act as potent Pfs inhibitors [97]. The observation suggests that inhibition of MTAN could be a possible strategy as it targets both AHL and AI-2 of the QS system [85].

The scientific literature claims that the inhibition of *S*-ribosylhomocysteinase (LuxS) might not affect growth and survival but could play a major role in

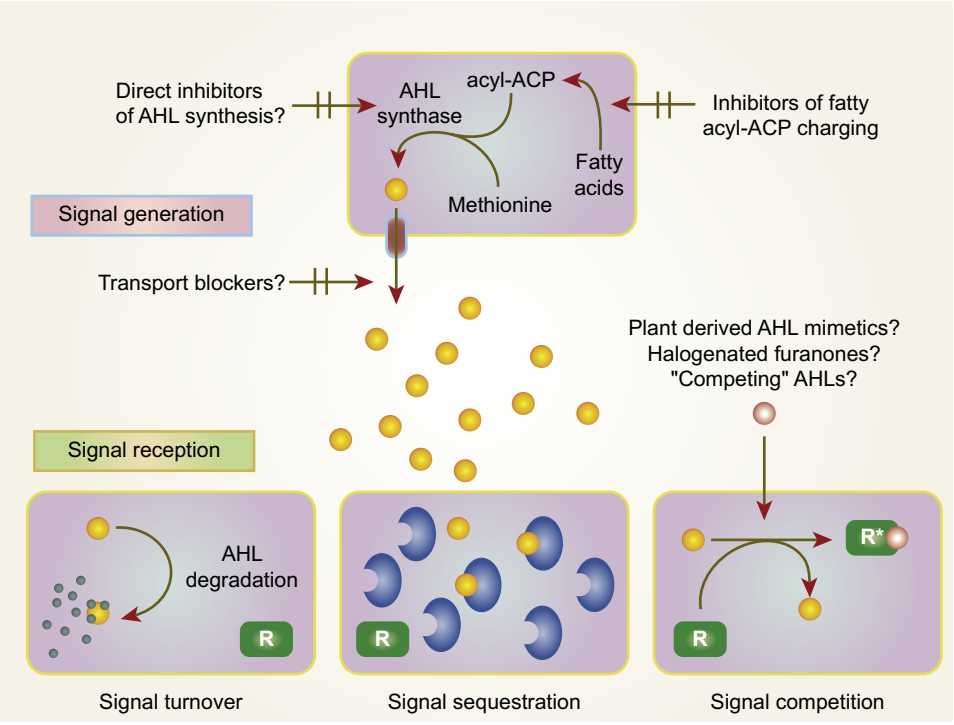


FIGURE 15.8 Potential targets and intervention strategies disrupting quorum sensing (QS) in bacteria. In principle, QS can be disrupted at two levels: signal generation or signal perception. For the former, one target would be to directly inhibit synthesis of *N*-acyl homoserine lactone (AHL) by the LuxI homolog (for example, by the application of a cell-permeable specific inhibitor), although this has yet to be accomplished. However, inhibitors that affect the synthesis of fatty acyl-acyl carrier protein (acyl-ACPs; one of the substrates for the AHL synthase) are known to reduce AHL production in vitro, so this approach has promise. Other targets for drug development are the transport proteins required by the long-chain AHLs to cross the cell envelope (in both directions). A different set of strategies has been developed to disrupt signal perception. These include degradation of the AHL molecule, sequestration of AHL, or competition for binding of the cognate signal molecule to its receptor by AHL-mimetic compounds. *Permission obtained from "Nature Publishing Group"© [91].*

QS inhibition. LuxS is responsible for the detoxification of the SAH and synthesize of 4,5-dihydroxy-2,3-pentanedione [85,98]. It will be important to mention that several substrate analogs like *S*-anhydroribosyl-L-homocysteine and *S*-homoribosyl-L-cysteine, (2*S*)-2-amino-4-[(2*R*,3*S*)-2,3-dihydroxy-3-*N*-hydroxy-carbamoylpropylmercapto] butyric acid, and (2*S*)-2-amino-4-[(2*R*,3*R*)-2,3-dihydroxy-3-*N*-hydroxy carbamoyl propyl mercapto] butyric acid are known to inhibit LuxS and could be further explored to address the problem of biofilm formation [99,100].

Hence, it is necessary to explore the natural bioresources for SAM and SAH analogs, which could serve as specific inhibitors of the QS signal generation, without interfering with the eukaryotic enzymes that may be utilizing a similar substrate [84,92].

15.5.2 Inhibition of AHL Signal Dissemination

Inactivation of cell-to-cell communication can also be achieved through the inactivation or complete degradation of the generated signal molecules. This can be achieved by different methods: chemical degradation, enzymatic destruction or metabolism of the AHLs. AHL signals are known to be inactivated by hydrolysis at alkaline pH [101]. According to reports, alkalinity is known to suppress the expression of QS-controlled genes and virulence factors [95].

The AiiA enzyme found in *Bacillus* species are known to behave as a catalyst, bringing about lactonolysis of AHL molecules [102,103]. Such AHL lactonases (homologs) are not limited to *Bacillus* species; they are also known to be present in *P. aeruginosa* (PAI-A), *Arthrobacter* sp, *Klebsiella pneumoniae*, *Agrobacterium tumefaciens*, and *Rhodococcus* sp. It has been observed that expression of AiiA in transgenic tobacco plants can make the plant less susceptible to infection by *Erwinia carotovora*, as compared to their wild-type counterparts, a phenomenon attributed to reduced release of AHLs [103,104]. However, AiiA enzyme-induced lactonolysis (opening of the ring structure) of AHL signal molecules are known to be reversed in acidic conditions [105].

The acyl-amide bond between the acyl chain and the lactone ring of AHLs gets hydrolyzed by AHL acylases, which ultimately leads to the production of fatty acid chain and a homoserine lactone moiety [106,107], and this breakdown is known to be nonreversible Figure 15.9. AHLs are also known to be inactivated by oxidoreductases, which are known to oxidize or reduce the acyl side chains [108]. However, these oxidoreductases are the least studied of all the AHL-targeting enzymes [109].

It has also been observed that the oxidized form of AHLs (3-oxo-C12 HSL) are able to react with oxidized halogen compounds, namely, hypobromous and hypochlorous acids. This phenomenon has been observed in *Laminaria digitata* (marine algae), which is known to secrete oxidized halogen compounds that are able to interfere with the QS signaling pathway of colonizing bacteria, thereby interfering with QS-controlled gene expression [110].

15.5.3 Inhibition of AHL Signal Reception

Inhibition of bacterial QS signaling may also be achieved through blockade or destruction of the receptor protein, i.e., LuxR homolog. Blockade of Lux-R receptor is a widely explored approach that has been utilized for inhibition of QS [95]. Competitive inhibitors (*N*-(phenyl sulfanyl acetyl)-L-HSL, *N*-(benzyl sulfanyl acetyl)-L-HSL, *N*-(phenethyl sulfanyl acetyl)-L-HSL, and *N*-(heptyl sulfanyl acetyl)-L-HSL) displaying structural similarity to the native AHL signals, are known to bind to the AHL-binding site of LuxR-type receptor,

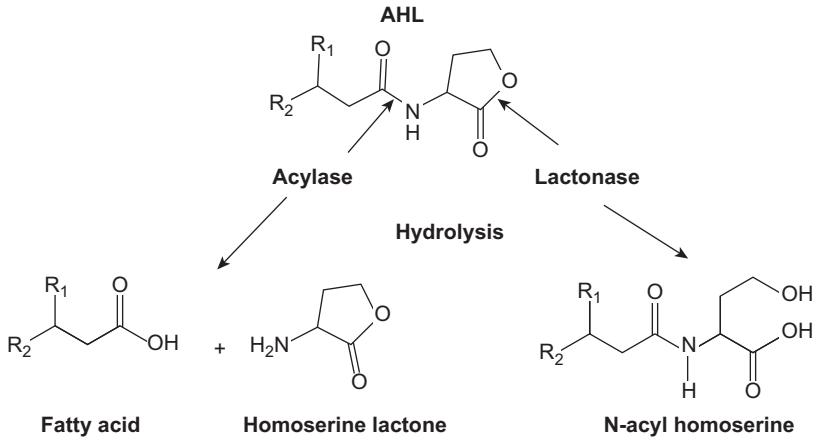


FIGURE 15.9 Enzymatic degradation of AHL by the acylases, which causes hydrolyse of the acyl-amide bond existing between the acyl chain and the lactone ring of AHLs.

thereby blocking downstream gene regulation [92,94]. Molecules with little or no structural similarity ((*Z*)-4-bromo-5-(bromomethylene)furan-2(5*H*)-one and (*Z*)-5-(bromomethylene)furan-2(5*H*)-one) are known to block the receptor in a noncompetitive fashion [111].

The designing of Lux-R blockers may be achieved by (i) substitutions in the acyl side chain of the lactone ring, (ii) substitutions or alterations in the lactone ring without altering the acyl side chain and (iii) extensive modifications in the acyl side chain and also in the lactone ring [95]. The acyl side chain may be modified in several ways, and it has been shown that the length of the side chain may be crucial for the biological activity. AHL analogs with a longer side chain may display higher inhibitory properties (Lux-R inhibition) as compared to those with a shorter side chain [112]. It has also been observed that the chirality of the homoserine lactone may be an important factor responsible for governing the biological activity.

According to available reports, certain macrolide antibiotics (at sublethal concentrations) are known to inhibit AHL-mediated signaling pathway as well as its synthesis in *P. aeruginosa* [113–115]. It still remains unclear as to how these antibiotics interfere or modulate the QS circuits since these compounds are known to inhibit protein synthesis at the ribosomal level.

15.6 NATURAL PRODUCTS AS A QS INHIBITOR

As bacteria have evolved, so did plants, in a manner so as to safeguard themselves from predators (herbivores) and pathogenic microbes. Thus, the antibacterial agents derived from the natural source (plants) may serve as an effective alternative, due to the presence of secondary metabolites, which are known to enjoy selectional advantages against the resistance organisms [116]. Since time immemorial, plant-derived compounds have been in use for the management of a variety of microbial infections [117]. Considering the therapeutic potential of natural products, there has been a renewed interest for evaluating the efficacy of natural products against the QS system. Recently, phytochemicals (particularly the secondary metabolites and natural peptides) have attracted increased scientific attention as they may serve as a useful source of anti-QS compounds [118–120].

According to the reports, spices such as garlic [121], ginger, cinnamon [122], clove [123], cumin [124] and turmeric [125] have been found to display QS-mediated biofilm inhibitory properties.

Turmeric (*Curcuma longa*) is a common dietary phytochemical that has been evaluated in QS-mediated biofilm formation studies, performed in *E. coli*, *P. aeruginosa* (PAO1), *P. mirabilis*, and *Serratia marcescens*. The findings suggest that curcumin derived from *C. longa* significantly inhibits the formation of biofilm by reducing exopolysaccharide as well as alginate production. Curcumin is also known to interfere with swimming and swarming motility in uropathogens [125], probably by interfering with the signal molecules of the QS system. Studies have also shown that curcumin enhances the susceptibility of the organism to conventional antibiotics [125].

Zingerone, one of the main chemical constituents of ginger (*Zingiber officinale*), displays the ability to inhibit as well as eradicate biofilms formed by *P. aeruginosa*. In a study conducted with ginger extract, pretreatment with the extract effectively reduced the production of EPS [126]. Zingerone was also found to produce significant reduction in swimming, swarming and twitching motility. The compound also increased the susceptibility of the organism to conventional antibiotics, when used in combination with the standard antibiotics such as ciprofloxacin [127].

Garlic (*Allium sativum*) was found to block QS-mediated biofilm formation and virulence development. DNA microarray-based transcriptomic analysis indicated the effective-ness of crude garlic extract on QS-regulated gene expression in *P. aeruginosa*. It was also observed that garlic extract displays the potential to inhibit QS (inhibition of LuxR-type receptor) [52], thereby downregulating QS-controlled gene expression. Moreover, treatment with garlic extract may also increase the susceptibility of biofilm toward tobramycin, probably by reducing the tolerance of the organism [94,121].

Emodin (extracted from rhubarb) was found to inhibit biofilms formed by *P. aeruginosa* and *Stenotrophomonas maltophilia*. This pure compound was found to decrease cell adherence and produce degradation of recombinant TraR (a transcriptional regulator similar to LasR) expressed in *E. coli* (BL21DE3) [128].

A number of polyphenols (hydroxycinnamic acid, rutin, epicatechin) have been found to block QS in *Chromobacterium violaceum* [129]. Similarly, furocoumarins from grapefruit and limonoids from orange seeds are known to interfere with AI-1 and AI-2 activities in *V. harveyi* [129]. Flavanones (naringenin and taxifolin) are known to inhibit QS (reduced AHL production) in *P. aeruginosa*, thereby reducing the expression of lasA, lasB, phzA1, and rh1A [129].

Essential oils are aromatic oily liquids from plant materials and are well known for their antibacterial properties [130]. Essential oils of *Piper bredemeyeri*, *Piper brachypodom*, *Piper bogotence*, *Gaultheria procumbens* L., *Achillea millefolium* (yarrow), *Syzygium aromaticum* (clove), *Coriandrum sativum* (coriander), *Cinnamomum verum* (cinnamon), thyme and *Origanum vulgare* (oregano) showed inhibitory effects on biofilm formation at sublethal concentrations [118,123,131–134]. Clove oil significantly reduced *las* and *rhl*-regulated

virulence factors (LasB, total protease, chitinase and pyocyanin). Moreover, it reduced swimming motility, exopolysaccharide production (sublethal concentration) and biofilm formation by *P. aeruginosa* (PAO1) and *Aeromonas hydrophila* [135]. The methanolic extract of marula (stem bark of *Sclerocarya birrea*) was found to display antibiofilm properties (evidence from biochemical and microscopic analysis). It was found to inhibit various QS-dependent virulence factors in *P. aeruginosa* (swarming motility protease and pyoverdin production) [136]. The methanolic root extract of *Buchanania lanzan* is known to reduce biofilm formation and also display the ability to disruption of biofilms produced by *P. aeruginosa* [137].

Traditional Chinese medicinal herbs have also been screened for antibiofilm properties against microbes like *C. violaceum* and *P. aeruginosa* [138]. According to reports, traditional Chinese medicinal herbs (*Prunus armeniaca*, *Prunella vulgaris*, *Nelumbo nucifera*, *Panax notoginseng* (root and flower), *Punica granatum*, *Areca catechu*, and *Imperata cylindrical*) were found to interfere with violacein (purple pigment) production in *C. violaceum* and swarming in *P. aeruginosa* [138]. Alcoholic extracts of some ornamental and medicinal plants of Egypt were investigated for anti-QS activity against *C. violaceum* [139]. Findings also reveal information about the effectiveness of some plants (leaves of *Adhatoda vasica* Nees, *Bauhinia purpurea* L., *Lantana camara* L., *Myoporum laetum* G. Forst., the fruits of *Piper longum* L., and aerial parts of *Taraxacum officinale* F.H. Wigg) against the QS system [139].

The medicinal plants of southern Florida have also been studied in detail for anti-QS properties. Aqueous extracts of *Conocarpus erectus*, *Callistemon viminalis*, and *Bucida buceras* were found to display antibiofilm properties along with significant inhibition of LasA protease, LasB elastase, and pyoverdin production [92]. Moreover, both *B. buceras* and *C. erectus* produced significant downregulation of *lasI*, *lasR*, *rhlI*, *rhlR* and reduced the concentrations of *N*-3-(oxododecanoyl)-L-homoserine lactone and *N*-(butanoyl)-L-homoserine lactone [41,92]. Antibiofilm activity of some South African medicinal plants has also been evaluated against *Listeria monocytogenes* biofilms [140]. The ethyl acetate extract of *Acacia karroo* and *Plectranthus ecklonii* showed higher antibiofilm properties as compared to the others [140]. Epicatechin, β-sitosterol and epigallocatechin, which were isolated from the ethyl acetate extract of *A. karroo* and *P. ecklonii*, exhibited higher disruption of biofilms formed by *L. monocytogenes* [140]. Table 15.2 summarizes the effects of different plants/plant products on QS-mediated biofilm formation.

A number of plants (legumes, pea, alfalfa) have been found to produce lactonase activity (AHL-degrading property). Plant-derived gamma aminobutyric acid has been found to cause degradation of AHL in *A. tumefaciens* [129,141].

15.7 SYNERGY WITH THE NATURAL PRODUCTS AND CONVENTIONAL ANTIBIOTICS

Many bacteria that are associated with plants or animals are known to utilize the QS system for intercellular communication [153]. Such relationship in plants may be beneficial (symbiotic; *Pseudomonas aureofaciens*) or detrimental (pathogenic; *E. carotovora*). The AHL-producing *P. aureofaciens* has been found to produce some antibiotics (utilizing the PhzR/I QS system) that have been found to be useful for the wheat plant [153].

Bacteria residing within a biofilm have been found to withstand antibiotics (up to a 1000-fold concentration) when compared to their planktonic counterparts [34]. As antibiotics are unable to penetrate the biofilms, the biofilm cover helps the cell to survive inside the biofilms for considerable period of time. The susceptibility of the bacteria to the antibiotics can be enhanced by altering (breaking/disrupting) the biofilm structure and exposing the cells to the antibiotic containing external medium. As mentioned previously, a combination of zingerone and ciprofloxacin demonstrated enhanced antibiofilm properties as compared to the individual molecules, displaying a synergistic role toward biofilm formation and thus the combination was found to eradicate the biofilms and reduce cell viability in a much efficient fashion [127]. Similarly, alpha-lipoyl andrographolide demonstrated synergistic effects on biofilm, when it was combined with azithromycin, ciprofloxacin, fosfomycin, streptomycin and gentamicin probably through inhibition of biofilm synthesis, as evident from the reduced production of EPS and pyocyanin [154]. In another study, terpenes (eugenol, menthol, and thymol) in combination with fluconazole produced synergistic activity on *C. albicans* biofilm [155]. Thymol with fluconazole showed better synergy as compared to the others. Curcumin was found to enhance the susceptibility of urinary tract pathogens (*E. coli*, *PAO1*, *P. mirabilis*, and *S. marcescens*) toward less sensitive antibiotics, namely, clindamycin, azithromycin, and erythromycin [125]. Therefore, synergistic combination could be useful, considering the fact that the concentration used in the combination is often found to be less than that of the individual minimum inhibitory concentration values, thus curbing the possibilities of resistant development by interfering with the biofilm integrity [125,127,155].

15.8 CONCLUSIONS

The world today is plagued with the growing menace of multidrug-resistant bacteria, a major concern for both public health professionals and the state exchequer. As evident from recent reports, hospital-acquired infections

TABLE 15.2 The effects of different plants on QS-mediated biofilm

Source	Extract (parts used)/Active ingredients	Nature of activity	Effective against (organism)	Reference
Croton nepetaefolius (Euphorbiaceae)	Casbane diterpene	Inhibit biofilm formation	*Streptococcus mutans*	[142]
Acacia karroo (Fabaceae)	Epigallocatechin, β-sitosterol	Reduction in cell numbers and no development of a biofilm	*Listeria monocytogenes*	[140]
Centratherum punctatum (Asteraceae)	Sesquiterpene lactone	Inhibit AHL production and elastase activity	*Pseudomonas aeruginosa*	[143]
Cuminum cyminum (Apiaceae)	Methyl eugenol	Reduce the AHL-dependent production of violacein, bioluminescence, and biofilm formation	*P. aeruginosa*, *Proteus mirabilis*, and *Serratia marcescens*	[124]
Garlic extract (Amaryllidaceae)	*p*-coumaric acid	Inhibit biofilm formation and the expression of bacterial virulence factor: Antagonized the activity of LuxR, AhyR, and TraR receptor	*P. aeruginosa*, *Escherichia coli*, *Agrobacterium tumefaciens*, *Chromobacterium violaceum*, and *Pseudomonas putida*	[55,121,144]
Curcuma longa (Zingiberaceae)	Curcumin	Attenuate the QS-dependent factors, such as exopolysaccharide production, alginate production, swimming and swarming motility	*E. coli*, *P. aeruginosa PAO1*, *P. mirabilis*, and *S. marcescens*	[125]
Zingiber officinale (Zingiberaceae)	Zingerone	Reduced swimming, swarming, and twitching motility, Inhibit biofilm formation	*P. aeruginosa*	[126,127]
Quercus infectoria G. Olivier (Fagaceae)	Tannic acid	Effect on bacterial cell surface hydrophobicity causes a reduction in biofilm formation	*Staphylococcus aureus*	[145]
Orange; *Citrus sinensis* (Rutaceae)	*O*-glycosylated flavanone, Naringin	Inhibit biofilm formation, swimming and swarming motility; induce the transcription levels of yenR, flhDC, and fliA gene	*C. violaceum*	[146]
Piper bredemeyeri, *Piper Brachypodom*, and *Piper bogotence* (Piperaceae)	Essential oil	Inhibit violacein production	*C. violaceum*	[131]
Coriandrum sativum (Apiaceae)	Essential oils	Affects biofilm formation	*Candida albicans*	[147]
Clove; *Syzygium aromaticum* (Myrtaceae)	Essential oils	Reduce *las*- and *rhl*-regulated virulence factors such as protease, chitinase, and pyocyanin production, swimming motility	*P. aeruginosa* and *Aeromonas hydrophila*	[135,148]
Bauhinia acuruana (Leguminosae)	Extract (branches, fruits)	Biofilm inhibition	*Staphylococcus epidermidis*	[149]
Pityrocarpa moniliformis (Leguminosae)	Extract (leaves)	Inhibit biofilm formation	*S. epidermidis*	[149]
Commiphora leptophloeos (Burseraceae)	Extract (stem bark)	Inhibit biofilm formation	*S. epidermidis*	[149]
Cocos nucifera Linn. (Arecaceae)	Husk fiber extract	Inhibit biofilm formation and EPS production	*Pseudomonas* sp., *Alteromonas* sp., and *Gallionella* sp.	[150]
Terminalia catappa (Combretaceae)	Methanolic extract (leaf)	Inhibit QS controlled violacein production; inhibit the maturation of biofilms	*C. violaceum*, *P. aeruginosa*	[151]

Continued

TABLE 15.2 The effects of different plants on QS-mediated biofilm—cont'd

Source	Extract (parts used)/Active ingredients	Nature of activity	Effective against (organism)	Reference
Sclerocarya birrea (Anacardiaceae)	Methanolic extract (stem bark)	Reduced swimming, motility, production of virulence factors: inhibit biofilm formation	*P. aeruginosa*	[136]
Buchanania lanzan Spreng (Anacardiaceae)	Methanolic extract (root)	Reduced biofilm formation	*E. coli*, *P. aeruginosa*	[137]
Areca catechu (Arecaceae)	Extract (seed)	Interfere with violacein production and swarming motility	*C. violaceum*, *P. aeruginosa*	[138]
Panax notoginseng (Araliaceae)	Extract (flower and root)	Interfere with violacein production and swarming motility; suppress the production of LasA and LasB, downregulated the synthesis of the AHL molecules	*C. violaceum*, *P. aeruginosa*	[138,152]
Ocimum sanctum (Lamiaceae) *Ananas comosus* (Bromeliaceae) *Musa paradiciaca* (Musaceae) *Manilkara zapota* (Sapotaceae)	Aqueous extracts	Inhibit AHL-mediated violacein production in *C. violaceum*; pyocyanin pigment, protease, elastase production and biofilm formation *P. aeruginosa*	*C. violaceum*, *P. aeruginosa*	[118]

pose a greater threat to human health, and the number of mortality is higher as compared to other health disorders [7,11]. Interestingly, a major proportion of infectious diseases are currently being linked to bacterial biofilms, particularly related to organisms like *S. aureus*, *E. coli*, *P. aeruginosa*, and such other disease-causing organisms. Even though the indiscriminate application of antibiotics coupled with slowing down of the discovery process has caused an alarming shrinkage of the antibiotic arsenal, however there has been a considerable development in the field of antibiofilm (QS inhibitors) drug discovery during the last couple of decades, as is evident from the growing number of publications and patent filings [109]. Furthermore, it is pertinent to suggest that QS inhibition (i.e., strategy for disruption of cell-to-cell communication) is likely to become a vital area of antibiotic drug discovery, since it deviates from the currently applied strategy, where cell destruction is considered as the ultimate goal. Moreover, antibiofilm agents, at the concentrations employed, demonstrate a reduced selective pressure on the invading organisms (reducing the possibility of resistance development) and are more targeted toward minimizing the virulence mechanism (virulence factor production), considered to be more effective and a safer approach for infection management.

Considering the immense untapped potential of the terrestrial plants (including lower plants, marine organisms, bacteria, and fungi), a well-orchestrated approach, involving genomics, metabolomics, proteomics, and bioinformatics, with a particular focus on the secondary metabolites, may ultimately prove to be highly beneficial to the scientific and medical community at large.

Finally, the injudicious application of antibacterials, commercial greed, and poor tolerance level of modern-day patients (through injudicious self-medication in common ailments such as flu and diarrhea), a situation conceived as the "Post Antibiotic Era," could easily be foreseen and this may well contribute towards the endangerment of the human civilization. Thus, it is time for clinicians, scientists, and policy makers to do some serious introspection and come forward with a concerted effort to formulate global strategies to save the earth.

Acknowledgments

The authors appreciate the support of Prof. A. Dasgupta (Jadavpur University) for the language editing.

References

[1] Sharma P, Tomar SK, Goswami P, Sangwan V, Singh R. Antibiotic resistance among commercially available probiotics. Food Res Int 2014;57:176–95.

[2] Bories G, Brantom P, Barberà TB, Chesson A, Cocconcelli PS, Debski B, et al. Technical guidance prepared by the panel on additives and products or substances used in animal feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. EFSA J 2008;732:1–15.

[3] Neu H. The crisis in antibiotic resistance. Science 1992;257: 1064–73.
[4] Russell A, Tattawasart U, Maillard J, Furr J. Possible link between bacterial resistance and use of antibiotics and biocides. Antimicrob Agents Chemother 1998;42:2151.
[5] Dong L, Yan H, Wang D. Antibiotic prescribing patterns in village health clinics across 10 provinces of western China. J Antimicrob Chemother 2008;62:410–5.
[6] Cohen M. Epidemiology of drug resistance: implications for a post-antimicrobial era. Science 1992;257:1050–155.
[7] Laxminarayan R, Heymann D. Challenges of drug resistance in the developing world. Br Med J 2012;344:e1567.
[8] Andersson DI, Hughes D. Antibiotic resistance and its cost: Is it possible to reverse resistance? Nat Rev Microbiol 2010;8: 260–71.
[9] Martinez J, Baquero F. Interactions among strategies associated with bacterial infection: pathogenicity, epidemicity, and antibiotic resistance. Clin Microbiol Rev 2002;15:647–79.
[10] Aiello AE, Larson E. Antibacterial cleaning and hygiene products as an emerging risk factor for antibiotic resistance in the community. Lancet Infect Dis 2003;3:501–6.
[11] Klevens R, Morrison M, Nadle J. Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. J Am Med Assoc 2007;298(15):1763–71.
[12] Howard D, Rask K. The impact of resistance on antibiotic demand in patients with ear infections. In: Laxminarayan R, editor. Battling resistance to antibiotics and pesticides: an economic approach. (Washington, DC): RFF Press; 2002. p. 119–33.
[13] Arias C, Murray B. The rise of the *Enterococcus*: beyond vancomycin resistance. Nat Rev Microbiol 2012;10:266–78.
[14] Rodríguez-Rojas A, Rodríguez-Beltrán J, Couce A, Blázquez J. Antibiotics and antibiotic resistance: a bitter fight against evolution. Int J Med Microbiol 2013;303:293–7.
[15] Davies J. Inactivation of antibiotics and the dissemination of resistance genes. Science 1994;264:375–82.
[16] Andersson D. Persistence of antibiotic resistant bacteria. Curr Opin Microbiol 2003;6:452–6.
[17] Livermore D. Bacterial resistance: origins, epidemiology, and impact. Clin Infect Dis 2003;36:S11–23.
[18] Fuente-Núñez C, Reffuveille F, Ferná ndez L, Hancock RE. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Curr Opin Microbiol 2013;16:580–9.
[19] Stewart P, Costerton J. Antibiotic resistance of bacteria in biofilms. Lancet 2001;358:135–8.
[20] Jolivet-Gougeon A, Bonnaure-Mallet M. Biofilms as a mechanism of bacterial resistance. Drug Discovery Today Technol 2014;11:49–56.
[21] Cragg G, Newman D. Natural products: a continuing source of novel drug leads. Biochim Biophys Acta 2013;1830:3670–95.
[22] Simões M, Simões L, Vieira M. A review of current and emergent biofilm control strategies. Food Sci Technol 2010;43:573–83.
[23] Palmer RJ, White D. Developmental biology of biofilms: implications for treatment and control. Trends Microbiol 1997;5(11): 435–40.
[24] Bradley D. A function of *Pseudomonas aeruginosa* PAO polar pili: twitching motility. Can J Microbiol 1980;26:146–54.
[25] Davies D, Geesey G. Regulation of the alginate biosynthesis gene algC in *Pseudomonas aeruginosa* during biofilm development in continuous culture. Appl Environ Microbiol 1995; 61(3):860–7.
[26] Davies D, Chakabarty A, Geesey G. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. Appl Environ Microbiol 1993; 59(4):1181–6.
[27] Parsek M, Greenberg E. Acyl-homoserine lactone quorum sensing in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. Proc Natl Acad Sci USA 2000;97(16):8789–93.
[28] Davey ME, O'Toole GA. Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 2000;64(4):847–67.
[29] Boyd A, Chakrabarty A. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Appl Environ Microbiol 1994;60: 2355–9.
[30] Hammond A, Miller K, Kruczek C, Dertien J, Colmer-Hamood J, Griswold J, et al. An in vitro biofilm model to examine the effect of antibiotic ointments on biofilms produced by burn wound bacterial isolates. Burns 2011;37:312–21.
[31] Flores G. Biofilm studies yield targets against cystic fibrosis. Drug Discovery Today 2002;7(23):1147–8.
[32] Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer K, et al. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J Clin Invest 2002;109(3):317–25.
[33] Stickler DJ. Bacterial biofilms in patients with indwelling urinary catheters. Nat Rev Urol 2008;5:598–608.
[34] Aslam S. Effect of antibacterials on biofilms. Am J Infect Control 2008;36(10):S175.e9–175.e11.
[35] Digiovine B, Chenoweth C, Watts C, Higgins M. The attributable mortality and costs of primary nosocomial bloodstream infections in the intensive care unit. Am J Respir Crit Care Med 1999;160:976–81.
[36] Walsh C. Molecular mechanisms that confer antibacterial drug resistance. Nature 2000;406:775–81.
[37] Mah T, O'Toole G. Mechanism of biofilm resistance to antimicrobial agents. Trends Microbiol 2001:34–9.
[38] Stewart P. Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrob Agents Chemother 1996;40: 2517–22.
[39] Nichols W, Dorrington S, Slack M, Walmsley H. Inhibition of tobramycin diffusion by binding to alginate. Antimicrob Agents Chemother 1988;32:618–23.
[40] Kumon H, Tomocika K, Matunaga T, Ogawa M, Ohmon H. A sandwich cup method for preparation assay of antimicrobial agents through *Pseudomonas* exopolysaccharides. Microbiol Immunol 1994;8:615–9.
[41] Adonizio A, Downum K, Bennett B, Mathee K. Anti-quorum sensing activity of medicinal plants in southern Florida. J Ethnopharmacol 2006;15:427–35.
[42] Shigeta M, Tanaka G, Komatsuzawa H, Sugai M, Suginaka H, Usui T. Permeation of antimicrobial agents through *Pseudomonas aeruginosa* biofilms: a simple method. Chemotherapy 1997;43: 340–5.
[43] Das J, Bhakoo M, Jones MV, Gilbert P. Changes in the biocide susceptibility of *Staphylococcus epidermis* and *Escherichia coli* cells associated with rapid attachment to plastic surfaces. J Appl Microbiol 1998;84:52–8.
[44] Anderl J, Franklin M, Stewart P. Role of antibiotic penetration limitation in *Klebsiella pneumonias* biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother 2000;44: 1818–24.
[45] De Beer D, Stoodley P, Roe F, Lewandowski Z. Effects of biofilm structure on oxygen distribution and mass transport. Biotechnol Bioeng 1994;43:1131–8.
[46] Costerton J, Lewandowski Z, Caldwell D, Korber D, Lappinscott H. Microbial biofilms. Ann Rev Microbiol 1995;49: 711–45.
[47] Tack K, Sabath L. Increased minimum inhibitory concentration with anaerobiasis for tobramycin, gentamycin and amikacin compared to latamoxey, piperacillin, chloramphenicol and clindamycin. Chemotherapy 1985;31:204–10.

[48] Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 2010; 35(4):322–32.
[49] Yang L, Haagensen J, Jelsbak L, Johansen H, Sternberg C, Høiby N. In situ growth rates and biofilm development of *Pseudomonas aeruginosa* populations in chronic lung infection. J Bacteriol 2008;190:2767–76.
[50] Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A. The rate of killing *Escherichia coli* by beta lactam antibiotics is strictly proportional to the rate of bacterial growth. J Gen Microbiol 1986;132: 1297–304.
[51] Anwar H, Costerton J. Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1990;34:1666–16671.
[52] Prigent-Combaret C, Vidal O, Dorel C, Lejeune P. Abiotic surface sensing and biofilm development regulation of gene expression in *Escherichia coli*. J Bacteriol 1999;181:5993–6002.
[53] Khalifa A, Moissenet D, Vu Thien H, Khedher M. Virulence factors in *Pseudomonas aeruginosa*: mechanisms and modes of regulation. Ann Biol Clin 2011;69(4):393–403.
[54] Miller M, Bassler B. Quorum sensing in bacteria. Ann Rev Microbiol 2001;55(1):165–99.
[55] Bodini S, Manfredini S, Epp M, Valentini S, Santori F. Quorum sensing inhibition activity of garlic extract and p-coumaric acid. Lett Appl Microbiol 2009;49(5):551–5.
[56] Swift S, Vaughan E, de Vos W. Quorum sensing within the gut ecosystem. Microb Ecol Health Dis 2000;12(1):81–92.
[57] Falcao J, Sharp F, Sperandio V. Cell-to-cell signaling in intestinal pathogens. Curr Issues Intest Microbiol 2004;5(1):9–18.
[58] Kendall M, Sperandio V. Quorum sensing by enteric pathogens. Curr Opin Gastroenterol 2007;23(1):10–5.
[59] Liu H-B, Koh K, Lee J, Kim J, Park S. Characterization of LasR protein involved in bacterial quorum sensing mechanism of *Pseudomonas aeruginosa*. Biotechnol Bioprocess Eng 2009;14:146–54.
[60] Venturi V. Regulation of quorum sensing in *Pseudomonas*. FEMS Microbiol Rev 2006;30:274–91.
[61] McDougald D, Rice S, Kjelleberg S. Bacterial quorum sensing and interference by naturally occurring biomimics. Anal Bioanal Chem 2007;387:445–53.
[62] Walters M, Sircili M, Sperandio V. AI-3 synthesis is not dependent on luxS in *Escherichia coli*. J Bacteriol 2006;168:5668–81.
[63] Sun J, Daniel R, Wagner-Dobler I, Zeng A. Is autoinducer-2 a universal signal for interspecies communication: a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways. BMC Evol Biol 2004;4:36.
[64] Lowery C, Dickerson T, Janda K. Interspecies and interkingdom communication mediated by bacterial quorum sensing. Chem Soc Rev 2008;37:1337–46.
[65] Struss AK, Pasini P, Flomenhoft D, Shashidhar H, Daunert S. Investigating the effect of antibiotics on quorum sensing with whole-cell biosensing systems. Anal Bioanal Chem 2012;402: 3227–36.
[66] Dong Y, Zhang L. Quorum sensing and quorum-quenching enzymes. J Microbiol 2005;43:101–9.
[67] Visick K, Skoufos L. Two-component sensor required for normal symbiotic colonization of *Euprymna scolopes* by *Vibrio fischeri*. J Bacteriol 2011;183(3):835–42.
[68] Waters C, Bassler B. Quorum sensing cell-to-cell communication in bacteria. Ann Rev Cell Dev Biol 2005;21:319–46.
[69] Pesci E, Pearson J, Seed P, Iglewski B. Regulation of las and rhl quorum sensing in *Pseudomonas aeruginosa*. J Bacteriol 1997;179: 3127–32.
[70] Brint J, Ohman D. Synthesis of multiple exoproducts in *Pseudomonas aeruginosa* is under the control of RhlR-RhlI, another set of regulators in strain PAO1 with homology to the autoinducer-responsive LuxR–LuxI family. J Bacteriol 1995;177: 7155–63.
[71] Whiteley M, Lee K, Greenberg E. Identification of genes controlled by quorum sensing *Pseudomonas aeruginosa*. Proc Natl Acad Sci USA 1999;96:13904–9.
[72] Seed P, Passador L, Iglewski B. Activation of the *Pseudomonas aeruginosa* lasI gene by LasR and the *Pseudomonas* autoinducer PAI: an autoinduction regulatory hierarchy. J Bacteriol 1995;177: 654–9.
[73] Amara N, Krom B, Kaufmann G, Meijler M. Macromolecular inhibition of quorum sensing: enzymes, antibodies, and beyond. Chem Rev 2011;111:195–208.
[74] Saenz H, Augsburger V, Vuong C, Jack R, Gotz F, Otto M. Inducible expression and cellular location of AgrB, a protein involved in the maturation of the staphylococcal quorum-sensing pheromone. Arch Microbiol 2000;174:452–5.
[75] Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, et al. The agr P2 operon: an autocatalytic sensory transduction system in *Staphylococcus aureus*. Mol Genet Genomics 1995;248: 446–58.
[76] Ji G, Beavis R, Novick R. Bacterial interference caused by autoinducing peptide variants. Science 1997;276:2027–30.
[77] Roux A, Payne SM, Gilmore MS. Microbial telesensing: probing the environment for friends, foes, and food. Cell Host Microbe 2009;6:115–24.
[78] George E, Muir TW. Molecular mechanisms of agr quorum sensing in virulent *Staphylococci*. ChemBioChem 2007;8:847–55.
[79] Antunes L, Ferreira R, Buckner M, Finlay B. Quorum sensing in bacterial virulence. Microbiology 2010;156:2271–82.
[80] Mayville P, Ji G, Beavis R, Yang H, Goger M, Novick R, et al. Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proc Natl Acad Sci USA 1999;96(4):1218–23.
[81] Bassler B, Greenberg E, Stevens A. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J Bacteriol 1997;179:4043–5.
[82] Taga M, Miller S, Bassler B. Lsr-mediated transport and processing of AI-2 in *Salmonella typhimurium*. Mol Microbiol 2003;50: 1411–27.
[83] Herzberg M, Kaye I, Peti W, Wood T. YdgG (TqsA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. J Bacteriol 2006;188:587–98.
[84] Xavier K, Bassler B. Regulation of uptake and processing of the quorum sensing autoinducer AI-2 in *Escherichia coli*. J Bacteriol 2005;187:238–48.
[85] Roy V, Adamsb B, We B. Developing next generation antimicrobials by intercepting AI-2 mediated quorum sensing. Enzyme Microb Technol 2011;49:113–23.
[86] Cagno R, Angelis M, Calasso M, Gobbetti M. Proteomics of the bacterial cross-talk by quorum sensing. J Proteomics 2011;74: 19–34.
[87] Van Houdt R, Aertsen A, Moons P, Vanoirbeek K, Michiels C. *N*-acyl-L-homoserine lactone signal interception by *Escherichia coli*. FEMS Microbiol Lett 2006;256:83–9.
[88] Riedel K, Hentzer M, Geisenberger O, Huber B, Steidle A, Wu H, et al. *N*-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology 2001;147:3249–62.
[89] Hornby J, Jensen E, Lisec A, Tasto J, Jahnke B, Shoemaker R. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl Environ Microbiol 2001;67: 2982–92.
[90] Oh K, Miyazawa H, Naito T, Matsuoka H. Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*. Proc Natl Acad Sci USA 2001;98:4664–8.

[91] Whitehead N, Welch M, Salmond G. Silencing the majority. Nat Biotechnol 2001;19:735–6.
[92] Adonizio A, Kong K, Mathee K. Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* by South Florida plant extracts. Antimicrob Agents Chemother 2008;52:198–203.
[93] Hentzer M, Givskov M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. J Clin Invest 2003;112:1300–7.
[94] Parsek M, Val D, Hanzelka B, Cronan JJ, Ep G. Acyl homoserine-lactone quorum-sensing signal generation. Proc Natl Acad Sci USA 1999;96:4360–5.
[95] Rasmussen T, Givskov M. Quorum sensing inhibitors: a bargain of effects. Microbiology 2006:895–904.
[96] Gutierrez J, Crowder T, Rinaldo-Matthis A, Ho M, Almo S, Schramm V. Transition state analogs of 5′-methylthioadenosine nucleosidase disrupt quorum sensing. Nat Chem Biol 2009;5: 251–7.
[97] Cornell K, Swarts W, Barry R, Riscoe M. Characterization of recombinant *Escherichia coli* 5′-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase: analysis of enzymatic activity and substrate specificity. Biochem Biophys Res Commun 1996;228: 724–32.
[98] Winzer K, Hardie K, Burgess N, Doherty N, Kirke D, Holden M, et al. LuxS: its role in central metabolism and the in vitro synthesis of 4-hydroxy-5-methyl-3(2H)-furanone. Microbiology 2002; 148:909–22.
[99] Alfaro J, Zhang T, Wynn D, Karschner E, Zhou Z. Synthesis of LuxS inhibitors targeting bacterial cell–cell communication. Org Lett 2004;6:3043–6.
[100] Shen G, Rajan R, Zhu J, Bell C, Pei D. Design and synthesis of substrate and intermediate analogue inhibitors of *S*-ribosylhomocysteinase. J Med Chem 2006;49:3003–11.
[101] Yates EA. *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. Infect Immun 2002;70:5635–46.
[102] Leadbetter J, Greenberg E. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*. J Bacteriol 2000;182:6921–6.
[103] Dong Y, Xu J, Li X, Zhang L. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proc Natl Acad Sci USA 2000;97:3526–31.
[104] Dong Y, Wang LH, Xu JL, Zhang HB, Zhang XF, Zhang LH. Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. Nature 2001;411:813–7.
[105] Camara M, Williams P, Hardman A. Controlling infection by tuning in and turning down the volume of bacterial small-talk. Lancet Infect Dis 2002;2:667–76.
[106] Lin Y, Xu J, Hu J, Wang L, Ong S, Leadbetter J, et al. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. Mol Microbiol 2003;47:849–60.
[107] Dong Y, Gusti A, Zhang Q, Xu J, Zhang L. Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. Appl Environ Microbiol 2002;68(4):1754–9.
[108] Uroz S, Chhabra S, Camara M, Williams P, Oger P, Dessaux Y. *N*-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities. Microbiology 2005;151:3313–22.
[109] LaSarre B, Federle M. Exploiting quorum sensing to confuse bacterial pathogens. Microbiol Mol Biol Rev 2013;77(1):73–111.
[110] Borchardt S, Allain E, Michels J, Stearns G, Kelly R, McCoy W. Reaction of acylated homoserine lactone bacterial signaling molecules with oxidized halogen antimicrobials. Appl Environ Microbiol 2001;67:3174–9.
[111] Kalia VC. Quorum sensing inhibitors: an overview. Biotechnol Adv 2013;31(2):224–45.
[112] Chhabra S, Stead P, Bainton N, Salmond G, Stewart G, Williams P, et al. Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* by analogues of *N*-(3-oxohexanoyl)-L-homoserine lactone. J Antibiot 1993;46:441–54.
[113] Tateda K, Comte R, Pechere J, Köhler T, Yamaguchi K, Delden C. Azithromycin inhibits quorum sensing in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2001;45:1930–3.
[114] Pechere J. Azithromycin reduces the production of virulence factors in *Pseudomonas aeruginosa* by inhibiting quorum sensing. Jpn J Antibiot 2001;54:87–9.
[115] Sofer D, Gilboa-Garber N, Belz A, Garber N. 'Subinhibitory' erythromycin represses production of *Pseudomonas aeruginosa* lectins, autoinducer and virulence factors. Chemotherapy 1999; 45:335–41.
[116] Butler M, Buss A. Natural products–the future scaffolds for novel antibiotics? Biochem Pharmacol 2006;12:919–29.
[117] Gibot S. Fighting the enemy properly. Crit Care Med 2004;32: 1223–4.
[118] Musthafa K, Ravi A, Annapoorani A, Packiavathy I, Pandian S. Evaluation of anti-quorum-sensing activity of edible plants and fruits through inhibition of the *N*-acyl-homoserine lactone system in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. Chemotherapy 2010;56:333–9.
[119] Daglia M, Stauder M, Papetti A, Signoretto C, Giusto G, Canepari P, et al. Isolation of red wine components with anti-adhesion and anti-biofilm activity against *Streptococcus mutans*. Food Chem 2010:1182–8.
[120] Kim J, YH K, YW S, Park S. Quorum sensing inhibitors from the red alga, *Ahnfeltiopsis flabelliformis*. Biotechnol Bioprocess Eng 2007;12:308–11.
[121] Bjarnsholt T, Jensen P, Rasmussen T, Christophersen L, Calum H, Hentzer M, et al. Garlic blocks quorum sensing and promotes rapid clearing of pulmonary *Pseudomonas aeruginosa* infections. Microbiology 2005;151:3873–80.
[122] Niu C, Afre S, Gilbert E. Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing. Lett Appl Microbiol 2006;43:489–94.
[123] Khan M, Zahin M, Hasan S, Husain F, Ahmad I. Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil. Lett Appl Microbiol 2009;49:354–9.
[124] Packiavathy I, Agilandeswari P, Musthafa K, Pandian S, Ravi A. Antibiofilm and quorum sensing inhibitory potential of *Cuminum cyminum* and its secondary metabolite methyl eugenol against gram negative bacterial pathogens. Food Res Int 2012;45:85–92.
[125] Packiavathy I, Priya S, Pandian S, Ravi A. Inhibition of biofilm development of uropathogens by curcumin – an anti-quorum sensing agent from *Curcuma longa*. Food Chem 2014;148:453–60.
[126] Kim H, Park H. Ginger extract inhibits biofilm formation by *Pseudomonas aeruginosa* PA14. Plos One 2013;8(9):E76106.
[127] Kumar L, Chhibber S, Harjai K. Zingerone inhibit biofilm formation and improve antibiofilm efficacy of ciprofloxacin against *Pseudomonas aeruginosa* PAO1. Fitoterapia 2013;90:73–8.
[128] Ding X, Yin B, Qian L, Zeng Z, Yang Z, Li H, et al. Screening for novel quorum-sensing inhibitors to interfere with the formation of *Pseudomonas aeruginosa* biofilm. J Med Microbiol 2011;60: 1827–34.
[129] Nazzaro F, Fratianni F, Coppola R. Quorum sensing and phytochemicals. Int J Mol Sci 2013;14:12607–19.
[130] Burt S. Essential oils: their antibacterial properties and potential applications in foods – a review. Int J Food Microbiol 2004;94(3): 223–53.

[131] Olivero J, Pajaro N, Stashenko E. Antiquorum sensing activity of essential oils isolated from different species of the genus *Piper*. Vitae 2011;18:77–82.
[132] Szczepanski S, Lipski A. Essential oils show specific inhibiting effects on bacterial biofilm formation. Food Control 2014;36: 224–9.
[133] Nikolíc M, Markovíc T, Mojovíc M, Pejind B, Savíc A, Períc T, et al. Chemical composition and biological activity of *Gaultheria procumbens* L. essential oil. Ind Crops Prod 2013:561–7.
[134] Jadhav S, Shah R, Bhave M, Ea P. Inhibitory activity of yarrow essential oil on *Listeria* planktonic cells and biofilms. Food Control 2013;29:125–30.
[135] Husain F, Ahmad I, Asif M, Tahseen Q. Influence of clove oil on certain quorum-sensing-regulated functions and biofilm of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. J Biosci 2013;38(5): 835–44.
[136] Sarkar R, Chaudhary S, Sharma A, Yadav K, Nema N, Sekhoachad M, et al. Anti-biofilm activity of marula – a study with the standardized bark extract. J Ethnopharmacol 2014; 154(1):170–5.
[137] Pattnaik A, Sarkar R, Sharma A, Yadav K, Kumar A, Roy P, et al. Pharmacological studies on *Buchanania lanzan* Spreng. A focus on wound healing with particular reference to anti-biofilm properties. Asian Pac J Trop Biomed 2013;3(12): 967–74.
[138] Koh K, Tham F. Screening of traditional chinese medicinal plants for quorum-sensing inhibitors activity. J Microbiol Immunol Infect 2011;44:144–8.
[139] Zaki A, Shaaban M, Hashish N, Amer M, Lahloub M. Assessment of anti-quorum sensing activity for some ornamental and medicinal plants native to Egypt. Sci Pharm 2013;81:251–8.
[140] Nyila M, Leonard C, Hussein A, Lall N. Activity of South African medicinal plants against *Listeria monocytogenes* biofilms, and isolation of active compounds from *Acacia karroo*. S Afr J Bot 2012;78:220–7.
[141] Chevrot R, Rosen R, Haudecoeur E, Cirou A, Cshelp BJ, Ron E, et al. GABA controls the lavel of quorum sensing signal in *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA 2006;103:7460–4.
[142] Sá N, Cavalcante T, Araú jo A, Santos H, Albuquerque M, Bandeira P, et al. Antimicrobial and antibiofilm action of Casbane Diterpene from *Croton nepetaefolius* against oral bacteria. Arch Oral Biol 2012;57:550–5.
[143] Amaya S, Pereira J, Borkosky S, Valdeza J, Bardón A, Arena M. Inhibition of quorum sensing in *Pseudomonas aeruginosa* by sesquiterpene lactones. Phytomedicine 2012;19:1173–7.
[144] Rasmussen TB, Bjarnsholt T, Skindersoe ME, Hentzer M, Kristoffersen P, Kö te M, et al. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. J Bacteriol 2005;187:1799–814.
[145] Chusri S, Phatthalung P, Voravuthikunchai S. Anti-biofilm activity of *Quercus infectoria* G. Olivier against methicillin-resistant *Staphylococcus aureus*. Lett Appl Microbiol 2012;54:511–7.
[146] Truchado P, Giménez-Bastida J, Larrosa M, Castro-Ibáñez I, Espín J, Tomás-Barberán F, et al. A inhibition of quorum sensing (QS) in *Yersinia enterocolitica* by an orange extract rich in glycosylated flavanones. J Agri Food Chem 2012;60(36): 8885–94.
[147] Furletti V, Teixeira I, Obando-Pereda G, Mardegan R, Sartoratto A, Figueira G, et al. Action of *Coriandrum sativum* L. Essential oil upon oral *Candida albicans* biofilm formation. Evid Based Complement Alternat Med 2011;2011:1–9.
[148] Chamdit S, Siripermpool P. Antimicrobial effect of clove and lemongrass oils against planktonic cells and biofilms of *Staphylococcus aureus*. Mahidol Univ J Pharm Sci 2012;39(2):28–36.
[149] Trentina D, Giordania R, Zimmerb K, da Silva A, da Silva M, Correiac M, et al. Potential of medicinal plants from the Brazilian semi-arid region (Caatinga) against *Staphylococcus* epidermidis planktonic and biofilm lifestyles. J Ethnopharmacol 2011;137: 327–35.
[150] Viju N, Satheesh S, Vincent S. Antibiofilm activity of coconut (*Cocos nucifera* Linn.) husk fibre extract. Saudi J Biol Sci 2013;20: 85–91.
[151] Taganna J, Quanico J, Perono R, Amor E, Rivera W. Tannin-rich fraction from *Terminalia catappa* inhibits quorum sensing (QS) in *Chromobacterium violaceum* and the QS-controlled biofilm maturation and LasA staphylolytic activity in *Pseudomonas aeruginosa*. J Ethnopharmacol 2011;134:865–71.
[152] Song Z, Kong K, Wu H, Maricic N, Ramalingam B, Priestap H, et al. Panax ginseng has anti-infective activity against opportunistic pathogen *Pseudomonas aeruginosa* by inhibiting quorum sensing, a bacterial communication process critical for establishing infection. Phytomedicine 2010;17(13):1040–6.
[153] Kievit TRD, Iglewski BH. Bacterial quorum sensing in pathogenic relationships. Infect Immun 2000;68(9):4839–49.
[154] Zeng X, Liu X, Bian J, Pei J, Dai H, Polyak S, et al. Synergistic effect of 14-alpha-lipoyl andrographolide and various antibiotics on the formation of biofilms and production of exopolysaccharide and pyocyanin by *Pseudomonas aeruginosa*. Antimicrob Agents chemother 2011;55(6):3015–7.
[155] Pemmaraju S, Pruthi P, Prasad R, Pruthi V. *Candida albicans* biofilm inhibition by synergistic action of terpenes and fluconazole. Indian J Exp Biol 2013;51:1032–7.

LIST OF ABBREVIATIONS

agr Accessory gene regulator
AHLs *N*-acylhomoserine lactones
AI-2 Autoinducer-2
AIP Auto-inducing oligopeptides
BHL *N*-(butanoyl)-L-homoserine lactone
BSIs Bloodstream infections
CF Cystic fibrosis
DPD 4,5-Dihydroxy-2,3-pentanedione
EPS Extracellular polymeric substances
LuxS *S*-ribosylhomocysteinase
MIC Minimum inhibitory concentration
MRSA Methicillin-resistant *Staphylococcus aureus*
MTA Methylthioadenosine
MTAN 5′-Methylthioadenosine nucleosidase
O-dDHL *N*-3-(oxododecanoyl)-L-homoserine lactone
QS Quorum sensing
SAH *S*-adenosyl homocysteine
SAM *S*-adenosylmethionine
SRH *S*-ribosylhomocysteine
UTI Urinary tract infection

CHAPTER

16

Clinical Effects of Caraway, a Traditional Medicine for Weight Loss

Mahnaz Kazemipoor[1], *Geoffrey A. Cordell*[2]

[1]Department of Science & Technology Studies, University of Malaya, Kuala Lumpur, Malaysia; [2]Natural Products Inc., Evanston, IL, USA

OUTLINE

16.1 INTRODUCTION

Health issues have become an indispensable aspect of human life, and the importance of wellness and fitness in modern and emerging societies around the world is established. Global health has become a fundamental element of foreign policy, and many governments now emphasize community health, and encourage institutions, researchers, and the media to develop, support, and publicize research projects related to health promotion and wellness [1]. This greater need for health awareness among societies brings focus to those factors which

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00016-7

influence, positively and negatively, both individual and societal health. Controlled clinical trials, which can assess the use of plant materials in the treatment and prevention of various diseases and human conditions, are also needed, particularly when the existing therapeutic modalities are not accessible due to cost or availability or present a clinical risk. The World Health Organization (WHO) has recognized this for many years [2] and has further encouraged countries to place the issues of both safety and efficacy as a priority in countries where traditional medicines and a variety of plant products are already present in the health care market place [3]. One of the recent initiatives of the Western Pacific Regional Office of WHO has been the development of a revised Regional Strategy for Traditional Medicine in the Western Pacific for the period 2011–2020 [4]. The strategic directions describe a range of stratified approaches to improve the quality, safety, and efficacy of traditional medicinal plants in health care in the. As such, the strategic directions recognize that the 27 countries in the region embrace a wide swath of economic development and research capacity. Cooperation within and between countries to address issues of quality, safety, and efficacy of botanical materials of known or standardized content is therefore strongly encouraged to enhance regional health care [3].

Another factor which has now emerged as being crucial to medicinal plant research and development is sustainability, and the term "sustainable medicine" has been developed [5–10] to describe the importance of considering the long-term use of both traditional medicines and synthetic drugs from a perspective of reliable and nondestructive sourcing for the future. This is of particular importance where population use of traditional medicines is expanding, where the globalization of products is increasing demand, or where climate change may impact areas for growing traditional medicines. In the research component of this scenario, preference is given to studies on those plant materials which are already established as sustainable commercial entities, or which are easily grown agronomically in order to derive an accessible (affordable and sustainable) product [11]. A further aspect concerning the sustainability of traditional medicine applies to the knowledge of the use of medicinal plants, and how that information is recorded, evaluated, and maintained from generation to generation of practitioner.

One of the major global health problems that have emerged as a result of improved economic status, the globalization of certain eating practices, and personal health awareness, is overweight and obesity. The terms overweight and obesity refer to the accumulation of excess fat in the body, and are clinically defined as a body mass index (BMI) higher than 25 and 30, respectively [12]. Since 1997, the WHO has warned that obesity is rapidly becoming a global epidemic, although it was not a noticeable health care concern during most of the twentieth century [13,14]. The use of the word "Globesity" in reports indicates the severity of the issue worldwide [15]. Studies reveal that half of the adult populations in the Organization for Economic Co-operation and Development countries are overweight, and 1/6th are obese. Based on WHO reports [16], in 2008, the global prevalence of overweight and obesity had reached over 1.4 billion in adults, and obesity has become a common disease existing in different social, economic, cultural, regional, and age groups. Consequently, this hidden health hazard is affecting communities and societies multidimensionally [17].

The consequences of obesity to society in terms of morbidity and mortality are enormous. Based on recent research, obesity is implicated as one of the leading causes of death worldwide, and is a well-established, threatening element for human health [18,19]. Furthermore, excess body fat can lead to the development of numerous, life-threatening, chronic diseases [20–22]. For example, being overweight significantly increases the risk of death from hypertension, dyslipidemia, type II diabetes, stroke, osteoarthritis, coronary heart disease, gallbladder disease, sleep apnea, respiratory problems, and endometrial, breast, prostate, and colon cancers. In other words, obesity causes a decline in life expectancy [23]. Accordingly, as a major risk factor for human health [24], urgent strategies are required to prevent further complications [25].

From an economic point of view, obesity and its related health consequences such as physician visits, hospitalization, and other related expenses, involve enormous costs currently, and for future health care [26–28]. As a result, the potential health and economic benefits of reducing excess weight are of considerable importance to public health systems [29]. In order to combat obesity and its health consequences, governments have adopted different policies which are essentially based on modifying lifestyle habits and increasing the health awareness of individuals. These programs mainly aim to promote healthy eating patterns and increase physical activity among people, especially school children. However, despite great efforts to fight this disease, "Globesity" remains a very challenging issue, and the management of obesity has become one of the crucial components of global and national health policies [30].

Today, weight control, in one form or another, is recognized as a common human concern. According to Weiss and colleagues [31], 51% of American adults above 20 years old had tried at some point to control their weight. This subject has attracted the attention of manufacturers, personal health advisors, physicians, patients, and especially governments, to find and develop

new approaches and improved solutions for the treatment and prevention of obesity. One attractive method of losing weight is the consumption of natural and synthetic antiobesity drugs, the long-term usage of these products is typically not under any medical supervision [32]. In addition, of the different weight loss pills available in the market, including Xenical (Orlistat), Phentermine/Fentermine, Meridia (Sibutramine), Adipex, Bontril, Didrex, Phentermine, and Tenuate, only two are approved by the United States Food and Drug Administration (USFDA): namely Orlistat and Sibutramine [33,34]. The long-term consumption of the present antiobesity products is not recommended, as they have exhibited several side effects, including gastrointestinal, psychiatric, and kidney problems which might be irremediable. Such negative symptoms may be due to changes in metabolic rate, and the metabolism of dietary intake [35,36].

Another important issue in the application of such dietary products is their efficacy, short term and long term. Some of these supplements might be effective only if taken along with a suitably modified weight loss diet and enhanced physical activity. Consequently, these remedies may be useful only over a short period of time, as the body usually adjusts quickly to most of these dietary supplements. These negative trends may misguide patients, wherein the products do not satisfactorily provide a long-term impact on weight loss, and are not tolerated on a chronic basis [37]. In this regard, the permitted promotional claims on dietary and slimming products sold to enhance weight loss are relevant, since they pertain to limiting patient expectations without a clinical evidence base.

16.1.1 Weight Loss Claims Regarding Dietary Supplements

The current methods being used for the treatment of obesity, such as synthetic antiobesity drugs, various dietary supplements, or bariatric/gastric bypass surgery are not satisfactory for addressing the issue of obesity on a long term, global basis because of high consumer cost, limitations of chronic usage, and unfavorable side-effects [38]. Therefore, obesity remains a major global health challenge, and accessible solutions for sustainable weight loss and prevention of weight gain are urgently needed [39]. There is a profound lack of scientific information on the rationale for using the presently available alternative therapies, such as dietary supplements, antiobesity drugs, and other slimming products. Hence, patients are confused in deciding between synthetic weight loss pills and slimming aids on one hand, and natural sources, such as medicinal plant products, on the other hand. Patients are therefore challenged in searching for a safe and effective method of long-term weight management.

Despite a variety of different treatment modern approaches for obesity, including surgery, weight loss pills, and dietary supplements, they do not satisfactorily impact weight loss, or are not tolerated by the body [40]. In addition, the high costs and the side-effects of these methods, drive patients and researchers to seek alternative approaches [41–45]. Can medicinal plants serve as a sustainable resource for standardized agents which can meet patient expectations for weight loss and provide long-term, consistent health benefits?

Many scientists and patients believe that treatment with medicinal plants may provide a safer, more reliable, and also cheaper, approach to addressing the issues of overweight and obesity, than the prevalent contemporary methods. However, despite the strong global market influence, and patient desperation for alternative antiobesity products and traditional medicinal plants, the awareness of the usefulness of these products is neither sufficient nor clearly perceived. In major part, this is because there is still doubt about their quality, standardization, safety, and efficacy for long-term human use [46,47]. In spite of several studies on the application of traditional medicinal plants for managing body weight, many challenging issues, including the safety and efficacy of antiobesity plants, remain, and there are continuing deficiencies in the deployment of natural approaches for treating obesity [40,48]. Consequently, seeking sustainable, natural product-based solutions, and examining a variety of natural sources for methods to safely and reliably treat obesity are important, albeit neglected, research targets. Success in developing such strategies will subsequently help to reduce the global health implications of obesity, particularly where access to alternative therapeutic approaches is limited. This chapter describes an approach to establishing the safety and efficacy of a plant-based material, caraway aqueous extract (CAE), for treating obesity and overweight, which might be considered as a natural alternative to the currently available dietary supplements and commercial products.

Caraway, particularly the fruit, is an ancient spice and flavoring material used in many parts of Europe, the Middle East, and Asia [49]. It is derived from the umbelliferous plant, *Carum carvi* L. (Apiaceae) [50], and is sometimes referred to as meridian fennel, even though another plant, *Foeniculum vulgare* Mill. (Apiaceae) has also been ascribed the name fennel. In Arabic, *C. carvi* is known as "Karawiya," and is used world-wide as a natural flavoring in various food products, including rye bread, curries, to flavor rice, in sauerkraut, in cheeses, and as a liquor. Previous studies have established an association between the moderate consumption of caraway oil/aqueous extract and other

caraway-derived preparations with a lower incidence of diabetes, dyslipidemia, hypertension, liver dysfunction, reproductive hormone imbalance, osteoporosis, cancer, and gastrointestinal and inflammatory diseases [51]. Evidence also shows that there is a relationship between the gut flora and obesity [52,53]. Consequently, plant materials such as caraway, which have intestinal relaxant and soothing effects [54] could also possess antiobesity properties.

A number of the components present in caraway, including the polyphenols and specific essential oil components have been attributed to possess anti-inflammatory, antihyperlipidemic, and antiobesity effects [55]. An association has been established between the moderate consumption of caraway-derived metabolites and a lower incidence of diabetes, dyslipidaemia, and inflammatory diseases [51]. A multitargeted, antiobesity effect of carvacrol—one of the major components of caraway—on animals was demonstrated through modifying the gene expressions associated with inflammation and adipogenesis [55]. However, there is no clinical scientific evidence which specifically focuses on exploring the possible role of caraway on weight loss. The aim was therefore to investigate the therapeutic potential of CAE on clinically obese and overweight human subjects.

The context of this study is that the use of natural remedies for inducing weight loss has increased dramatically over the last few decades, and typically involve the inclusion of particular medicinal plants in the diet on a regular basis to assist an individual to lose weight gradually [40]. Most of the antiobesity medications studied presently are based on plants used in traditional medicine, as they have been found to be more acceptable than the synthetic medications [46]. One example is the weight loss reported in animals and humans treated with "WeighLevel," a combination of four medicinal plants used in traditional Arabic and Islamic medicine, including the leaves of *Alchemilla vulgaris* L. (Rosaceae), *Olea europaea* L. (Oleaceae), and *Mentha longifolia* L. (Lamiaceae), the seed extract of *Cuminum cyminum* L. (Apiacaeae), and other ingredients [56]. Caraway tends to be more widely used for weight loss purposes, especially in the countries of the Middle-East region. One of the reasons is historical. In Islamic traditional references, such as Khorasani's *Makhzan al-Advieh*, and Ibn Sina's *Canon of Medicine* (980−1037 AD), the consumption of CAE is recommended specifically for weight loss [57,58].

In traditional medicine resources, caraway is recommended as a remedy for a variety of health problems, especially digestive disorders [59]. Moreover, based on a patented natural supplement formula, combinations of carminative herbs, including caraway, have been used to reduce the adverse effects of weight loss drugs, such as orlistat and oral lipase inhibitors [60]. Caraway seed acts as a carminative, and adding this herb to the diet helps in preventing or relieving flatulence. The carminative volatile oils present in caraway induce a relaxant effect on the movements of the intestine muscle [61−63]. Such an effect will synergistically aid in digestion, which, in turn, has a direct effect on food absorption and calorie intake. In addition, using this spice will provide healthful and therapeutic effects for the patient, and will improve the taste and flavor of the final product [49]. Hence, adding caraway to the recipe of food products, may lead food technologists towards novel formulations in the production of functional food preparations.

Despite a significant number of in vitro and in vivo studies on the constituents of caraway and their remedial effects (discussed in the next section), there are limited clinical studies on the effects of this plant on weight loss [64]. This study introduces an alternative, natural product-based approach for weight loss which is potentially cheaper and healthier to consume, and with minimum human health risks. It is hoped that the results of this research will lead to additional studies which eventually will help patients shift from a temporary weight loss solution to a dietary practice that is long-lasting and sustainable. The findings of this study may be a useful indicator for patients who are not satisfied with the currently available slimming products, and are still seeking a suitable, safe, and natural alternative. Incorporating natural products with potent antiobesity properties into a daily human dietary regimen would be a safe, effective, consistent, and inexpensive method for both the treatment and prevention of obesity.

16.1.2 Background Literature on Caraway

In this section, various aspects of the background of caraway are described. Among the topics covered here include the use, the definition and classification of caraway, the botanical description and morphology, the etymology, and the geographical distribution, cultivation, and regions of production. Attention is then turned to the ethnopharmacological and therapeutic applications of caraway, the chemical compounds and the biological activities, and the safety and toxicological evaluations of caraway-based products. The Apiaceae family is a collection of typically aromatic plants having hollow stems comprised of more than 434 genera and 3780 species. Among the well-known members of the family are anise, asafoetida, caraway, carrot, celery, coriander, cumin, dill, fennel, parsley, parsnip, and sea holly [65−67].

Caraway is defined as the dried ripe fruit of the biennial, usually white-flowered, aromatic Old World herb

FIGURE 16.1 Caraway plant in flower (© Copyright Mel Harte, 2010).

FIGURE 16.3 Dried caraway fruits (often termed caraway seeds) (http://www.discoverlife.org/ap/copyright.html).

(*C. carvi* L., Apiaceae), also known as Persian cumin or meridian fennel, and is one of the ancient cultivated plants of Asia, Africa, and Europe [68]. The fruits (also known, erroneously, as "seeds") are used extensively as a mild spice and flavoring for culinary purposes in many cuisines. Caraway seeds are also widely used in various systems of traditional medicine, and the aromatic constituents have been studied for their health beneficial effects [69,70]. The plant resembles a carrot plant with feathery leaves and is slender, branched, and hollow-stemmed, 30–80 cm in height. The dried, brown fruits are hard, crescent-shaped achenes, around 2 mm long, with five pale ridges [71]. Caraway flowers, and caraway ripened and dried fruits are shown in Figures 16.1–16.3 [72] (http://www.discoverlife.org/ap/copyright.html), and in sketched form in Figure 16.4. The fruits have a pleasant odor, and an aromatic flavor and sharp taste; they are similar to cumin, with which they are sometimes confused [70]. In Middle East countries they are distinguished by their color; caraway is known as black zeereh and cumin seeds known as green zeereh [74].

FIGURE 16.2 Caraway plants with ripening fruits (© Les Mehrhoff, 2008–2010.

Several other plants of family Apiaceae are often mistaken for caraway (*C. carvi*) due to their similarity in odor or appearance, including anise, fennel, cumin, black cumin, and black caraway [75,76]. Table 16.1 shows the appearance and common name of some of these aromatic plants.

There is little, incomplete, and yet complex information known about the etymology of *C. carvi*. The *carvi* term is initiated from the Arabic *al-karwiya* seeds, whereas the word *carum* most likely originated from the Latin word *Caria*, an ancient city in Asia Minor, where caraway may have been used in early times. The usage of "carum carvi" probably dates back to very ancient times based on the finding of caraway seeds by archaeologists in the artifacts of primitive civilizations in Europe [79,80]. The English use of the word caraway was initiated over

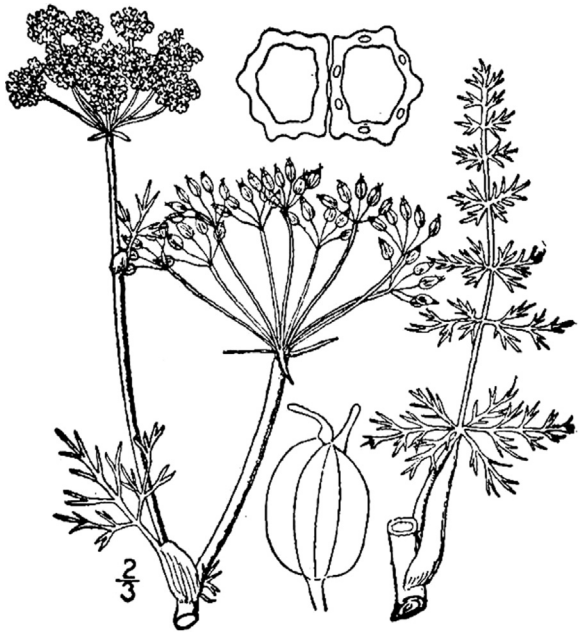


FIGURE 16.4 *Carum carvi* L. [73] (http://www.discoverlife.org/ap/copyright.html).

TABLE 16.1 Other Aromatic Plants Mistaken for *Carum carvi* due to Their Resemblance in Name or Appearance [77,78]

Appearance	Scientific name	Common name	Other names
	*Carum carvi* L.	Caraway seeds/Persian cumin	Jiraa, zeera siyaah, kamoon, kamoon-roomi
	*Cumin cyminum* L.	Cumin	Safed jeeraa, kamun
	*Foeniculum vulgare* Mill.	Fennel seeds/sweet cumin	Perum jeerakam
	*Pimpinella anisum* L.	Aniseed, anise	Saumph, aneesun
	*Bunium persicum* (Boiss.) B. Fedtsch.	Black caraway	Jirak, jiraa siyah, kamoon-armani, shahi jeera, kaala jeera

1400 years ago. The original term is *cuminum* (cumin) in Latin, and karon in the Greek, which is transformed to carum (which now means caraway). Some synonyms for caraway in different parts of the world are indicated in Table 16.2 [66,81].

The origin of caraway, as one of the oldest recognized spice plants, is unknown. However, the names Persian caraway and Roman cumin suggest that it was introduced into global commerce and usage from Persia or Europe. It has been cultivated since olden times, and is found growing wild all over central Asia, especially in Iran, the Himalayas, northern and central Europe, the Balkans, and North Africa. By 1806 it was being cultivated in North America, where it was used as a flavoring to season bread and cheese, and as a pickling spice, to prepare the liquor Kummel, and to give flavor to sausages. Presently, it is mainly cultivated in North Africa, especially Egypt, and in Finland, the Netherlands, Eastern and Northern Europe, and Germany, the Mediterranean regions, Russia, Iran, Indonesia, and North America [59,70,82,83].

Even though the plant is indigenous to Asia, Europe, and Northern Africa, the plants are cultivated in several different regions throughout the world, this plant is better adapted to cooler climates than other Apiaceae species. Caraway plants are grown widely as a winter plant and as a summer crop in different regions, such as in Northern France, and the north Himalayan region in India [70,77]. In warmer regions, it is cultivated in the winter months as an annual, and in temperate climates it is cultivated as a summer annual or biennial plant. There is still some domestic production of caraway in Europe, including Germany and Scandinavia, even though it is mostly imported from Egypt, and the cultivation of caraway as a spice crop has been expanding in the United States and Canada [83,84].

For thousands of years, caraway has been used, particularly in China, Egypt, Roman Britain, the Sumer

TABLE 16.2 Synonyms of Caraway in Different Languages

Language	Alternative expression
Albanian	Qimnoni
Arabic	Taghde, Roman kommon, Al-Karvia, Al-Karawya, Kammun Armani, Karawiaa, Karawiya
Armenian	Chaman
Azeri	Adi Cirə
Belarusian	Kmen
Brazil	Alcarávia
Bulgarian	Kim
Burmese	Ziya
Catalan	Comi de Prat
Chinese	Fang Feng, Ge Lü Zi, Yuan Sui (Mandarin), Goht Leuih Ji (Cantonese)
Czech	Kmín, Kmín Kořenný, Kmín Luční
Danish	Almindelig Kommen, Karve, Kommen, Vild Kommen
Dutch	Karwij, Karwijzaad, Wilde komijn
English	Caraway, Persian cumin, Carum, Meridian fennel, Wild cumin, Carvies, Carroway
Estonian	Harilik Köömen
Finnish	Kumina, Saksan Kumina, Tavallinen, Kumina
French	Anis des pré, Carvi, Cumin des Prés, Kummel
German	Gemeiner Kümmel, Wiesenkümmel
Hindi	Vilayati jeera, foreign cumin
Italian	Caro, Carvi, Cumino dei prati, Kümmel
Norwegian	Karve
Persian (Farsi)	Black zeereh, Kermani zireh, Roman cumin
Romanian	Chimen
Russian	Тмин, Tmin
Spanish	Alcarahueya, Alcarave, Alcaravia, Carvi, Comino de prado
Swedish	Kummin
Turkish	Frenk kimyonu, Frankish cumin

area, and India, as a culinary seasoning [85], and may have been in use in Europe longer than any other spice. It was used by the Early Greeks in their recipes, and also recommended and prescribed by Dioscorides for healing purposes [70]. It is one of the most important species used as a wild food plant in the Eurasia regions and Estonia [86], and is utilized in the cuisines of the Middle East, India, Central Europe, and North Africa, especially Tunisia and Yemen [87] for its pungent liquorice flavor [88], which is derived from the essential oils, especially carvone and limonene. This culinary flavoring is found in sauerkraut, casseroles, curries, and other foods such as specific cheeses, different liqueurs, breads, cakes, desserts, and salads [89], as well as a spicy marinade for meats, dumplings, and goose [71]. In the Middle East, caraway pudding is a popular dessert during Ramadan, and the seeds are added as a spice in Persian cuisine to foods such as bread, yogurt, pickles, sauces, and salads [90]. Caraway seed oil is also used as a fragrance component in soaps, lotions, and perfumes.

From a therapeutic perspective, caraway is known as a traditional medicinal plant with a long history of healing [51,85,91]. It is believed to have many of the medicinal properties similar to dill, fennel, and anise, and is reported to have potent antispasmodic, antiseptic, aromatic, carminative, digestive, and stimulant properties. It has been used by chewing the raw dried fruits, and also in the form of drink or tea through brewing, decoction, distillation, fermentation, or as a tisane [92–96]. Therapeutic use of caraway products has been widely known in different ethnomedical systems from Northern Europe to the Mediterranean regions, Russia, Iran, Indonesia, and North America, where the use continues as a primary component of traditional treatments [59]. Traditionally, it was believed to warm and stimulate a cold, languid stomach. In folklore, it is used for the treatment of stomach complications, dysentery, uterine problems, internal wounds, and ulcers [97]. The fruits have also been applied in the form of a condiment, and the oil for the treatment of colds, coughs, sore throat, fever, bronchitis, gingivitis, and gastrointestinal complaints [98].

According to the ancient Persian-Islamic references in the ninth and tenth century AD, such as Rhazes' book *al-Hāwī fi al-Tibb* (Comprehensive Book on Medicine), Ibn Dawoud Dinawari's book *Kitab al Nibat* (*Book of Plants*), and Avicenna's *Al-Qanun fi'al-Tibb* (*The Rules of Medicine*), caraway is acclaimed to have healing properties. Greek physicians prescribed caraway oil or seeds for "pale-faced girls." Also, Romans consumed caraway to relieve indigestion [91]. Caraway is well-known for its carminative and stomach-calming properties, being mostly used for alimentary problems [58,99,100]. In Iranian traditional medicine, caraway has been recommended as an antiseptic, antispasmodic, antiparasitic, lactigenic, hypolipidemic, antiflatulence, carminative, and for digestive complaints [101]. Also in Iran, caraway is considered energizing, carminative and astringent, and its healing properties have been applied to gastrointestinal, gynecological, and respiratory conditions, and recommended for the treatment of toothache, diarrhea, and epilepsy [74,90]. Today, in the Middle East region, it is considered a medicinal plant commonly used for losing weight due to its ability to soothe the stomach, and improve digestion [100–103]. In Iranian-Islamic

traditional references such as *Makhzan Al-Advieh*, regular consumption of caraway extract is prescribed for losing weight [57], and today, it is sold as an antiobesity product in Iran's markets.

In the herbal remedies of India and Ayurveda, the fruits of caraway are mostly prescribed as a carminative, eupeptic, antispasmodic, and astringent, and applied for the treatment of mild gastrointestinal ailments, such as stomach-ache, bloating, indigestion, cramp, and flatulence due to its stomach-strengthening properties [77,104]. Caraway aqueous brewed extract is commonly used for pediatric conditions, especially digestive complaints. Also, in Ayurveda, it is claimed to improve the absorption of other plants, and to promote the function of vital organs, such as the liver. Extracts have also been used in bronchopulmonary conditions, as a cough therapy, and also as an analgesic. Vapors from caraway seeds are known to provide relief in patients suffering from back pain and rheumatism [69,105–107]. In Tibetan traditional medicine, caraway is considered to have a pungent taste and a warming influence due to its hot nature [87]. In the Moroccan system of folk medicine, the fruits of caraway are well-known as a stimulant, being consumed as a galactagog to stimulate milk production in lactating mothers, and to stimulate menstruation (emmenagog) in women, as a digestive, also to increase sexual desire (aphrodisiac), and urine flow (diuretic) [108]. It has been prescribed for healing hyperglycemia, hypertension, also heart and renal diseases [109–113]. In addition, it has been used to treat flatulent colic in infants, and for relieving stomach complaints, being frequently applied to flavor children's medicines [88,114,115]. In Jordanian traditional medicine therapies, caraway is used as a home remedy to treat different gastrointestinal and respiratory problems [116]. In the traditional medicine of Poland, caraway is known as a therapy for alimentary disorders, flatulence, and as a galactagog plant, while in Russia, it is recommended as a cure for pneumonia. In Great Britain and the United States, it is considered as a stomachic and carminative. In the Malay Peninsula, caraway is one of the important medicinal plants used during confinement, and in Indonesia, it is regarded as the therapy for inflamed eczema. Also, in India, it has been commonly used traditionally as a female fertility regulating agent [59,69,117].

A large number of experimental studies investigating the chemical and biological properties of various caraway preparations have been reported attempting to correlate the chemistry and the biological activities of this plant. From a chemical perspective, the aqueous and oil extracts of the roots of caraway have afforded a variety of phenolic and aromatic compounds, including different flavonoids, iso-flavonoids, flavonoid glycosides, monoterpenoids, such as carvone and its derivatives, glucosides, lignins, and alkaloids, as well as polyacetylenic compounds [118–123]. A number of phytonutrients have been found in caraway seeds, including different vitamins, amino acids, proteins, and minerals, also starch, sugars and other carbohydrates, tannins, phytic acid, and dietary fibers [124]. The other constituents present in this plant are fatty acids (saturated and unsaturated), triacylglycerol, polysaccharides, and lignin [125,126]. Carvone and limonene are usually reported as the main phytochemicals present in caraway seeds. The other important compounds extracted usually from hydro/steam distillation include: carvacrol, α-pinene, γ-terpinene, linalool, carvenone, and *p*-cymene [127–129]. Analysis of the caraway seed essential oils showed they varied in different regions and climates [130]. Detected phytochemicals are mostly phenolic and aromatic compounds, including monoterpenes (hydrocarbons/oxygenated), oxygenated sesquiterpenes, aldehydes, ketones, and esters [51].

As discussed earlier, since ancient times, caraway and its derivatives have been widely and commonly applied as traditional medicinal plants for treating different health problems in different cultures. A variety of the therapeutic properties of caraway mentioned in traditional medicine have been investigated and confirmed experimentally. Recently, significant developments in the pharmacological assessments of caraway have been reported resulting in several recommended therapeutic activities for this plant. The main reported pharmacological actions include antioxidant, antimicrobial, anticarcinogenic/antimutagenic, antidiabetic/hypoglycemic, hypolipidemic/antihyperlipidemic, diuretic, estrogenic/antiosteoporotic, and immunomodulatory. Caraway is also reported to have other functional properties, including: larvicidal, antibacterial, and antifungal activities, and health promoting effects on the central nervous system with adaptogenic property (antistress), as well as carminative and laxative for gastrointestinal conditions, antidyspeptic, antiulcerogenic, anti-asthmatic, antitussive, and antispasmodic activity. Furthermore, it is used industrially, in cosmetics, and also as fumigant, molluscicide, insecticidal, or pesticide [87].

The diversity of bioactivities expressed by caraway preparations is attributed to the multiplicity of bioactive constituents, hence its description as having a "hot" nature in some medical systems, attributed to the high content of essential oils, flavonoids, and phenolic compounds present [59,62,77]. However, linking a certain biological activity to a particular compound, which is the classical Western reductionist approach, has remained a challenging issue. It is more probable that multiple targeting (network pharmacology) of individual compounds and the synergy between and within specific types of phytochemicals is responsible for the diverse notable pharmacological properties.

Nonetheless, the biological actions reported for caraway compounds support the traditional medicinal properties [51,68].

The safety of a traditional medicine is frequently assumed based on historical use, and rarely scientifically established through a well-constructed clinical trial. This is especially an issue when different preparations are made which do not follow traditional methods, an aqueous extract, rather than an expressed, or steam-distilled, essential oil for example. Possible therapy constraints (dose, route of administration, etc.) must therefore be deliberated as limiting aspects based on clinical evidence. Presenting products of this plant as a safe and harmless substitute therefore involves examination of the probable side-effects of consuming caraway. Research shows that a combination of caraway seed oil and peppermint oil (50 and 90 mg, respectively) has shown skin allergy, burning sensation with eructation, and nausea in sensitive patients with functional dyspepsia [131]. In addition, due to the blood glucose-lowering activity of caraway, diabetics and patients taking drugs, plant medicines, or supplements with hypoglycemic property should consume this plant with caution. Such negative reports are rare, and no scientifically valid clinical trial supports the claims regarding the negative reactions of caraway essential oil in humans [132]. On the other hand, several studies have described the safety of caraway. In an animal study, the effects of different doses of caraway seed powder (30, 60, and 90 mg/kg body weight) on the formation of aberrant crypt foci in 1,2-dimethylhydrazine-induced colon cancer in rats were examined, with the result that no clinical signs of toxicity were detected in the treated rats [133].

The potential hepatoprotective ability of caraway oil extract was assessed through the carbon-tetrachloride-induced hepatotoxicity test in mice. The findings showed that this plant extract probably exhibits a hepatoprotective effect through maintaining the activity of xenobiotic detoxifying enzymes, including glutathione *S*-transferase, and glutathione peroxidase (GSH-Px), lowering GSH, and inhibiting lipid peroxidation [134,135]. While these findings support the safety and tolerability of caraway extract, additional safety studies are necessary to define the suitable dosage, guidelines, cautions, and other recommendations for the usage of various caraway preparations.

16.2 MATERIALS AND METHODS

16.2.1 Study Design and Study Outcomes

Taking a clinical trial approach, 110 overweight and obese women aged 20–55 were enrolled at different fitness centers in the city of Yazd, located in the centre of Iran. CAE is consumed regularly in Iran, especially in Yazd, for losing weight. Furthermore, as the rate of obesity is mostly higher in females, and women are usually more interested to attend weight loss programs than men [17], overweight and obese women were selected as the study population. Among the recruited candidates, only healthy women with BMI of more than 25 were included and screened for the dietary intervention program. Pregnant and lactating women, and individuals who suffered from specific health problems, were specifically excluded from this intervention. Also excluded were individuals with hypo/hyperthyroidism, a significant history or current presence of type I or II diabetes mellitus, or who were hypertensive (systolic BP 140 and/or diastolic BP 90), had clinically significant endocrine, hepatic, renal, or cardiovascular disease, such as impaired liver function, chronic renal disease, primary dyslipidemia, myopathy, or patients presently using drugs affecting metabolism or appetite. Individuals who have maintained a weight loss of 41 kg in the preceding 3 months, have the habit of not eating meals at regular intervals, have participated in another investigational study within the past 30 days, have a history of alcohol or drug abuse within the past year, have a history of sleep disorders, clinical depression, or other psychiatric or psychological conditions, and who are abnormally obese were also excluded. In general, individuals presenting with any medical condition or the use of any medication that could interfere with the conduct of the study, or which placed the prospective subject at risk, or who had a known allergy or sensitivity to any of the "active" or "placebo" product ingredients, were excluded.

This study was carried out in Iran, as an example of an emerging country with a high outbreak of obesity and overweight. Importantly, the people in Iran are familiar with the use of caraway seeds or its derivatives, as a flavoring agent for culinary purposes, and also of its remedial benefits, especially for decreasing weight in the form of water extract. Furthermore, caraway products are affordable and easily available in Iran, especially at the Yazd market. For gathering the required data, three methods were used: questionnaire, face-to-face interview, and a physical examination of the prospective study subjects. To evaluate the effects of caraway intake, volunteers were randomized into test and control groups, and they were asked to consume either the prepared caraway product or the placebo preparation daily for 12 weeks. The possible changes in body composition, anthropometric indices, blood profile, and vital parameters were investigated before and after intervention. Moreover, food intake status and physical activity level were measured during this period to ensure the harmony between the test and control groups. In order to reduce the possibility of allergic reactions due to the

consumption of caraway, only eligible females who were familiar with caraway were selected for the intervention treatment.

16.2.2 Preparation of Herbal Extract and Placebo

The caraway extract samples (0.1 w/v) obtained from the Baharan Company, Yazd, Iran (Industrial Ministry License no. 28/1232 and Health Ministry License no. 35/10500) were extracted from the seeds of caraway through steam distillation. The placebo was prepared by dissolving edible caraway essence (Givaudan Flavours Co., Kemptthal, Switzerland) in drinking water (1% g/L) which was identical with caraway extract in appearance and flavor. Subjects were provided with measured bottles and were asked to dissolve 30 mL of the placebo or caraway extract with 30 mL of water.

16.2.3 Analysis of Phytochemicals Using Gas Chromatography–Mass Spectrometry

The phytochemical constituents present in CAE were identified using gas chromatography–mass chromatography (GC–MS) analysis with a flame ionization detector, and extracted by Headspace solid phase microextraction (HS-SPME) with subsequent hexane extraction. The capillary gas chromatographic profiles of the CAE constituents were reported as their retention time compared with the MS of standard compounds.

16.2.4 Statistical Analysis

Values for each participant were standardized for each dependent variable to remove outliers using Z-scores, and the normal distribution was tested using the Kolmogorov–Smirnov test. Student's *t*-test, with a 99% confidence interval, was applied to identify the significant differences in values between groups, and the paired *t*-test was used to examine mean differences within each group during the 3-month treatment period. All statistical analyses were performed using SPSS software version 18.0.0 (SPSS Inc., Chicago, IL, USA), and all data are expressed as mean ± standard deviation (SD); p values less than 0.01 were considered to be significant and equal variances were assumed.

16.3 RESULTS

16.3.1 Follow-up of Subjects Involved in the Clinical Trial

Of the 110 overweight and obese females enrolled for the study, 70 were eligible for enrolment in the clinical trial. Sixty of the initially enrolled participants completed the full 12-week treatment regimen, of them, 10 subjects dropped out during the intervention (Figure 16.5).

16.3.2 Demographic and Baseline Characteristics of the Study Population

The demographic and baseline features of the participants in the study are illustrated in Table 16.3. Approximately 54% of the participants were overweight, and 46% were obese. All of the participants had abdominal obesity (waist circumference, WC, >88 cm). The average values (mean ± SD) of age, body weight, and BMI of the subjects were 37.1 ± 8.6 years, 75.4 ± 11.7 kg, and 29.8 ± 4.1 kg/m^2, respectively. The subjects had an average (mean ± SD) 44 ± 2.6 (kcal/kg/day) of physical activity level (PAL) and 7.9 ± 1.4 h sleep during a 24 h period.

There were no remarkable differences in any of the demographic variables measured, including age, height, weight, BMI, and bone mass between the study and control groups. Similarly, no significant differences were observed in sleep hours, PAL, basic metabolic rate (BMR), active metabolic rate (AMR), resting energy expenditure (REE), and total daily energy expenditure (TDEE) measurements between the CAE group and the placebo group at baseline. Also, no significant differences were observed in the average levels of body composition, anthropometric indices, and clinical and para-clinical assessments between the two groups at baseline.

16.3.3 Changes in Variables within and between Groups after the 12-Week Intervention Period

16.3.3.1 Effect of CAE on Body Composition

The changes in body composition variables over the 12 weeks of the trial for the CAE and placebo groups are summarized in Table 16.4. Following the 12 weeks of treatment, the mean body weight, BMI, and body fat (BF) percentage, had significantly decreased compared with placebo, whereas in the placebo group, these values increased slightly, although significant changes were not noticeable. Therefore, CAE possibly had a positive effect on inducing weight and fat loss.

In the CAE group there was an average weight loss of 1.9 kg, while in the placebo group, an average weight gain of 0.81 kg was observed. Also, the BMI showed a significant drop of −0.8 in the CAE group, while in the placebo group, the BMI increased slightly (+0.2). The BF percentage showed a significant reduction of −0.7% in the CAE group, while in the placebo group BF was

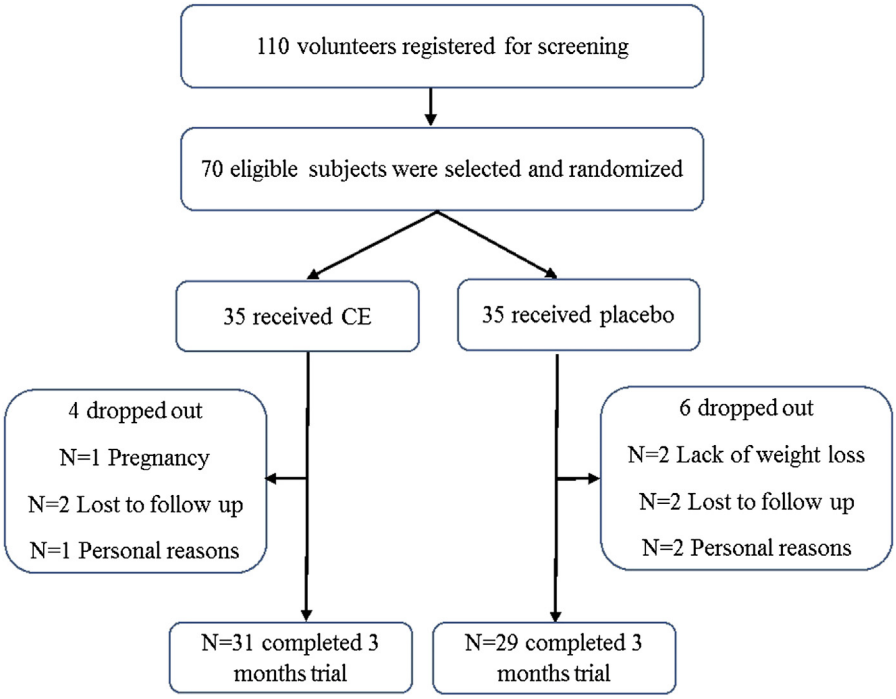


FIGURE 16.5 Flow chart of data collection and follow-up.

TABLE 16.3 Demographics and Baseline Characteristics of the Study Population Randomized to the Placebo or Caraway Aqueous Extract (CAE) Groups (n = 35)

	Placebo group	CAE group	
Variables	**Mean ± SD**	**Mean ± SD**	**p-value**
Age (years)	37.0 ± 7.9	37.2 ± 9.3	0.91
Height (cm)	158.2 ± 4.9	159.7 ± 6.2	0.25
Weight (kg)	74.9 ± 11.7	76.0 ± 11.8	0.70
BMI (kg/m^2)	30.4 ± 4.7	29.2 ± 3.4	0.24
Bone mass (kg)	7.8 ± 1.1	8.1 ± 1.1	0.35
Sleep (h/day)	7.9 ± 1.6	7.9 ± 1.3	0.97
PAL (kcal/kg/day)	43.7 ± 2.5	44.4 ± 2.8	0.26
BMR (kcal/m^2/h)	1474.4 ± 123.5	1488 ± 154.4	0.69
AMR (kcal/m^2/h)	2241.4 ± 216.3	2176.6 ± 260.5	0.30
REE (kcal)	1453.2 ± 133.3	1503.1 ± 127.4	0.15
TDEE (kcal)	2236.1 ± 206.9	2308.3 ± 193.2	0.17

Abbreviations: BMI, body mass index; PAL, physical activity level; BMR, basic metabolic rate; AMR, active metabolic rate; REE, resting energy expenditure; TDEE, total daily energy expenditure.

increased slightly (0.2%). In contrast, body muscle (BM) percentage increased significantly in the CAE group after treatment (0.2%), while in the placebo group, no significant changes were observed. Also, the difference in growing BM after the 12-week intervention was considerably higher in the subjects consuming CAE, compared to the placebo group. The percentage of body water (BW) reduced was slight in both the CAE and placebo groups (−0.01% and −0.2%, respectively), and was not remarkable, either within, or between, groups.

16.3.3.2 *Effect of CAE on Anthropometric Indices*

The anthropometric indices which were measured are also presented in Table 16.4. During the 12-week intervention, the waist circumference (WC) and the waist to hip ratio (WHR), were reduced significantly only in the CAE group, while the average levels of thigh circumference (TC) and mid-upper arm circumference (MUAC) showed significant reduction in both treatment groups. In addition, the level of reduction in all body size measurements and anthropometric indices were noticeable between the CAE group and the placebo group. After the intervention period, the WC in the CAE group showed a considerable drop of −6.2 cm, while in the placebo group the reduction was not significant (−0.1 cm). Also, the WHR was reduced (−0.03 cm) in the CAE group, while no noticeable changes were observed in the placebo group. The reduction in TC was noticeable in both the CAE and placebo groups (−5.4 cm and −1.9 cm, respectively) after treatment, and the difference in reduction between the two groups was significant. Mid-upper arm circumference (MUAC) showed a remarkable reduction of 2.2 cm in the CAE

TABLE 16.4 Body Composition and Anthropometric Indices Measured (Mean ± SD) at Baseline and after the 12-Week Treatment

	Week 0		Week 12	
Variables	**Placebo group (n = 29)**	**CAE group (n = 31)**	**Placebo (n = 29)**	**CAE group (n = 31)**
BODY COMPOSITION				
Weight (kg)	72.0 ± 10.7	76.9 ± 12.2	72.8 ± 10.8	75.0 ± 12.2[a]
BMI (Kg/m^2)	28.3 ± 2.6	30.7 ± 4.7	28.5 ± 2.8	29.9 ± 4.7[a]
BF (%)	33.8 ± 2.4	35.4 ± 3.6	34.0 ± 2.5	34.7 ± 3.7[a]
BM (%)	31.8 ± 1.3	31.4 ± 1.6	31.8 ± 1.3	31.6 ± 1.6[a]
BW (%)	48.3 ± 1.9	47.2 ± 2.6	48.1 ± 1.8	47.2 ± 2.7
ANTHROPOMETRIC INDICES				
WC (cm)	91.3 ± 7.3	96.0 ± 10.2	91.2 ± 7.9	89.8 ± 8.6[a]
WHR	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1[a]
TC (cm)	59.7 ± 4.5	61.7 ± 5.8	57.9 ± 4.6[b]	56.3 ± 5.6[b,a]
MUAC (cm)	31.0 ± 3.4	32.4 ± 3.3	30.2 ± 3.2[b]	30.2 ± 2.7[b,a]

[a] $p < 0.01$ *significantly different from baseline compared to placebo.*
[b] $p < 0.01$ *significantly different from baseline within group.*

group, while the drop in MUAC in the placebo group was only 0.8 cm, and the decrease in MUAC during the intervention was significantly higher in CAE group compared to the placebo group. Accordingly, the CAE showed greater efficacy than did placebo for each of the primary outcome variables with respect to the anthropometric indices and body composition measurements, except for BW.

16.3.3.3 *Effect of CAE on Clinical and Biochemical Variables*

The mean values (mean ± SD) of blood markers, including blood glucose, liver function and kidney function tests, lipid profile, and complete blood cell count (CBC) tests in both groups measured at baseline and after 12 weeks treatment were calculated, and the results are shown in Table 16.5. After 12 weeks of treatment, all of the variables showed either slight or significant changes in both groups. Overall, no statistically significant differences were observed either within or between groups in any of the variables, except for the CBC test. After the 12-week treatment, the red blood cell (RBC) level showed a clinically significant rise, whereas the PDW showed a remarkable drop in the CAE group compared to the placebo group.

16.3.3.4 *Effect of CAE on Para-Clinical Variables*

Para-clinical variables, including diastolic blood pressure (DBP), systolic blood pressure (SBP), and heart rate (HR), as markers of heart function, were measured at baseline and after 12 weeks of treatment, and the mean values are summarized in Table 16.5. In the CAE group, the SBP showed a slight rise of 0.7 ± 5.4 mmHg after the treatment period, whereas no remarkable changes were observed in the placebo group. Also, the DBP increased slightly in the CAE group (0.4 ± 4.7 mmHg), and in the placebo group a minor decrease was observed (−0.8 ± 4.7 mmHg). However, the differences were not noticeable either within or between treatment groups during the 12-week intervention. The average HR showed a minor decrease in both the CAE and placebo groups (−0.6 ± 4 bpm and −0.8 ± 3.8 bpm, respectively) after the 12-week treatment. No statistically significant changes were observed in either of the treatment groups. Hence, consumption of CAE did not show any clinically significant effects on BP and HR as markers of the heart function.

16.3.3.5 *Safety Issues and Adverse Events*

As mentioned above, no clinically significant changes or adverse events were observed in any of the biochemical variables and para-clinical measurements (HR, DBP, and SBP) also, no remarkable and/or undesirable changes were evidenced in the function of any of the vital organs, including heart, liver, and kidney functions, as well as urine and blood profile parameters, during the 12 weeks of treatment with CAE (Table 16.5). Also, during the 12 weeks of CAE intervention, no clinically significant opposing effects were reported by any of the subjects. No clinically significant effects from baseline were observed in the general health status between the placebo and the CAE groups. In addition, of the 60 participants who completed the study, only the subjects in the placebo group experienced mild skin allergy

TABLE 16.5 Differences (Mean ± SD) of Clinical and Para-Clinical Parameters between and within Groups during Intervention [136]

Variables		Week 0		Week 12	
BLOOD SERUM ASSESSMENTS	**Reference range**	**Placebo group (n = 29)**	**CAE group (n = 31)**	**Placebo group (n = 29)**	**CAE group (n = 31)**
FBS	70–110 mg/dL	91.6 ± 7.4	91.2 ± 12.0	93.7 ± 3.4	95.2 ± 11.8
LIVER FUNCTION TEST					
AST, SGOT	5–40 IU/L	16.9 ± 4.5	16.8 ± 4.4	16.6 ± 1.6	17.6 ± 7.8
ALT, SGPT	5–40 IU/L	16.1 ± 4.8	16.6 ± 4.7	16.3 ± 5.9	16.3 ± 4.9
ALP	64–306 IU/L	173.1 ± 28.0	181.5 ± 36.6	148.0 ± 30.9	163.0 ± 46.3
Bili, D	<0.25 mg/dL	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Bili, T	0.2–1.1 mg/dL	1.1 ± 0.5	1.2 ± 0.6	1.0 ± 0.5	1.1 ± 0.6
KIDNEY FUNCTION TEST					
Creat	0.4–1.5 mg/dL	0.9 ± 0.2	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
UA	3–6 mg/dL	4.1 ± 1.1	4.2 ± 1.0	4.2 ± 0.7	4.2 ± 0.5
Urea	10–50 mg/dL	28.9 ± 7.0	29.6 ± 7.7	28.4 ± 3.3	28.9 ± 8.3
LIPID PROFILE					
TC	<200 mg/dL	183.3 ± 22.6	209.3 ± 29.9	190.4 ± 51.9	199.0 ± 25.1
TG	<150 mg/dL	121.9 ± 41.5	112.8 ± 35.1	145.0 ± 50.4	124.4 ± 42.6
HDL-Chol	>46 mg/dL	53.0 ± 9.9	55.9 ± 9.6	51.7 ± 7.7	56.7 ± 10.1
LDL-Chol	<130 mg/dL	106.7 ± 17.7	123.9 ± 28.7	110.8 ± 41.9	125.8 ± 25.9
LDL/HDL	<2.8	2.1 ± 0.5	2.3 ± 0.8	2.2 ± 0.9	2.3 ± 0.6
Chol/HDL	<4.3	3.5 ± 0.6	3.9 ± 1.1	3.8 ± 1.2	3.7 ± 1.0
CBC TEST					
WBC	4–10 10^3/μL	6.8 ± 1.6	6.4 ± 1.4	6.9 ± 1.4	6.9 ± 1.8
RBC	3.6–6.1 10^6/μL	4.8 ± 0.5	4.5 ± 0.3	4.6 ± 0.3	4.7 ± 0.3[a]
HGB	11.5–18.8 (g/dL)	12.4 ± 0.9	12.8 ± 0.7	12.7 ± 0.6	13.3 ± 0.6[b]
HCT	34–54 (%)	38.7 ± 3.0	39.0 ± 2.9	38.7 ± 2.4	40.8 ± 1.5
MCV	80–100 fL	84.7 ± 11.4	87.7 ± 4.2	84.6 ± 6.3	87.1 ± 5.9
MCH	27–36 (pg)	28.3 ± 2.1	28.8 ± 1.5	27.8 ± 1.8	28.5 ± 1.6
MCHC	32–36 (g/dL)	32.8 ± 1.1	32.6 ± 1.1	32.9 ± 0.3	32.8 ± 1.1
PLT	150–450 10^3/μL	272.5 ± 60.5	228.9 ± 40.3	252.7 ± 63.4	232.8 ± 52.7
RDW	11.6–14.6 (%)	13.3 ± 0.8	13.1 ± 1.2	13.1 ± 0.5	13.5 ± 1.0
PDW	7–20 fL	14.0 ± 3.3	13.8 ± 2.9	15.9 ± 0.9	12.0 ± 2.7[a]
MPV	6–13 fL	9.5 ± 1.1	8.7 ± 1.2	9.5 ± 1.2	9.7 ± 1.41
URINE TEST					
USG	10.015–1.025 g/mL	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.006
PARA-CLINICAL VARIABLES					
DBP	60–90 mmHg	74.3 ± 6.0	75.5 ± 7.9	71.0 ± 7.6	75.9 ± 6.80
SBP	90–140 mmHg	111.3 ± 10.3	112.7 ± 10.4	111.3 ± 9.5	113.4 ± 11.21
HR	60–100 bpm	75.2 ± 8.7	78.1 ± 9.1	74.5 ± 8.6	77.5 ± 8.11

Abbreviations: FBS, Fasting blood sugar; AST, SGOT, Aspartate transaminase, glutamate oxaloacetate transaminase; ALT, SGPT, Alanine transaminase, glutamate pyruvate transaminase; ALP, Alkaline phosphatase; Bili, D, Bilirubin Direct; Bili, D, Bilirubin total; Creat, Creatinine; UA, Uric Acid; TC, Total cholesterol; TG, Triglyceride; HDL-Chol, High density lipoprotein cholesterol; LDL-Chol, Low density lipoprotein cholesterol; LDL/HDL, Low density lipoprotein to High density lipoprotein cholesterol ratio; Chol/HDL, Cholesterol to HDL ratio; CBC, Complete blood cell count; WBC, White blood cell; RBC, Red blood cell; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; PLT, Platelets count; RDW, Red cell distribution width; PDW, Platelet distribution width; MPV, Mean platelet volume; USG, Urine-specific gravity; DBP, Diastolic blood pressure; SBP, Systolic blood pressure; HR, Heart rate; mg/dL: Milligram/desi litre; g/dL, gram/desi litre; μL, microliter; IU/L, International unit per litre; fL, femtoliters (10^{-15}L); pg, picograms; Bpm, beats per minute; mmHg, millimeter of mercury.

[a]*Significant difference from baseline compared to placebo ($p < 0.01$), independent samples* t*-test.*

[b]*Significant difference from baseline within group ($p < 0.01$), paired samples* t*-test.*

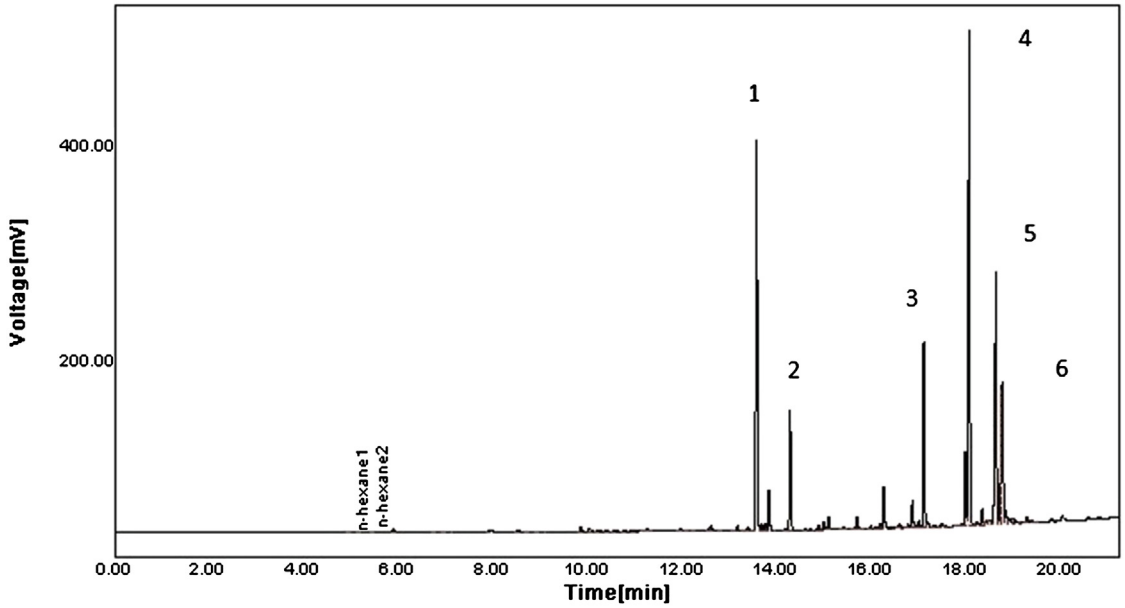


FIGURE 16.6 Chromatogram of caraway aqueous extract (CAE) infusion extracted by HS-SPME. CAE volatiles obtained by steam distillation, followed by hexane extraction include: 1: limonene, 2: γ-terpinene, 3: *trans*-carveol, 4: carvone, 5: thymol, 6: carvacrol.

reactions to the placebo product. However, no significant adverse effects were reported during the physical examinations. Also, there were no reported side-effects or complaints regarding the tolerability of the intake of CAE.

16.3.4 Detection of Phytochemicals Using GC–MS

The analytical results of the phytochemicals extracted into the CAE are shown in the gas chromatogram (Figure 16.6). The predominant ingredients detected from the GC–MS analysis were a range of volatile and phenolic compounds, especially simple monoterpenes, including limonene, γ-terpinene, *trans*-carveol, carvone, carvacrol, and thymol.

16.4 DISCUSSION

16.4.1 Analysis of CAE Phytochemicals

The initial objective of this study was to identify the major phytochemical constituents present in the steam-distilled caraway extract using GC–MS analysis. The results showed that most of the constituents detected from the GC–MS analysis were a range of different volatile and phenolic compounds, especially simple monoterpenes, including limonene, γ-terpinene, *trans*-carveol, carvone, carvacrol, and thymol, and the phenylpropene derivative, anethole. The structures of the isolated metabolites are shown in Figure 16.7 (http://webbook.nist.gov). With regard to the results of the GC–MS analysis in this study, these findings are consistent with the major compounds characterized from the caraway extract as reported in previous studies [128–130,135,137–141].

16.4.2 Effect of CAE on Body Composition, and Anthropometric Indices

The second objective of this study was to determine the role of CAE on weight loss, and identify the impact of CAE intake on body composition, and anthropometric indices in overweight and obese women. Hence, the weight-lowering activity of caraway as an established traditional medicinal plant in Iran was studied in a triple-blind, placebo-controlled, clinical trial in Iranian overweight and obese females. As nutrition and exercise are the two major components of life-style in the control of body weight, participants were enrolled who were habitually performing aerobics during the whole period of the intervention, without changing their dietary habits and lifestyle patterns.

This study showed that consumption of CAE may result in reasonable antiobesity effects. Similar findings were observed in a recent animal study reporting that 0.1% carvacrol, as one the major constituents of caraway, can prevent obesity and induce weight and fat loss in mice fed with high fat diet [55]. Also, several animal studies in normal and diabetic rats have proved the beneficial effects of CAE (20 mg/kg) on treating a number of health problems such as hyperglycemia [142], and

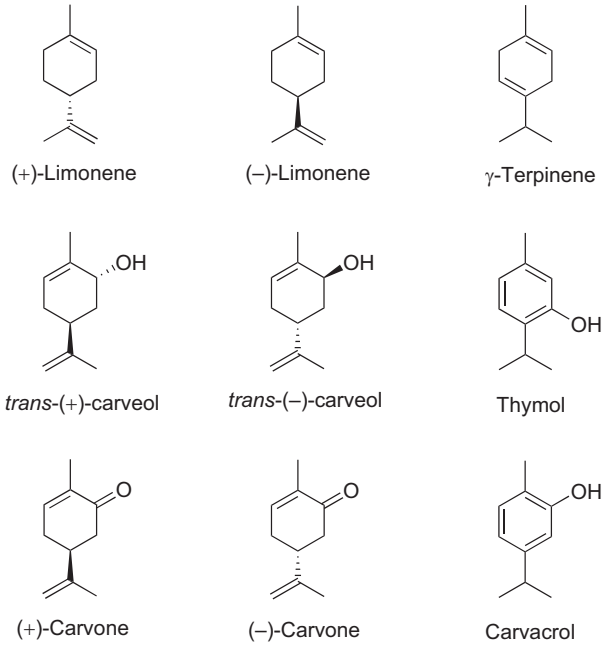


FIGURE 16.7 Structures of the extracted metabolites.

hyperlipidemia [143] which are recognized as common health consequences of obesity. Together, this clinical trial suggests a possible phytotherapeutic approach and natural medication for the application of CAE in the management of obesity.

In this study, changes in body composition, and anthropometric indices in healthy, overweight, and obese females after the 12-week consumption of CAE or placebo were evaluated. Clinically significant improvement in all the mentioned parameters after the treatment period was observed. Among the body composition variables, total body weight and fat percentage showed significant reduction in participants consuming CAE. These findings support the study hypothesis that consumption of caraway seed extract could be of value in reducing weight and fat percentage in humans. It is probable that body weight and fat loss might be linked to anti-oxidant, anti-inflammatory, and antibacterial properties of caraway phytochemicals, especially phenolic compounds, such as carvacrol, and the unsaturated fatty acids (UFA) [130]. The intake of such bioactive ingredients with high antimicrobial activity, prevents the proliferation of pathogenic microorganisms, and thus enhances the growth and multiplying of beneficial gut bacteria [137,144]. These interactions will possibly improve the balance of gut microbial ecology and alter gastrointestinal (GI) microbiome towards higher beneficial gut bacteria which could further improve digestion and absorption of the ingested food providing homeostasis in GI tract [145–147]. On the other hand, gut microflora (GM) could affect the host metabolism through regulating the expression of human genes. Hence, the interplay between GM, gene expression, and the host metabolic activity may have an effect on obesity and weight changes. This assumption is in line with the recent findings demonstrating that GM composition could have mutual interplay with the host, programming and affecting metabolism in human body altering body composition [148,149]. The suggested mechanism of action is explained in Figure 16.8 [64].

In this procedure, the gut microflora, which were altered following the antimicrobial actions of the present bioactive compounds, may induce the expression of specific genes involved in fat metabolism, which consequently leads to constraining inflammation in adipose tissue, and preventing adipogenesis [55,150]. Previous studies indicated that the balanced gut microbiota prevents macrophage infiltration into adipose tissue causing interruption in the transformation of preadipocytes to mature adipocytes. This procedure will subsequently lead to constraint in adipogenesis and differentiation of adipocytes [151]. These illustrations reinforce the possible role of the anti-inflammatory properties of the compounds present in CAE in weight and fat loss. Also, this hypothesis is in line with the findings of recent studies reporting that inflammation is related to body fat [152]. Hence reducing the fat mass, which occurred in this study, might also be linked with anti-inflammatory reactions occurring in the human body.

Furthermore, UFAs could induce fatty acid oxidation resulting in lipolysis and fat loss [153,154]. In addition, caraway essential oils and phenolic compounds could inhibit lipid peroxidation and enhance apoptosis in preadipocytes due to their antioxidant properties [135]. These compounds can diminish adipose tissue and body fat mass through inhibiting adipogenesis, inducing apoptosis in preadipocytes, and stimulating lipolysis in adipocytes [155–157]. In addition, previous studies have proposed that an aqueous extract of caraway could display lipolytic activity due to reducing intestinal lipid absorption, by binding with bile acids. A similar mechanism might be appropriate to describe the observed fat-lowering activity of CAE. Also, caraway may facilitate weight and fat loss through inhibiting lipid biosynthesis by altering lipid metabolism which may contribute to the regulation of body fat [143]. Accordingly, the weight-lowering property of caraway may be mediated by the improvement of digestion, absorption, and lipid metabolism. In conclusion, this trial revealed that daily consumption of CAE for 12 weeks evokes an advantageous effect on overweight and obesity. This outcome supports its consumption by the Iranian population for the treatment and management of obesity. It also implies that intake of caraway could

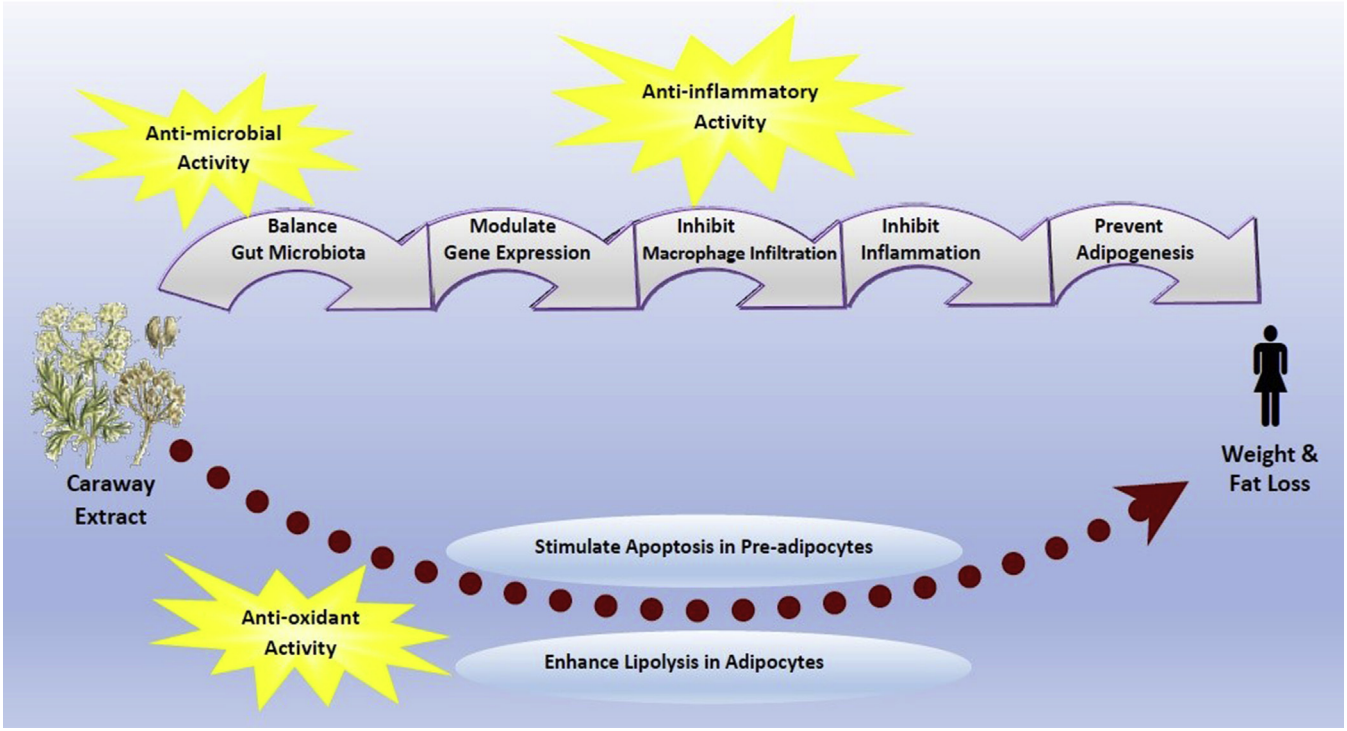


FIGURE 16.8 The possible metabolic actions of caraway extract on the human body during weight loss [64].

be useful in decreasing the complications of obesity, such as diabetes mellitus and cardiovascular diseases.

Altogether, the health benefits of CAE lay in the potential contribution of this plant-based product to the overall intake of potent phytochemicals, especially phenolic and volatile compounds. However, supplementary studies are required to examine the effects of these constituents in treating obesity and overweight at the molecular level. Also, the actual sites of this activity, the detailed mechanisms of action, and the specific bioactive components of caraway involved should be determined.

With regard to the body composition changes, body weight and fat were decreased meaningfully, and are consistent with a recent animal study demonstrating the potent antiobesity activity of carvacrol in obese mice [55]. In contrast, no significant alterations were observed in the BW percentage in participants during the study. These findings show that the intake of CAE should not have any adverse effects on body health. Usually, the percentage of water will be changed during various disease states, and assessing BW is essential in determining the occurrence and progression of diseases [158].

However, two previous animal studies have reported that administration of caraway oil (10 mg/kg body weight) and the aqueous extract (1 g/kg body weight) could increase weight and alleviate weight loss, along with decreasing blood sugar in diabetic rats [159,160]. This finding can be justified in that weight loss is one of the clinical issues of diabetes mellitus, which might occur due to imbalances in body metabolism. In this case, administration of caraway oil or aqueous extract could regulate the body metabolism, normalize blood sugar, and bring body weight to the normal level. Hence, although this report is in contrast with these findings, it reinforces the demonstration in the present study of the beneficial effect of caraway intake on restoring body weight to the normal level.

On the other hand, BM percentage showed a slight development in the CAE group, whereas there were no significant changes in muscle percentage in the placebo group during the course of the study. Thus physical activity did not have an interfering effect on decreasing body weight and fat percentage. Accordingly, these outcomes suggest that the positive alterations in body composition were probably related to the phytochemicals present in CAE, and not essentially with the exercise, even though it was expected that physical activity would probably have a synergistic effect on lowering body weight and body fat in those study participants consuming CAE. Furthermore, the bioproducts that have been formed during lipolysis were probably transformed into muscle induced by exercise, synergistically, and the loss in body fat and rise in muscle mass is most likely due to physiological adaptations to physical activity [37,161,162].

With regard to the alterations in body size, as expected, all anthropometric indices showed a notable

decrease in those study participants consuming CAE compared to the placebo group. The findings show that the WC, the waist to hip ratio, the thigh circumference, and the MUAC decreased significantly after the 12-week consumption of CAE. As mentioned previously, demographic values showed that all of the participants had WC greater than borderline, showing abdominal obesity at baseline. The WHR and WC are the major determinants and markers of central adiposity, and are recognized as the major risk factors for the health related issues, such as cardiovascular problems, ischemic stroke, cancer mortality, myocardial infarction, diabetes, impaired fibrinolytic activity, etc. [163–168]. Consequently, reducing WHR and WC through consumption of CAE may be of value in the prevention and treatment of abdominal obesity and its complications. On the other hand, the body size values did not show any significant reduction in the placebo group during the course of the study, except for the thigh circumference and the MUAC, which indicates that exercise did not have any interfering or synergistic effect on the reduction of abdominal obesity and visceral fat.

Anthropometric indices are known as beneficial indicators for evaluating the adiposity of an individual, and worthy markers of the health and nutritional status of an individual [169]. The results of the anthropometric assessments are in line with the outcomes of body composition measures, indicating that there is a link between a reduction in body size, body fat, and weight, as lowering the WHR and WC reflect a reduction in visceral fat. Also, these results show a possible contribution of CAE in lowering abdominal fat. Overall, these findings indicate that the balancing, improvement, and normalization in body size and body composition have occurred simultaneously. Together, these results provide complementary and supplementary data which support the study hypothesis regarding the potential antiobesity activity of caraway essential oil/aqueous extract ingredients for human.

16.4.3 Effect of CAE on Clinical and Para-Clinical Variables and Safety Issues

The third objective of this study was to examine the safety and tolerability of CAE intake for humans. There appears to be no triple-blind clinical study on the safety and tolerability of any caraway preparations taken alone. A few studies have examined the safety of caraway oil and as a combination with other phytochemicals, but not as a single aqueous extract preparation [170]; May et al. [171]. The study illustrates that daily consumption of CAE induces weight loss with no clinically significant adverse events or serious side effects. During this clinical trial, the intake of CAE did not show any side effects on major vital organ functions, including the heart, liver, and kidney. Also, no clinically significant changes were observed on urine specific gravity, serum blood glucose level, and lipid profile. Overall, no remarkable adverse events were observed in the clinical and para-clinical valuations with either treatment during the 12-week intervention period, and the CAE was well-tolerated by all of the participants. These findings may support other opinions which have recommended caraway as a safe natural product with several therapeutic effects for humans (May et al., 2000) [59,135,171].

Caraway and its byproducts have a long history of folk usage, and are widely-used plant-based products [88,172]. There are a number of in vitro and in vivo studies which report different therapeutic potentials and biological activities for caraway oil/aqueous extract and its derivatives, for example antimicrobial, anti-inflammatory, antioxidant, anticancer, and antispasmodic effects [51,61,145]. However, studies on the safety and tolerability of caraway seed and its extracts in humans are few. In this research, the safety and tolerability of CAE were studied in healthy, overweight and obese women in a randomized, triple-blind, placebo-controlled clinical trial during 3 months. After the treatment period, no severe adverse events were observed in any of the participants with respect to the cardiovascular, hepatic, renal, or hemopoietic systems. Also, the results revealed no significant changes in the clinical and para-clinical variables. As a result, this CAE may be regarded as being well-tolerated, and safe to consume with no problematic effects for humans [136]. The findings concerning the adverse effects of CAE intake were as expected, and in agreement with those of the earlier studies, indicating that there are no known and recognized cautions, adverse events, or drug interactions associated with the consumption of caraway [173,174].

The findings of the blood CBC test indicated that the average level of RBC improved noticeably while the mean values of PDW showed a remarkable reduction in subjects consuming CAE as compared with the placebo group after the 12-week intervention. On the other hand, HG levels showed a significant increase only in the CAE group, which supports the findings related to the remarkable rise observed in the RBC levels. These alterations display the potential valuable influence of CAE as a phytotherapeutic agent for the treatment of anemia. It is possible that treatment with CAE may well compensate for the low RBC level in patients suffering from anemia. These judgments are in line with an earlier animal study which suggested that CAE (2.5 g/100 mL) may have protective properties against anemia through stimulating the absorption of iron in the gastrointestinal tract [175]. Also, the reduction in the PDW levels in patients consuming CAE is in line with a previous study which

reported the antiplatelet properties of carvacrol [145]. Prior studies revealed that hyperthyroidism is linked to a rise in MPV (mean platelet volume) levels and a decline in PDW levels [176]. In addition, administration of a hydro-alcoholic extract of caraway (1600 mg/kg) displayed a hyperthyroidism influence through escalation in T3 and T4 levels, and a reduction in TSH levels [177]. Also, the weight of rats in the caraway extract group was remarkably lower than the control group, which shows the weight lowering effect of caraway extract.

Thyroid hormones are recognized as determining factors of the basal metabolic rate and energy expenditure. Therefore, modifying thyroid hormone levels affects thermogenesis in the body leading to major changes in body composition, reducing body fat, and body weight [178–180]. In line with these findings, the present outcomes propose a moderate antihypothyroidism influence of CAE in humans. This plant preparation may influence hypothyroidism homeostasis, which could possibly give rise to an enhanced metabolic rate and, in consequence, a reduction of body weight and fat percentage [136]. This is in agreement with the body composition changes observed in this study. The probable mechanism of action and effect of consumption of CAE on biochemical variables are shown in Figure 16.9. Altogether, this study suggests that CAE, as a potential antiobesity plant, can be consumed in moderation and regularly as part of a healthy, balanced diet for normalizing body composition without any severe adverse effects.

This study is the first triple-blind clinical trial examining the effects of consumption of CAE on body composition and anthropometric indices, along with a physical activity program, and investigating the antiobesity effect of CAE in overweight and obese females during 12 weeks of treatment. Moreover, this study has three additional strengths. Firstly, participants consuming CAE showed noteworthy reductions in body weight, body fat, and also body size, as compared with the placebo group, although they did not change their dietary habits. Secondly, this interventional study was a randomized, triple-blinded, placebo-controlled clinical trial which improves the precision and accuracy of the outcomes and decreases probable bias in the results.

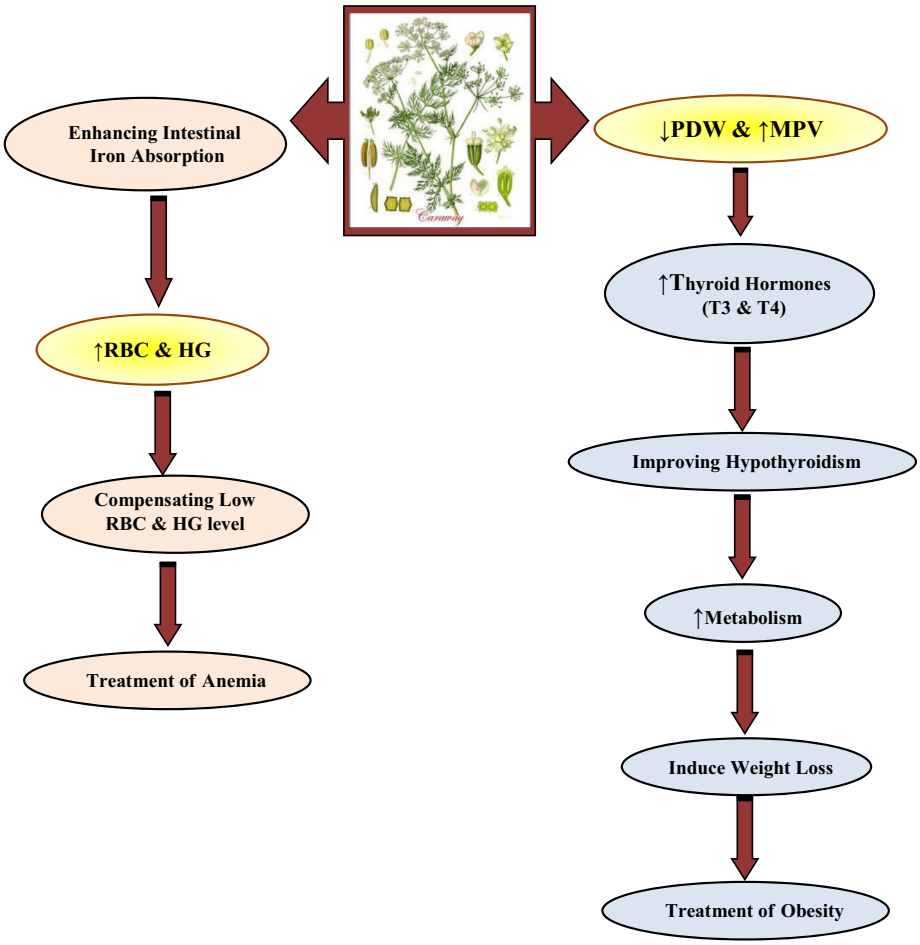


FIGURE 16.9 Plausible CAE effects in the treatment of anemia, hypothyroidism, and obesity [136].

Thirdly, this is the first evidence-based study demonstrating the safety and tolerability of a caraway extract in humans.

16.5 CONCLUSIONS

This research was carried out to examine the efficacy and safety of a caraway extract, as an example of a traditional, antiobesity medicinal plant, on healthy obese and overweight women in a randomized, triple blind, placebo controlled, clinical trial during a 12-week period. The results showed that consumption of CAE could be of practical value in the management of obesity. According to previous reports, antiobesity plants are able to induce weight loss through different mechanisms, including suppressing appetite, inducing lipolysis, inhibiting lipogenesis, and enhancing metabolism [40]. In addition, some of the potent antiobesity plants can induce weight loss through two or more mechanisms [181,182]. From the findings of this study discussed previously, it is proposed that CAE can induce weight loss through regulating multiple mechanisms including lipolysis, lipogenesis, and metabolism. Also, it can be concluded that the antiobesity properties of CAE are due to the presence of the bioactive compounds present. From the evidence presented in this study and in previous reports, it is hypothesized that the antiobesity activity from the prebiotic effect of CAE in the gastrointestinal tract through modulating gut microflora [64]. The mechanism of action of these phytochemical constituents needs to be defined at the molecular level. At the same time, efforts to make available this evidence and pursue the expanded human use of this traditional medicinal plant, and develop the consumption of caraway and its derivatives as a sustainable dietary practice, together with exercise, as a part of a healthy lifestyle, should be continued.

The outcomes of this study indicate that a daily, single dose of CAE for 12 weeks did not show any remarkable negative events in healthy, overweight and obese women. Treatment with CAE was on the whole, well-tolerated with no informed problematic effects. In addition, the noticeable rise in RBC and reduction in PDW afforded by the 12-week administration of the caraway extract suggests a potential use of this plant-derived extract in the management of anemia and hypothyroidism, respectively, which requires further investigation [136]. However, additional studies, with different doses and preparations of CAE in other national and ethnic groups, with multiple genders, with larger population, in wider age groups, with a variety of health problems, and over a longer extended period of time, are recommended to determine more accurate data about the safety, tolerability, efficacy, and applicability of CAE, and to further improve the importance and worth of these primary clinical findings. It is hoped that the findings of this study will be useful for the management and fight against obesity and overweight using natural sources with potent antiobesity activity to improve the quality of life of the world's population.

16.6 SUMMARY

The scientific examination of medicinal plants could lead to new findings in the development of natural antiobesity therapies. By examining an extract of caraway as a potent traditional plant for losing weight, this study will contribute to the options for treatment using either chemical or traditional medicinal plant approaches that would help people with obesity to make the right decision. Moreover, analysis and identification of the bioactive ingredients of this herb may lead to a natural antiobesity product based on a novel formula. Conducting this study improves and addresses important health problems related to obesity by the application of scientific methods to the study of traditional medicinal plants. This is the essence of the evidence-based approach. In addition, this clinical methodology could serve as a template for evaluating the currently available weight loss products. This would prevent further harmful, and even irremediable effects of overdosage or misusage of such products. This study presents clinical research-based data on the impact of caraway on the management or treatment of obesity, and indicates that this plant-based medicine may have a high market potential.

Acknowledgment

The authors are grateful to Prof. Toh Seok Kam, Department of Chemistry, University of Malaya, for assistance in the preparation of the structure diagrams for this article.

References

[1] Farr C, Virchow K. Towards a common definition of global health. Lancet 2009;373:1993–5.
[2] World Health Organization. WHO traditional medicine strategy 2002–2005. WHO/EDM/TRM/2002.1. Geneva: WHO; 2002.
[3] World Health Organization. WHO traditional medicine strategy 2014–2023. Geneva: WHO; 2014.
[4] World Health Organization. The regional strategy for traditional medicine in the western Pacific (2011–2020). Manila, Republic of the Philippines: WHO, Regional Office for the Western Pacific; 2012.
[5] Cordell GA. Sustainable drugs and global health care. Quím. Nova 2009;32(5):1356–64.
[6] Cordell GA. Phytochemistry and traditional medicine – a revolution in process. Phytochem Lett 2011a;4(4):391–8.
[7] Cordell GA. Plant medicines key to global health. Chem Eng News 2011b;89(26):52–6.

[8] Cordell GA. Sustainable medicines and global health care. Planta Med 2011c;77(11):1129–38. http://dx.doi.org/10.1055/s-0030-1270731.
[9] Cordell GA. Phytochemistry and traditional medicine – the revolution continues. Phytochem Lett 2014. http://dx.doi.org/10.1016/j.phytol.2014.06.002.
[10] Cordell GA, Colvard MD. Natural products and traditional medicine: turning on a paradigm. J Nat Prod 2012;75(3):514–25.
[11] Cordell GA. New strategies for traditional medicine. In: Rai M, Cordell GA, Martinez J, Marinoff M, Rastrelli L, editors. Medicinal plants: biodiversity and drugs. Boca Raton (FL): CRC Press; 2012. p. 1–45.
[12] World Health Organization. Obesity: preventing and managing the global epidemic, report of a WHO consultation (WHO technical report series 894). Geneva: WHO; 2000.
[13] Auld MC, Powell LM. Economics of food energy density and adolescent body weight. Economica 2009;76(304):719–40.
[14] Caballero B. The global epidemic of obesity: an overview. Epidemiol Rev 2007;29(1):1–5.
[15] Delpeuch F, Maire B, Monnier E, Holdsworth M. Globesity: a planet out of control. London: Earthscan; 2009.
[16] World Health Organization. Obesity and overweight. Fact Sheet No. 311. Geneva: WHO Media Centre; 2011.
[17] Yach D, Stuckler D, Brownell KD. Epidemiologic and economic consequences of the global epidemics of obesity and diabetes. Nat Med 2006;12(1):62–6.
[18] Barness LA, Opitz JM, Gilbert-Barness E. Obesity: genetic, molecular, and environmental aspects. Am J Med Genet A 2007;143(24):3016–34.
[19] Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual causes of death in the United States, 2000. J Am Med Assoc 2004;291(10):1238–45.
[20] Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of US adults. N Engl J Med 2003;348(17):1625–38.
[21] Guh D, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis A. The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. BMC Public Health 2009;9(88):1–20.
[22] Shehzad A, Ha T, Subhan F, Lee Y. New mechanisms and the anti-inflammatory role of curcumin in obesity and obesity-related metabolic diseases. Eur J Nutr 2011;50(3):151–61. http://dx.doi.org/10.1007/s00394-011-0188-1.
[23] Olshansky SJ, Passaro DJ, Hershow RC, Layden J, Carnes BA, Brody J, et al. A potential decline in life expectancy in the United States in the 21st century. N Engl J Med 2005;352(11):1138–45.
[24] Kopelman PG. Obesity as a medical problem. Nature 2000;404(6778):635–43.
[25] James PT, Rigby N, Leach R. The obesity epidemic, metabolic syndrome and future prevention strategies. Eur J Cardiovasc Prev Rehabil 2004;11(1):3–8.
[26] Colditz GA. Economic costs of obesity and inactivity. Med Sci Sports Exerc 1999;31(11):S663–7.
[27] Picot J, Jones J, Colquitt J, Gospodarevskaya E, Loveman E, Baxter L, et al. The clinical effectiveness and cost-effectiveness of bariatric (weight loss) surgery for obesity: a systematic review and economic evaluation. Health Technol Assess 2009;13(41):1–190. 215–357, iii–iv.
[28] Wolf AM, Colditz GA. Current estimates of the economic cost of obesity in the United States. Obes Res 1998;6(2):97–106.
[29] Sofi F, Abbate R, Gensini GF, Casini A. Accruing evidence on benefits of adherence to the mediterranean diet on health: an updated systematic review and meta-analysis. Am J Clin Nutr 2010;92(5):1189–96.
[30] Nestle M, Jacobson MF. Halting the obesity epidemic: a public health policy approach. Public Health Rep 2000;115(1):12–24.
[31] Weiss EC, Galuska DA, Khan LK, Serdula MK. Weight-control practices among US adults, 2001–2002. Am J Prev Med 2006;31(1):18–24.
[32] Blanck HM, Khan LK, Serdula MK. Use of nonprescription weight loss products. J Am Med Assoc 2001;286(8):930–5.
[33] Padwal RS, Majumdar SR. Drug treatments for obesity: orlistat, sibutramine, and rimonabant. Lancet 2007;369(9555):71–7.
[34] Weigle DS. Pharmacological therapy of obesity: past, present, and future. J Clin Endocrinol Metab 2003;88(6):2462–9.
[35] Blanck HM, Serdula MK, Gillespie C, Galuska DA, Sharpe PA, Conway JM, et al. Use of nonprescription dietary supplements for weight loss is common among Americans. J Am Diet Assoc 2007;107(3):441–7.
[36] Rucker D, Padwal R, Li SK, Curioni C, Lau DCW. Long term pharmacotherapy for obesity and overweight: updated meta-analysis. Br Med J 2007;335(7631):1194–9.
[37] Pittler MH, Ernst E. Dietary supplements for body-weight reduction: a systematic review. Am J Clin Nutr 2004;79(4):529–36.
[38] Balsiger BM, Murr MM, Poggio JL, Sarr MG. Bariatric surgery: surgery for weight control in patients with morbid obesity. Med Clin North Am 2000;84(2):477–89.
[39] Fouad M, Rastam S, Ward K, Maziak W. Prevalence of obesity and its associated factors in Aleppo, Syria. Prev Control 2006;2(2):85–94.
[40] Kazemipoor M, Radzi CWJWM, Cordell GA, Yaze I. Safety, efficacy and metabolism of traditional medicinal plants in the management of obesity: a review. Int J Chem Eng Appl 2012;3(4):228–92. http://dx.doi.org/10.7763/IJCEA.2012.V3.201.
[41] Chang J. Medicinal herbs: drugs or dietary supplements? Biochem Pharmacol 2000;59(3):211–9.
[42] Chaput JP, St-Pierre S, Tremblay A. Currently available drugs for the treatment of obesity: sibutramine and orlistat. Mini Rev Med Chem 2007;7(1):3–10.
[43] Clegg A, Colquitt J, Sidhu M, Royle P, Walker A. Clinical and cost effectiveness of surgery for morbid obesity: a systematic review and economic evaluation. Int J Obes 2003;27(10):1167–77.
[44] Pittler MH, Ernst E. Complementary therapies for reducing body weight: a systematic review. Int J Obes 2005;29(9):1030–8.
[45] Pittler MH, Schmidt K, Ernst E. Adverse events of herbal food supplements for body weight reduction: systematic review. Obes Rev 2005;6(2):93–111.
[46] Kumari P, Singh N, Bhatia V, Chawla, Kumar D. Herbal fight for obesity: a review. Int J Pharm Res Dev 2011;3(4):25–8.
[47] Smyth S, Heron A. Diabetes and obesity: the twin epidemics. Nat Med 2006;12(1):75–80.
[48] Dara L, Hewett J, Lim JK. Hydroxycut hepatotoxicity: a case series and review of liver toxicity from herbal weight loss supplements. World J Gastroenterol 2008;14(45):6999–7004.
[49] Mariaca RG, Berger TFH, Gauch R, Imhof MI, Jeangros B, Bosset JO. Occurrence of volatile mono- and sesquiterpenoids in highland and lowland plant species as possible precursors for flavor compounds in milk and dairy products. J Agric Food Chem 1997;45(11):4423–34.
[50] Hammer K, Lehmann CO, L'errino P. A check-list of the Libyan cultivated plants including an inventory of the germplasm collected in the years 1981, 1982 and 1983. Gen Res Crop Evol 1988;36(3):475–527.
[51] Johri RK. *Cuminum cyminum* and *Carum carvi*: an update. Pharmacogn Rev 2011;5(9):63–72. http://dx.doi.org/10.4103/0973-7847.79101.
[52] Angelakis E, Armougom F, Million M, Raoult D. The relationship between gut microbiota and weight gain in humans. Future Microbiol 2012;7(1):91–109.
[53] Armougom F, Henry M, Vialettes B, Raccah D, Raoult D. Monitoring bacterial community of human gut microbiota reveals

an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. PLoS One 2009;4(9):e7125.
[54] Al-Essa MK, Shafagoj YA, Mohammed FI, Afifi FU. Relaxant effect of ethanol extract of *Carum carvi* on dispersed intestinal smooth muscle cells of the guinea pig. Pharm Biol 2010;48(1): 76–80. http://dx.doi.org/10.3109/13880200903046161.
[55] Cho S, Choi Y, Park S, Park T. Carvacrol prevents diet-induced obesity by modulating gene expressions involved in adipogenesis and inflammation in mice fed with high-fat diet. J Nutr Biochem 2012;23(2):192–201. http://dx.doi.org/10.1016/j.jnutbio.2010.11.016.
[56] Said O, Saad B, Fulder S, Khalil K, Kassis E. Weight loss in animals and humans treated with "Weighlevel," a combination of four medicinal plants used in traditional Arabic and Islamic medicine. Evid Based Complement. Alternat Med 2011:1–6. http://dx.doi.org/10.1093/ecam/nen067.
[57] Aghili Khorasani MH. Makhzan al Adviah. Tehran (Iran): Tehran: Safa Publication. Bavardaran Press, Research Institute for Islamic and Complementary Medicine, Iran University of Medical Sciences; 2001.
[58] Nasser M, Tibi A, Savage-Smith E. Ibn Sina's Canon of Medicine: 11th century rules for assessing the effects of drugs. J R Soc Med 2009;102(2):78–80. http://dx.doi.org/10.1258/jrsm.2008.08k040.
[59] Sadowska A, Obidoska G. Pharmacological uses and toxicology of caraway. In: Németh É, editor. Caraway; the genus *Carum*. Amesterdam (The Netherlands): Taylor & Francis e-Library; 2003. p. 186–96.
[60] Thompson RJ. Method and composition of a carminative herb or natural supplement to decrease the adverse effects of orlistat, and oral lipase inhibitor. Manchester: U.S. Patent US20080069906 A1; March 20, 2008. Issue.
[61] Alhaider A, Al-Mofleh I, Mossa J, Al-Sohaibani M, Rafatullah S, Qureshi S. Effect of *Carum carvi* on experimentally induced gastric mucosal damage in wistar albino rats. Int J Pharmacol 2006;2(3):309–15.
[62] Charles DJ. Caraway. In: Antioxidant properties of spices, herbs and other sources. Norway (IA, USA): Springer; 2013. p. 199–206.
[63] Plant OH, Miller GH. Effects of carminative volatile oils on the muscular activity of the stomach and colon. J Pharmacol Exptl Therapeut 1926;27(2):149–64.
[64] Kazemipoor M, Radzi CWJBWM, Hajifaraji M. Antiobesity effect of caraway extract on overweight and obese women: a randomized, triple-blind, placebo-controlled clinical trial. Evid Based Complement Alternat Med 2013;2013. http://dx.doi.org/10.1155/2013/928582.
[65] Bennett BC. Economic botany: twenty-five economically important plant families. Encyclopedia of life support systems. Oxford (UK): UNESCO-EOLSS Publishers; 2010.
[66] Brechbill GO. The spice notes of fragrance. (New Jersey, USA): Fragrance Books Inc.; 2012.
[67] Papini A, Banci F, Nardi E. Molecular evidence of polyphyletism in the plant genus *Carum* L. (Apiaceae). Genet Mol Biol 2007; 30(2):475–82.
[68] Khan IA, Abourashed EA. Leung's encyclopedia of common natural ingredients used in food, drugs and cosmetics. 3rd ed. Hoboken (New Jersey): John Wiley & Sons, Inc.; 2011.
[69] Perry LM, Metzger J. Medicinal plants of east and southeast Asia: attributed properties and uses. Massachusetts and London: MIT Press; 1980.
[70] Prance GS, Nesbitt M, editors. The cultural history of plants: spices. New York: Routledge; 2005.
[71] Vaughan J, Geissler C. The new oxford book of food plants. London (UK): Oxford University Press; 2009.
[72] Discover life organization *Carum carvi* L. (caraway). http://www.discoverlife.org/20/q.
[73] Britton NL, Brown A. An illustrated flora of the northern United States, Canada and the British possessions. 2nd ed., vol. 2. New York (USA): Charles Scribner's Sons; 1913.
[74] Ghazanfar SA. Medicinal and aromatic plants–Arabia and Iran. Encyclopedia of life support systems. Oxford (UK): UNESCO-EOLSS; 2011. http://www.eolss.net.
[75] The plant list. 2013. http://www.theplantlist.org/ [accessed 01.01.14].
[76] Natural Resources Conservation Service; United States Department of Agriculture: USDA-NRCS. Plants classification report: family apiaceae. Greensboro, NC 27401–4901 USA: National Plant Data Team; 2013. http://plants.usda.gov/java/ClassificationServlet?source=profile&symbol=Apiaceae&display=31.
[77] Khare CP. Indian medicinal plants: an illustrated dictionary. New Delhi (India): Springer; 2008.
[78] Zaid H, Rayan A, Said O, Saad B. Cancer treatment by Greco-Arab and Islamic herbal medicine. Open Nutraceut J 2010;3:203–13.
[79] Gledhill D. The names of plants. 4th ed. Cambridge University Press; 2008.
[80] Sekerci Ö. The eastern origin of English words. J Lang Linguist Stud 2007;3(1).
[81] Skeat WW. Principles of English etymology: the native element. Oxford, London: Clarendon Press; 1892.
[82] Levetin E, McMahon K. Plants and society. WCB. 2nd ed. Boston (Massachusetts, USA): McGraw Hill; 1999.
[83] Rosengarten JF. The book of spices. Wynnewood (Philadelphia): Livingston Publishing Co.; 1969.
[84] Sher H, Alyemeni MN, Faridullah. Cultivation and domestication study of high value medicinal plant species (its economic potential and linkages with commercialization). Afr J Agric Res 2010;5(18):2462–70.
[85] Jones FA. Herbs: useful plants. J R Soc Med 1996;89(12):717.
[86] Kalle R, Soukand R. Historical ethnobotanical review of wild edible plants of Estonia (1770s–1960s). Acta Soc Botan Polon 2012;81(4):271–81. http://dx.doi.org/10.5586/asbp.2012.033.
[87] Lim T. Carum carvi. In: Edible medicinal and non-medicinal plants, vol. 5, fruits. Dordrecht: Springer Science + Business Media; 2013. p. 6–18.
[88] Halberstein RA. Medicinal plants: historical and cross-cultural usage patterns. Ann Epidemiol 2005;15(9):686–99. http://dx.doi.org/10.1016/j.annepidem.2005.02.004.
[89] McKenna TK. Food of the Gods: the search for the original tree of knowledge: a radical history of plants, drugs, and human evolution. New York: Random House; 1999.
[90] Zargari A. Medicinal plants. 5th ed., vol. 1. Tehran: Tehran University Publications; 1995.
[91] Kenner D, Requena Y. Botanical medicine: a European professional perspective. Brookline (MA, USA): Paradigm Publications; 2001.
[92] Bown D. New encyclopedia of herbs and their uses. The herb society of America. New York: Dorling Kindersley; 2001.
[93] Chevallier A. Encyclopedia of herbal medicine. London: Dorling Kindersley; 2000.
[94] Chopra RN, Nayar SL, Chopra IC. Glossary of Indian medicinal plants (including the supplement). New Delhi: Council of Scientific & Industrial Research, Publications & Informations Directorate, CSIR; 1986.
[95] Grieve M. A modern herbal: the medicinal, culinary, cosmetic and economic properties, cultivation and folklore of herbs, grasses, fungi, shrubs, & trees with all their modern scientific uses, vol. 2. New York: Courier Dover Publications; 1971.
[96] Sastri BN. The wealth of India: a dictionary of Indian raw materials and industrial products, vol. 4. New Delhi: Council of Scientific and Industrial Research (CSIR), Publications and Information Directorate; 1956.

[97] Fahad S, Bano A. Ethnobotanical and physiological studies of some endangered plant species collected from two different altitudes in Gilgit Baltistan. Pak J Bot 2012;44:165–70.
[98] Dachler M. Alimentary, culinary and industrial uses of caraway. In: Németh É, editor. Caraway; the genus *Carum*. Amsterdam (The Netherlands): Taylor & Francis e-Library; 2005. p. 174–85.
[99] Saad B, Said O. Greco-Arab and islamic herbal medicine: traditional system, ethics, safety, efficacy, and regulatory issues. Hoboken (New Jersey): John Wiley & Sons, Inc. Publication; 2011a.
[100] Saad B, Said O. Tradition and perspectives of Greco-Arab and islamic herbal medicine. In: Dasgupta A, Hammett-Stabler CA, editors. Herbal supplements: efficacy, toxicity, interactions with western drugs, and effects on clinical laboratory tests. Hoboken (New Jersey): John Wiley & Sons, Inc. Publication; 2011b. p. 209–53.
[101] Mikaili P, Shayegh J, Asghari MH, Sarahroodi S, Sharifi M. Currently used traditional phytomedicines with hot nature in Iran. Ann Biol Res 2011;2(5):56–68.
[102] Dasgupta A, Hammett-Stabler CA. Herbal supplements: efficacy, toxicity, interactions with western drugs, and effects on clinical laboratory tests. Hoboken (New Jersey): John Wiley & Sons, Inc.; 2011.
[103] Saad B, Azaizeh H, Said O. Arab herbal medicine. In: Watson RR, Preedy VR, editors. Botanical medicine in clinical practice. CAB International; 2008. p. 31–9.
[104] Balch PA. Herbal prescription for common health problems: indigestion. In: Prescription for herbal healing: an easy-to-use a–z reference to hundreds of common disorders and their herbal remedies, vol. 317. Torquay (UK): Penguin. Avery; 2002. p. 537.
[105] Joshi SG. Medicinal plants: family apiaceae. 1st ed. Oxford and IBH Publishing Co. Pvt. Ltd; 2000.
[106] Mhaskar K, Blatter E, Caius J. Kiritikar and Basu's illustrated Indian medicinal plants, their usages in ayurveda and unani medicines, vol. 5. New Delhi: Sri Satguru publications; 2000.
[107] Sivarajan V, Balachandran I. Ayurvedic drugs and their plant sources. New Delhi: Oxford and IBH Publishing; 1994.
[108] Lahlou S, Tahraoui A, Israili Z, Lyoussi B. Diuretic activity of the aqueous extracts of *Carum carvi* and *Tanacetum vulgare* in normal rats. J Ethnopharmacol 2007;110(3):458–63. http://dx.doi.org/10.1016/j.jep.2006.10.005.
[109] Eddouks M, Khalidi A, Zeggwagh NA. Pharmacological approach of plants traditionally used in treating hypertension in Morocco (Approche pharmacologique des plantes utilisées traditionnellement dans le traitement de l'hypertension artérielle au Maroc). Phytothérapie 2009;7(2):122–7.
[110] Eddouks M, Maghrani M, Lemhadri A, Ouahidi ML, Jouad H. Ethnopharmacological survey of medicinal plants used for the treatment of diabetes mellitus, hypertension and cardiac diseases in the south-east region of Morocco (Tafilalet). J Ethnopharmacol 2002;82(2–3):97–103.
[111] El Amrani F, Rhallab A, Alaoui T, El Badaoui K, Chakir S. Ethnopharmacological survey of some plants used for the treatment of diabetes in the region of Meknès-Tafilalet (Morocco). Phytothérapie 2010;8(3):161–5.
[112] Jouad H, Haloui M, Rhiouani H, El Hilaly J, Eddouks M. Ethnobotanical survey of medicinal plants used for the treatment of diabetes, cardiac and renal diseases in the North centre region of Morocco (Fez–Boulemane). J Ethnopharmacol 2001;77(2):175–82.
[113] Tahraoui A, El-Hilaly J, Israili ZH, Lyoussi B. Ethnopharmacological survey of plants used in the traditional treatment of hypertension and diabetes in south-eastern Morocco (Errachidia province). J Ethnopharmacol 2007;110(1):105–17. http://dx.doi.org/10.1016/j.jep.2006.09.011.
[114] Bnouham M. Medicinal plants with potential galactagogue activity used in the moroccan pharmacopoeia. J Comp Integr Med December 2010;7(1). http://dx.doi.org/10.2202/1553-3840.1268.
[115] Reynolds J. Essential oils and aromatic carminatives. Martindale – the extra pharmacopeia. 28th ed. London (UK): Royal Pharmaceutical Society; 1996. p. 670–6.
[116] Barakat Abu-Rmailah B, Afifi F. Treatment with medicinal plants in Jordan. Dirasat Med Biol Sci 2000;27(1):53–74.
[117] Kumar D, Kumar A, Prakash O. Potential antifertility agents from plants: a comprehensive review. J Ethnopharmacol 2012;140(1):1–32. http://dx.doi.org/10.1016/j.jep.2011.12.039.
[118] Kunzemann J, Herrmann K. Isolation and identification of flavon(ol)-*O*-glycosides in caraway (*Carum carvi* L.), fennel (*Foeniculum vulgare* Mill.), anise (*Pimpinella anisum* L.), and coriander (*Coriandrum sativum* L.), and of flavon-*C*-glycosides in anise. I. Phenolics of spices (author's transl). Z Lebensm Unters Forsch 1977;164(3):194–200.
[119] Matsumura T, Ishikawa T, Kitajima J. New *p*-menthanetriols and their glucosides from the fruit of caraway. Tetrahedron 2001;57(38):8067–74.
[120] Matsumura T, Ishikawa T, Kitajima J. Water-soluble constituents of caraway: aromatic compound, aromatic compound glucoside and glucides. Phytochemistry 2002a;61(4):455–9.
[121] Matsumura T, Ishikawa T, Kitajima J. Water-soluble constituents of caraway: carvone derivatives and their glucosides. Chem Pharm Bull 2002b;50(1):66–72.
[122] Najda A, Dyduch J, Brzozowski N. Flavonoid content and antioxidant activity of caraway roots (*Carum carvi* L.). Veg Crop Res Bull 2008;68(1):127–33.
[123] Nakano Y, Matsunaga H, Saita T, Mori M, Katano M, Okabe H. Antiproliferative constituents in Umbelliferae plants II. Screening for polyacetylenes in some Umbelliferae plants, and isolation of panaxynol and falcarindiol from the root of *Heracleum moellendorffii*. Biol Pharm Bull 1998;21(3):257–61.
[124] Al-Bataina BA, Maslat AO, Al-Kofahi MM. Element analysis and biological studies on ten oriental spices using XRF and Ames test. J Trace Elem Med Biol 2003;17(2):85–90.
[125] Reiter B, Lechner M, Lorbeer E. The fatty acid profiles – including petroselinic and *cis*-vaccenic acid – of different Umbelliferae seed oils. Lipid/Fett 1998;100(11):498–502.
[126] Seidler-Lozykowska K, Baranska M, Baranski R, Krol D. Raman analysis of caraway (*Carum carvi* L.) single fruits. Evaluation of essential oil content and its composition. J Agric Food Chem 2010;58(9):5271–5.
[127] Raal A, Arak E, Orav A. The content and composition of the essential oil found in *Carum carvi* L. commercial fruits obtained from different countries. J Essent Oil Res 2012;24(1):53–9. http://dx.doi.org/10.1080/10412905.2012.646016.
[128] Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Rezaee M-B, Jaimand K, Alinezhad S, Saberi R, et al. Chemical composition and antiaflatoxigenic activity of *Carum carvi* L., *Thymus vulgaris* and *Citrus aurantifolia* essential oils. Food Control 2009;20(11):1018–24. http://dx.doi.org/10.1016/j.foodcont.2008.12.007.
[129] Simic A, Rancic A, Sokovic MD, Ristic M, Grujic-Jovanovic S, Vukojevic J, et al. Essential oil composition of *Cymbopogon winterianus* and *Carum carvi* and their antimicrobial activities. Pharm Biol 2008;46(6):437–41. http://dx.doi.org/10.1080/13880200802055917.
[130] Laribi B, Kouki K, Bettaieb T, Mougou A, Marzouk B. Essential oils and fatty acids composition of Tunisian, German and Egyptian caraway (*Carum carvi* L.) seed ecotypes: a comparative study.

Ind Crops Prod 2013;41(0):312–8. http://dx.doi.org/10.1016/j.indcrop.2012.04.060.
[131] May B, Kohler S, Schneider B. Efficacy and tolerability of a fixed combination of peppermint oil and caraway oil in patients suffering from functional dyspepsia. Aliment Pharmacol Ther 2000;14(12):1671–7. http://dx.doi.org/10.1046/j.1365-2036.2000.00873.x.
[132] Nicotra G. Phytotherapy of functional dyspepsia. Herbs for gastric discomfort. Agro Food Ind Hi-Tech 2012;23(5):24–7.
[133] Kamaleeswari M, Deeptha K, Sengottuvelan M, Nalini N. Effect of dietary caraway (*Carum carvi* L.) on aberrant crypt foci development, fecal steroids, and intestinal alkaline phosphatase activities in 1,2-dimethylhydrazine-induced colon carcinogenesis. Toxicol Appl Pharmacol 2006;214(3):290–6.
[134] Naderi-Kalali B, Allameh A, Rasaee MJ, Bach HJ, Behechti A, Doods K, et al. Suppressive effects of caraway (*Carum carvi*) extracts on 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin-dependent gene expression of cytochrome P450 1A1 in the rat H4IIE cells. Toxicol In Vitro 2005;19(3):373–7.
[135] Samojlik I, Lakic N, Mimica-Dukic N, Dakovic-Svajcer K, Bozin B. Antioxidant and hepatoprotective potential of essential oils of coriander (*Coriandrum sativum* L.) and caraway (*Carum carvi* L.) (Apiaceae). J Agric Food Chem 2010;58(15):8848–53. http://dx.doi.org/10.1021/jf101645n.
[136] Kazemipoor M, Radzi CWJBWM, Hajifaraji M, Cordell GA. Preliminary safety evaluation and biochemical efficacy of a *Carum carvi* extract: results from a randomized, triple-blind, and placebo-controlled clinical trial. Phytother Res 2014. http://dx.doi.org/10.1002/ptr.5147.
[137] Iacobellis NS, Lo Cantore P, Capasso F, Senatore F. Antibacterial activity of *Cuminum cyminum* L. and *Carum carvi* L. essential oils. J Agric Food Chem 2005;53(1):57–61.
[138] Park IK, Kim JN, Lee YS, Lee SG, Ahn YJ, Shin SC. Toxicity of plant essential oils and their components against *Lycoriella ingenua* (Diptera: Sciaridae). J Econ Entomol 2008;101(1):139–44.
[139] Richter J, Schellenberg I. Comparison of different extraction methods for the determination of essential oils and related compounds from aromatic plants and optimization of solid-phase microextraction/gas chromatography. Anal Bioanal Chem 2007;387(6):2207–17. http://dx.doi.org/10.1007/s00216-006-1045-6.
[140] Rivera LL, Vilarem G, Gomez RS, Estrada MJ, Feijoo JAV. Water soluble fractions of caraway (*Carum carvi* L.). Essent Oil Bol Latinoam Caribe Plant Med Aromat 2010;9(6):495–500.
[141] Seo S-M, Kim J, Lee S-G, Shin C-H, Shin S-C, Park I-K. Fumigant antitermitic activity of plant essential oils and components from ajowan (*Trachyspermum ammi*), allspice (*Pimenta dioica*), caraway (*Carum carvi*), dill (*Anethum graveolens*), geranium (*Pelargonium graveolens*), and litsea (*Litsea cubeba*) oils against Japanese termite (*Reticulitermes speratus Kolbe*). J Agric Food Chem 2009;57(15):6596–602. http://dx.doi.org/10.1021/jf9015416.
[142] Eddouks M, Lemhadri A, Michel JB. Caraway and caper: potential anti-hyperglycaemic plants in diabetic rats. J Ethnopharmacol 2004;94(1):143–8. http://dx.doi.org/10.1016/j.jep.
[143] Lemhadri A, Hajji L, Michel JB, Eddouks M. Cholesterol and triglycerides lowering activities of caraway fruits in normal and streptozotocin diabetic rats. J Ethnopharmacol 2006;106(3):321–6. http://dx.doi.org/10.1016/j.jep.2006.01.033.
[144] Hawrelak JA, Cattley T, Myers SP. Essential oils in the treatment of intestinal dysbiosis: a preliminary *in vitro* study. Altern Med Rev 2009;14(4):380–4.
[145] Can Baser K. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. Curr Pharm Des 2008;14(29):3106–19.
[146] Michiels J, Missotten J, Fremaut D, DeSmet S, Dierick N. *In vitro* dose-response of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. Livest Sci 2007;109(1–3):157–60. http://dx.doi.org/10.1016/j.livsci.2007.01.132.
[147] Upreti RK, Kannan A, Pant A. Alterations in rat gut bacteria and intestinal epithelial cells following experimental exposure of antimicrobials. FEMS Immunol Med Microbiol 2008;54(1):60–9.
[148] Backhed F. Programming of host metabolism by the gut microbiota. Ann Nutr Metab 2011;58:44–52. http://dx.doi.org/10.1159/000328042.
[149] Musso G, Gambino R, Cassader M. Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. Annu Rev Med 2011;62:361–80. http://dx.doi.org/10.1146/annurev-med-012510-175505.
[150] Lombardo YB, Chicco AG. Effects of dietary polyunsaturated n-3 fatty acids on dyslipidemia and insulin resistance in rodents and humans. A review. J Nutr Biochem 2006;17(1):1–13. http://dx.doi.org/10.1016/j.jnutbio.2005.08.002.
[151] Cani PD, Delzenne NM. The gut microbiome as therapeutic target. Pharmacol Ther 2011;130(2):202–12. http://dx.doi.org/10.1016/j.pharmthera.2011.01.012.
[152] Wesseltoft-Rao N, Holven KB, Telle-Hansen VH, Narverud I, Iversen PO, Hjermstad MJ, et al. Measurements of body fat is associated with markers of inflammation, insulin resistance and lipid levels in both overweight and in lean, healthy subjects. ESPEN J 2012;7(6):e234–40. http://dx.doi.org/10.1016/j.clnme.2012.10.002.
[153] Iyer A, Fairlie DP, Prins JB, Hammock BD, Brown L. Inflammatory lipid mediators in adipocyte function and obesity. Nat Rev Endocrinol 2010;6(2):71–82. http://dx.doi.org/10.1038/nrendo.2009.264.
[154] Kalupahana NS, Claycombe KJ, Moustaid-Moussa N. (n-3) fatty acids alleviate adipose tissue inflammation and insulin resistance: mechanistic insights. Adv Nutr 2011;2(4):304–16. http://dx.doi.org/10.3945/an.111.000505.
[155] Hsu CL, Yen GC. Effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes. J Agric Food Chem 2007;55(21):8404–10.
[156] Hsu CL, Yen GC. Phenolic compounds: evidence for inhibitory effects against obesity and their underlying molecular signaling mechanisms. Mol Nutr Food Res 2008;52(1):53–61. http://dx.doi.org/10.1002/mnfr.200700393.
[157] Rayalam S, Della-Fera MA, Baile CA. Phytochemicals and regulation of the adipocyte life cycle. J Nutr Biochem 2008;19(11):717–26.
[158] Glenda Winson SK. Management of HIV-associated diarrhea and wasting. J Assoc Nurses AIDS Care 2001;12(Suppl.):55–62. http://dx.doi.org/10.1016/S1055-3290(06)60158-1.
[159] Ene A, Nwankwo E, Samdi L. Alloxan-induced diabetes in rats and the effects of black caraway (*Carum carvi* L.) oil on their body weight. Res J Med Med Sci 2007;2(2):48–52.
[160] Haidari F, Seyed-Sadjadi N, Taha-Jalali M, Mohammed-Shahi M. The effect of oral administration of *Carum carvi* on weight, serum glucose, and lipid profile in streptozotocin-induced diabetic rats. Saudi Med J 2011;32(7):695–700.
[161] Nissen SL, Sharp RL. Effect of dietary supplements on lean mass and strength gains with resistance exercise: a meta-analysis. J Appl Physiol 2003;94(2):651–9.
[162] Panton LB, Rathmacher JA, Baier S, Nissen S. Nutritional supplementation of the leucine metabolite beta-hydroxy-beta-methylbutyrate (HMB) during resistance training. Nutrition 2000;16(9):734–9. http://dx.doi.org/10.1016/s0899-9007(00)00376-2.

[163] Dagenais GR, Yi Q, Mann JF, Bosch J, Pogue J, Yusuf S. Prognostic impact of body weight and abdominal obesity in women and men with cardiovascular disease. Am Heart J 2005;149(1):54–60.
[164] Landin K, Stigendal L, Eriksson E, Krotkiewski M, Risberg B, Tengborn L, et al. Abdominal obesity is associated with an impaired fibrinolytic activity and elevated plasminogen activator inhibitor-1. Metabolism 1990;39(10):1044–8.
[165] Lee CMY, Huxley RR, Wildman RP, Woodward M. Indices of abdominal obesity are better discriminators of cardiovascular risk factors than BMI: a meta-analysis. J Clin Epidemiol 2008; 61(7):646–53.
[166] Suk S-H, Sacco RL, Boden-Albala B, Cheun JF, Pittman JG, Elkind MS, et al. Abdominal obesity and risk of ischemic stroke: the Northern Manhattan stroke study. Stroke 2003;34(7):1586–92.
[167] Wang Y, Rimm EB, Stampfer MJ, Willett WC, Hu FB. Comparison of abdominal adiposity and overall obesity in predicting risk of type 2 diabetes among men. Am J Clin Nutr 2005;81(3):555–63.
[168] Zhang X, Zhang G, Zhang H, Karin M, Bai H, Cai D. Hypothalamic IKK [beta]/NF-[kappa] B and ER stress link overnutrition to energy imbalance and obesity. Cell 2008;135(1):61–73.
[169] Mandal GC, Bose K, Kozieł S. Impact of social class on body fatness among rural pre-school Bengalee Hindu children of Arambagh, West Bengal, India. J Comp Hum Biol 2011;62(3):228–36. http://dx.doi.org/10.1016/j.jchb.2011.03.001.
[170] Madisch A, Holtmann G, Mayr G, Vinson B, Hotz J. Treatment of functional dyspepsia with a herbal preparation: a double-blind, randomized, placebo-controlled, multicenter trial. Digestion 2004;69(1):45–52.
[171] Westphal J, Horning M, Leonhardt K. Phytotherapy in functional upper abdominal complaints – results of a clinical study with a preparation of several plants. Phytomedicine 1996;2(4):285–91.
[172] Guarrera PM, Savo V. Perceived health properties of wild and cultivated food plants in local and popular traditions of Italy: a review. J Ethnopharmacol 2013;146(3):659–80. http://dx.doi.org/10.1016/j.jep.2013.01.036.
[173] Blumenthal M. The complete German commission E monographs, therapeutic guide to herbal medicines: American botanical council. Austin (TX): Thieme; 1999.
[174] Capasso F, Gaginella TS, Grandolini G, Izzo AA. Plants and the digestive system. Phytotherapy 2003:251–94.
[175] El-Shobaki F, Saleh Z, Saleh N. The effect of some beverage extracts on intestinal iron absorption. Z Ernahrungswiss 1990; 29(4):264–9.
[176] Ford H, Carter J. Haemostasis in hypothyroidism. Postgrad Med J 1990;66(774):280–4.
[177] Dehghani F, Panjehshahin MR, Vojdani Z. Effect of hydroalcoholic extract of caraway on thyroid gland structure and hormones in female rat. Iran J Vet Res 2010;11(4):337–41.
[178] Johnstone AM, Murison SD, Duncan JS, Rance KA, Speakman JR. Factors influencing variation in basal metabolic rate include fat-free mass, fat mass, age, and circulating thyroxine but not sex, circulating leptin, or triiodothyronine. Am J Clin Nutr 2005; 82(5):941–8.
[179] Kim B. Thyroid hormone as a determinant of energy expenditure and the basal metabolic rate. Thyroid 2008;18(2):141–4. http://dx.doi.org/10.1089/thy.2007.0266.
[180] Moreno M, de Lange P, Lombardi A, Silvestri E, Lanni A, Goglia F. Metabolic effects of thyroid hormone derivatives. Thyroid 2008;18(2):239–53.
[181] Yun JW. Possible anti-obesity therapeutics from nature – a review. Phytochemistry 2010;71(14–15):1625–41. http://dx.doi.org/10.1016/j.phytochem.2010.07.011.
[182] Kazemipoor M, Radzi CWJWM, Cordell GA, Iman Yaze. Potential of traditional medicinal plants for treating obesity: a review. In: International Conference on Food Science and Nutrition (ICNFS), Singapore, 23rd to 24th July 2012. IPCBEE, IACSIT Press; p. 164–9. doi:arXiv:1208.1923.

LIST OF ABBREVIATIONS

ALP Alkaline phosphatase
ALT, SGPT Alanine transaminase, glutamate pyruvate transaminase
AMR Active metabolic rate
AST, SGOT Aspartate transaminase, glutamate oxaloacetate transaminase
BF Body fat
BM Body muscle
BMI Body mass index
BMR Basic metabolic rate
BP Blood pressure
BW Body water
CBC Complete blood cell count
Chol/HDL Cholesterol to HDL ratio
CWE Caraway water extract
DBP Diastolic blood pressure
FBS Fasting blood sugar
FID Flame ionization detector
g/kg Gram/kilogram
GC–MS Gas chromatography-mass spectrometry
GI Gastrointestinal
GM Gut microflora
HCT Hematocrit
HDL-C High density lipoprotein cholesterol
HGB Hemoglobin
HR Heart rate
IU/L International unit per liter
LDL-C Low density lipoprotein cholesterol
MCH Mean corpuscular hemoglobin
MCHC Mean corpuscular hemoglobin concentration
MCV Mean corpuscular volume
mg/dL Milligram/desi liter
MPV Mean platelet volume
MUAC Mid-upper arm circumference
PAL Physical activity level
PDW Platelet distribution width
PLT Platelets count
RBC Red blood cell
RDW Red cell distribution width
REE Resting energy expenditure
SBP Systolic blood pressure
SD Standard deviation
TC Thigh circumference
TDEE Total daily energy expenditure
TG Triglyceride
UA Uric acid
UFA Unsaturated fatty acids
USG Urine-specific gravity
WBC White blood cell
WC Waist circumference
WHR Waist to hip ratio

CHAPTER

17

Challenges in Identification of Potential Phytotherapies from Contemporary Biomedical Literature

Indra Neil Sarkar

University of Vermont, Burlington, VT, USA

OUTLINE

17.1 DESCRIPTION OF MEDICINAL USES OF PLANTS

The study of natural sources, such as plants or herbs [1], for potential medicinal uses has been the foundation for many contemporary treatments [2,3]. Perhaps the most well-known example of a plant-based medicine ("phytotherapy") is that of Willow bark (*Salix* spp.) used in the 5th century BC by Hippocrates to alleviate pain symptoms [4,5]. The active ingredient (salicin) for this phytotherapy was later isolated (in 1828) and gave rise to a medicine to be known as "aspirin," providing the foundation for what are now known as nonsteroidal anti-inflammatory drugs [4–7].

The establishment of the earliest academic botanical gardens (in 1544 at the University of Pisa) was for the study of medicinal plants [8–10]. In England, Nicholas Culpeper collated one of the earliest systematic listing of phytotherapies in his seminal work, the *Complete Herbal* [11]. Subsequently, John Bartram, hailed as the first American botanist, established the first botanical garden (in 1728) in the United States with the intention to collect and study the medicinal properties of plants [10,12]. The importance of phytotherapies, which were commonly prepared by apothecaries (considered to be the precursor to modern-day pharmacists), formed the nidus for the "Eclectic" branch of medicine in the United States. As such, faculty of early American medical schools with botanical medicine departments often made use of botanical gardens (among the first being the Elgin gardens that was established in 1801 by Dr. David Hosack of Columbia University's College of Physicians and Surgeons [10,13–15]). By 1909, there were eight Eclectic medical schools (those that primarily focused on the teaching medicine that utilized botany-based approaches) in the United States [16]. However, the general under-resource of Eclectic medical schools led to the ultimate recommendation of their closure [16–18]. From 1939 (when the last Eclectic medical school closed in the United States) onward, there was

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00017-9

a decline in the use of phytotherapeutics in general medical practice, which increasingly relied on the use of synthetic drugs. Despite the general decline of the regular use of phytotherapies in Western medicine, much of the world's population relies on medical philosophies that involve a strong phytotherapeutic component [19].

That much of the world's population makes use of phytotherapies for possible remedies suggests that there may be botanical treatments that contemporary medicine may not be aware of. Thus, while advances in pharmacochemistry throughout the twentieth century have led to a broad spectrum of pharmacopeia, which forms the basis of pharmacology knowledge used in contemporary medicine [20], there still remains significant opportunity to discover new therapeutics from natural sources, such as plants [21–25]. There continues to be general interest in identifying potential natural therapies for many ailments, such as H1N1 Influenza [26]. More formally, the National Cancer Institute at the United States National Institutes of Health has had a natural product screening program (the Developmental Therapeutics Program [27]), which includes plant-based products, since 1955 [28–31]. However, the process of identification, analysis, and validation of potential phytotherapeutic candidates (e.g., Taxol [32,33] [isolated from *Taxus brevifolia*]) across disjointed spheres of knowledge remains a laborious and time-consuming process [34–40]. In general, attempts to link previously recorded phytotherapies from detailed legacy ethnobotany reports have resorted to manual approaches [41]. As highlighted in a 2012 review, there are significant opportunities to develop informatics approaches for identifying phytotherapies from available data [42].

Appreciating that there may be description of naturally sourced therapies in archived sources, recent years have seen the establishment of significant digitization initiatives [43–45]. As such, the increased availability of ethnobotanical and biomedical knowledge in digital formats suggests that there may be the potential to leverage automated techniques to facilitate the phytotherapy discovery process [46]. Specifically, the growing digital corpus of plant knowledge might enable computational approaches to identify potential therapeutic uses of candidate phytotherapies as well as possible toxicity concerns that may need to be addressed before considering therapeutic viability. Simply put, there are three major corpora that one might consider as potential sources of potential phytotherapeutic knowledge: (1) biomedical literature—the peer-reviewed literature is the primary source of expert knowledge about potential uses, clinical trials, and descriptions of adverse events related to potential phytotherapies; (2) ethnobotanical literature—generally developed as an artifact of in-depth ethnobotanical studies of particular populations that have leveraged plant-based therapies; and (3) historical literature—catalogs of how plants were used to treat specific ailments, often organized as "herbals," which date back to the earliest days of recorded medicine. This chapter is specifically focused on biomedical literature, but many of the methodologies could potentially be applied to the other types of corpora. The choice of biomedical literature is largely because of the computational infrastructure that has been developed to support its use in support of modern medicine.

17.2 BIOMEDICAL LITERATURE

Biomedical literature represents the recorded wisdom of biomedical practice, based on the interpretation and study of data ranging from molecular to clinical to public health. The largest publicly available citation database is the MEDLINE database created and maintained by the United States National Library of Medicine (NLM) at the United States National Institutes of Health. With an annual growth of more than 500,000 entries [47], primarily from peer-reviewed sources across 39 languages, MEDLINE represents a significant portion of biomedical knowledge. It is important to acknowledge that there are a number of other sources for biomedical knowledge; however, MEDLINE is the most cited and the most studied. Its popularity in biomedical knowledge discovery research is largely because the entire archive is electronically accessible, either by Web services (called Entrez Utilities) or by lease (free for most academic use).

MEDLINE is the latest incarnation of the NLM collection of gathered biomedical knowledge, which started as *Index Medicus* in 1879 by John Shaw Billings, who was the founding director of the precursor of the NLM (the Library of the Surgeon General) [75–77]. The choice of possible source journals is determined by a formal NLM committee (the "Literature Selection Technical Review Committee"). Alongside MEDLINE, the Medical Subject Headings (MeSH) taxonomy provides a keyword set for indexing biomedical content [48]. Subject matter experts manually determine which MeSH descriptors to assign to individual records within MEDLINE. MEDLINE is commonly accessed on the Web using PubMed [49], also provides a common interface for other major biomedical literature sources, including PubMedCentral (PMC).

As of this writing, MEDLINE contains more than 21 million citations (the latest count can be seen as the number of results from: http://www.ncbi.nlm.nih.gov/pubmed/?term="medline"[sb]). Of these citations, more than 515,000 are indexed with the MeSH descriptor for plants (PLANTS: http://www.ncbi.nlm.nih.gov/pubmed/?term="plants"[mh]), suggesting that there may be a corpus of literature focused on plants

that may warrant further investigation. It is important to note that much of this literature may be focused on plant diseases rather than plant-based therapies. A more focused search using the MeSH descriptor "Phytotherapy" reveals that there are more than 29,000 indexed citations (PHYTOTHERAPY: http://www.ncbi.nlm.nih.gov/pubmed/?term="phytotherapy"[mh]). An essential limitation of MEDLINE records that must be acknowledged is that they only contain key metadata that are made available from publishers (e.g., title, journal name, authors, abstract). Therefore, it may also be valuable to consider free full-text sources, such as the aforementioned PMC that is indexed in MEDLINE. PMC has more than 62,000 and 1600 publications that are, respectively, indexed with the MeSH descriptors "Plants" and "Phytotherapy" (PLANTS: http://www.ncbi.nlm.nih.gov/pmc/?term="plants"[mh]; PHYTOTHERAPY: http://www.ncbi.nlm.nih.gov/pmc/?term="phytotherapy"[mh]).

These cursory searches reveal that there may indeed be sequestered knowledge about medicinal uses of plants. However, they also reflect only those citations that are primarily about potential phytotherapies as determined by MEDLINE indexers. MeSH-based inferences are potentially powerful and do offer a first step toward navigating potential knowledge that may be embedded within MEDLINE. Studies have shown that MeSH-based inferences may also miss deeper relationships that are contained within MEDLINE. The remaining sections of this chapter describe the methodologies used for extracting putative knowledge relationships, with particular attention to phytotherapy discovery relative to MEDLINE and PMC. For the rest of this chapter, the term "biomedical literature" will primarily be referencing that which is indexed or available from MEDLINE or PMC. However, one should not restrict oneself to this strict definition; the approaches described here may be applied to any corpus of literature, biomedical, or otherwise.

17.3 CONCEPT RECOGNITION

With the exception of MeSH descriptors, which reflect an abstract view of the content of a given article indexed in MEDLINE, putative knowledge is embedded in natural language, which may be deciphered using computational approaches. Natural Language Processing (NLP) is the general term for describing machine-based approaches for interacting with humans. Classically, NLP is divided into two major types: (1) Natural Language Generation (NLG)—which constitutes approaches for conversion of machine-centric data into interpretable language understandable by humans; and (2) Natural Language Understanding (NLU)—which is comprised of techniques to decipher human-generated language into a systematic form that can be subjected to subsequent computation. A detailed discourse on the details of NLP techniques is outside the scope of the present discussion, but there are a number of excellent resources for the interested reader [50,51]. The subsequent discussion will focus on the use of NLU to extract two types of entities from text: (1) taxonomic names and (2) biomedical concepts. These represent two major types of entities that can be used to form the basis for phytotherapy knowledge discovery from narrative sources such as those in MEDLINE.

17.3.1 Taxonomic Name Recognition

Phytotherapeutic knowledge can collectively be anchored around the principle that all therapeutic information will be described in the context of a specified plant species. The reference to plants may be singular (i.e., using a single plant as the primary source of a described therapy) or plural (i.e., using an amalgam of plant materials that are sources from multiple plants). For MEDLINE or PMC, there are approximately 1100 distinct plant-associated MeSH descriptors that a given citation can be tagged with; this is far less than the total possible number of therapeutic plants that may have therapeutic potential. Therefore, there is a significant opportunity to leverage computational approaches for identifying plant-associated knowledge contained within biomedical literature.

Setting aside the many biological challenges with accurate name identification, such as due to nomenclatural changes, taxonomic revisions, and incorrect identification, the identification of organism names in text is fraught with numerous challenges. The general form of algorithms used for the identification of organism names is termed Taxonomic Name Recognition (TNR) systems [52]. TNR approaches are a type of Named Entity Recognition (NER) algorithms, which are a distinct type of NLU approaches to identify meaningful concepts in text. TNR algorithms are of three major classes: (1) dictionary based; (2) rule based; and (3) model based.

Dictionary-based approaches use lists of words that consist of known organism names to identify possible organism names (e.g., LINNAEUS [53] and SPECIES [54]). Alternative dictionary approaches have been used that make use of a dictionary of words that are known *not* to be organism names (e.g., TaxonGrab [55]). Rule-based systems rely on a set of systematic rules to identify potential organism names. Rule-based systems make use of structural rules about organism names that may be derived using machine learning approaches (e.g., FAT [56])—for example, all organism

species names begin with a genus name that is capitalized and a species name that is not (e.g., *Arabidopsis thaliana*). There are major challenges with both dictionary- and rule-based approaches. While there is continued progress in collecting and cataloging organism names, dictionary approaches are hindered by the lack of a comprehensive list of organism names (which includes all possible variations of organism names) that is publicly available. Structural rules make many assumptions about how organism names are represented in text—for example, some passages of text that are machine-accessible may no longer have the styling associated with them. To address these challenges, language-based models have also been developed to identify organism names. These model-based systems first analyze a set of known organism names in context, from which a set of learning rules are developed (e.g., NetiNeti [57]). These learned rules are then used to develop a mathematical model that is able to identify those phrases identified in text that are statistically likely to be an organism name.

Recall and precision are the standard metrics used to evaluate many NLU systems, including TNR systems. Recall is defined as the ability of a system to identify all possible candidates ((True Positives)/(True Positives + False Negatives)). Precision is defined as the correctness of identified candidates ((True Positives)/((True Positives) + (False Positives))).Typically, rule-based systems perform with higher efficiency (which can be an important considering when analyzing large volumes of text) in computational performance compared to model-based systems and are generally more precise (i.e., the ability to accurately identify known organism names) than model-based systems. Model-based systems tend to have slightly higher performance in terms of recall (i.e., the ability to reliably identify all potential organism names) but may be less efficient when processing very large collections of text.

Practically, the majority of TNR systems developed to date have been reported to perform at greater than 95% recall and 95% precision. Therefore, the choice of rule-based versus model-based approaches will depend greatly on context and perhaps on individual research preferences. TNR systems have also been developed that combine a number of approaches and are optimized for particular information retrieval tasks (e.g., identification of gene names associated with species names, as done by the SR4GN system [58]). Most TNR systems focus on the identification of scientific names; there is less emphasis on vernacular names [59]. To date, vernacular name identification has been incorporated into TNR systems using lookup rules [53]. While TNR systems have been shown to perform well, there are still many opportunities for improvement and future development [59].

There are many nuances of how organism names appear in text. Many of the challenges are due to biological reasons as well as being generated through artifacts of the publication process. To date, TNR systems have been developed for the purpose of identifying potential taxonomic names. This is distinct from identifying a taxonomic concept (e.g., distinguishing between the intended conifer or wasp when encountering name *Agathis montana*). Ideally, disambiguation of taxonomic concepts would be possible if the full taxonomic names are used in literature (e.g., with authorities: *A. montana* de Laub for the conifer and *A. montana* Shest for the wasp). However, the reality is that—especially in biomedical literature—that most authors do not use full taxonomic names with authorities. The disambiguation of taxonomic concepts therefore remains an area of significant opportunity in the development of TNR systems.

TNR by no means is a perfect solution, but the high levels of performance suggest that it can be used as a powerful means for identifying a significant set of plant-related knowledge without requiring manual analysis of volumes of test. The reality of more than 500,000 articles indexed in MEDLINE per annum necessitates the use of TNR systems, even without perfect performance. If the plant of interest is a MeSH descriptor, then MeSH indexed articles might still be an appropriate place to start with the identification of valid literature for the plant of interest. However, TNR applications may facilitate the identification of other articles that perhaps mention the plant but are not salient enough to warrant MeSH indexing. Perhaps of greater significance is the potential that TNR systems offer in the identification of literature about plants that are not indexed by a MeSH descriptor.

17.3.2 Biomedical Concept Recognition

In contrast to TNR algorithms, the identification of canonical biomedical concepts (e.g., diseases, signs, symptoms, and drugs) is a mature area of research. Many of the early systems were designed with the intention to identify biomedical concepts of interest from dictated clinical notes [60]. Other systems were specifically designed to identify biomedical concepts from within biomedical literature sources, such as those indexed by MEDLINE [51,60].

Perhaps the most notable of NLU systems for analyzing biomedical literature is the MetaMap application, developed at the NLM at the National Institutes of Health [61]. MetaMap aims to identify biomedical concepts described in a passage of text that can be mapped to the Unified Medical Language System (UMLS) Metathesaurus [62]. The UMLS Metathesaurus

is a collection of more than 100 controlled vocabularies used across biomedicine, representing more than one million concepts that span from molecules to populations. Technically, there are important distinctions that one should make between a terminology (a list of terms), controlled vocabulary (a set of terms with definitions), and ontologies (a controlled vocabulary that is organized hierarchically). But it is increasingly common in recent years to refer to all sets of terms, vocabularies, or ontologies as just "ontologies." Without delving into too much detail, this can be an acceptable compromise as terminologies and controlled vocabularies can be considered flat hierarchies.

Considering the volume of biomedical literature that requires systematic indexing within MEDLINE, the MetaMap application was designed as an integral part of the Medical Text Indexer (MTI) initiative at the NLM [63]. Working in tandem with the NLM Index Section, which is the primary group responsible for the indexing of MEDLINE content, the MTI process is designed to provide a set of candidate MeSH descriptors for indexing. The general success of MetaMap has made it one of the more popular tools for biomedical concept identification. A major advantage of using MetaMap is that, in addition to MeSH, it is capable of mapping concepts found within a passage of text to any that are in the UMLS. This means that it is potentially capable of identifying over one million biomedical concepts (which does include the concepts associated with MeSH descriptors). The UMLS concepts can then be filtered by a specified semantic type, which are organized into a taxonomy of high-level concepts that was designed to facilitate the organization of UMLS concepts. For example, if one is interested in only disease or syndrome concepts, then MetaMap can be instructed to only return results associated with the appropriate semantic type ("Disease or Syndrome").

A number of other systems have been developed in recent years that represent a complement of approaches and potential mapping target concepts. Of particular note is the National Center for Biomedical Ontology (NCBO) Annotator, which is a freely accessible Web service that maps to concepts from over 370 source ontologies (which includes many that are also part of the UMLS) [64]. The details of how NCBO Annotator and MetaMap differ are outside the scope of the present discussion. However, it is important to note that the mapping predicted by NCBO Annotator and MetaMap, which are both freely accessible, are indeed complementary to one another [65]. Especially in the case where searching for concepts that may be embedded in natural language text is unclear, it is prudent to explore both NCBO Annotator and MetaMap as a possible source of mapping for biomedical concepts.

It is certainly true that contained within the set of source ontologies will be a set of organisms, so the need for the aforementioned TNR systems may seem unnecessary. However, with the exception of NCBI Taxonomy [66], there are to date no complete sets of organism names included in the collections used as the source for NCBO Annotator or MetaMap. NCBI Taxonomy only contains those organisms that are associated with entries in GenBank, which is the public repository of molecular sequence data maintained at the National Center for Biotechnology Information at the NLM (GenBank is part of the International Nucleotide Sequence Database Consortium that enables synchrony between the other major molecular sequence repositories in the globe: the DNA Data Bank of Japan and the European Nucleotide Archive [67]). In total, NCBI Taxonomy consists of approximately 150,000 organisms (represented about 10% of described organisms) [66]. There have been some efforts for developing consolidated organism lists, include those specific for plants (http://www.theplantlist.org). Until a complete list of organism names is regularly included in a biomedical ontology repository, tools like MetaMap or NCBO Annotator will be significantly limited in the identification of organism names by biomedical concept identification NLU systems. Furthermore, most TNR systems often leverage a set of linguistic rules that differ from more traditional NLU systems like MetaMap and NCBO Annotator (e.g., for scientific names, TNR systems are specifically tuned to identify Latin words, whereas NLU tools like MetaMap and NCBO Annotator are designed for English linguistic patterns). Nonetheless, it can be expected that as increased vernacular names are available in cataloged biomedical ontology archives that English rules would enable current biomedical NLU systems to identify them. Indeed, dictionary-lookup TNR systems have been shown to perform with high performance [54].

Researchers can rely on a continually improving and mature NLU infrastructure for identification of biomedical concepts from natural language text. In contrast to TNR systems, which are still very much in early development and demonstration, biomedical concept recognition has been well tested in many contexts, with particularly good performance with the identification of biomedical concepts from biomedical knowledge sources like MEDLINE. Nonetheless, it is still important to underscore that NLU systems are not perfect and may be prone to missing concepts or erroneously classifying concepts. Because biomedical concept identification tools like MetaMap and NCBO Annotator are built on significant ontology cataloging infrastructure, they offer a plausible solution for biomedical concept recognition that can keep up with the growing volume of biomedical literature.

17.4 IDENTIFYING POTENTIAL PLANT–THERAPY RELATIONSHIPS

Once one has identified the array of taxonomic names and biomedical concepts an essential next step is determining the relationship between them. In contrast to traditional ethnobotanical surveys, where identification of potential phytotherapies is determined from careful ethnographic study of how botanicals are used for treatment ailments, computational predictions are based almost exclusively on previously reported data that are available in published resources. Therefore, it is essential to underscore that computational prediction of potential phytotherapies is only based on what has already been reported; computational ethnobotanical predictions are not designed to serve as a complete proxy for in-depth ethnobotanical surveys. Despite this potential limitation with computational approaches, there is significant opportunity to leverage existing archives of biomedical knowledge.

Within biomedicine, the potential to identify possible new knowledge has been demonstrated through the identification of correlations from within published literature. The most famous example of identifying new knowledge from studying literature is attributed to Swanson. Swanson identified relationships between cod liver oil and blood viscosity as one set of direct inferences from published literature [68]. By studying another set of biomedical literature, it was also observed that there was a reported correlation between Raynaud's Syndrome and an increase in blood viscosity. Through the transitive property, Swanson was able to suggest that cod liver oil might have a positive therapeutic effect on Raynaud's Syndrome by reducing blood viscosity. Subsequent clinical trials were able to validate this initial hypothesis, which was based exclusively on a study of biomedical literature.

The demonstration of the potential opportunity to identify new therapies from studying biomedical literature by Swanson provides much inspiration and promise for the development of computational approaches. The ARROWSMITH system, developed at the University of Illinois at Chicago embodies the systematic process pioneered by Swanson [69]. Without going too much into the detail of how such a system works, it is useful to consider two types of relationships that are described in literature: (1) direct—those relationships that are embodied by a specific description of how two entities are related (e.g., cod liver oil *reduces* blood viscosity); and (2) indirect—those relationships that can be inferred based on combinations of direct relationships (e.g., since code liver oil *reduces* blood viscosity *and* Raynaud's Syndrome would benefit from a *reduction* in blood viscosity *suggests* that cod liver oil might be used to treat Raynaud's Syndrome). While the principle of discovering new inferences is conceptually straightforward, the actual implementation can be difficult. What is important about the inspiring Swanson example is that the initial direct relationships were identified through a manual process. Through a human (Swanson) reading a set of articles, direct relationships were identified. The subsequent step of inferencing also required some human judgment to ascertain what relationships could be linked and thus lead to a new relationship. Computational approximations of these two tasks require natural language understanding and inferencing capabilities that can be challenging.

The previous section on Concept Recognition focused primarily on entity recognition, but it is important to also consider an important next step in many NLU systems: predication extraction. Predications are defined a Subject–Predicate–Object *triples* [70]. Predications are constructed in NLU systems through a combination of lexical, syntactic, and semantic analysis. Lexical analysis is focused on the identification of concepts (e.g., concept recognition systems such as described before). Syntactic analysis focused on the grammatical context of the identified concepts (e.g., noun, verb, adjective). Finally, semantic analysis focuses on identifying the meaning of concepts. The identification of triples from text can be a challenging task largely because of the variety of nuances associated with language. Nonetheless, there has been significant progress in recent years with respect to systems that are able to identify semantic predications from text. Perhaps the most notable is the SemRep system [71] from the NLM, which underpins Semantic MEDLINE [72] (http://skr3.nlm.nih.gov/SemMed/index.html). Currently, systems like Semantic MEDLINE allow one to browse through the identified predications from a set of literature (e.g., from a given PubMed search).

Considering predications as the collection of direct relationships, it is conceivable to search for indirect relationships such as those inspired by Swanson's observation of the potential for use of t he transitive property to infer possible new relationships (i.e., if A is related to B, and if B is related to C, then A is indirectly related to C). The mere identification of such possible inferences then requires a filtering process to identify those that are more likely to be putative inferences, versus those that are only linked due to generally stochastic nature of inferences based on reported knowledge relationships. Two general approaches can be used for ranking new inferences: (1) probabilistic and (2) model based. Probabilistic approaches aim to determine the potential significance of two concepts being related based on a statistical measure of correlation. One such statistical test is the conditional probability test, where the

probability of a concept A occurring with a concept C is conditional on the combined occurrence with B. By contrast, model-based approaches aim to develop a mathematical representation of the possible relationship between two concepts based on the relationships identified in a defined set. For example, the relationship between a concept A and a concept C is modeled within a set of documents relative to a concept B, but also accounting for how often the concept B is associated with other potentially related concepts.

It is important to point out that probabilistic versus model-based approaches rank putative correlations with different goals in mind. Probabilistic approaches aim to address the question: "how statistically significant is the relationship?"; model-based approaches are focused on addressing a different question: "how important is the relationship relative to the realm of possible relationships?" For the purposes of identifying putative relationships between identified plants and possible therapeutic uses, it has been shown that a model-based approach outperforms a probabilistic approach for identifying relationships of potential validity [73].

The validation of predicted findings is a difficult task, especially in the area of phytotherapy discovery. This is largely because there are limited "gold standard" data sets on to which benchmarking can be done to determine the efficacy and reliability of a computational method. Therefore, it must be emphasized that determining the merit of a given methodology (probabilistic versus model-based) should really only be done in the context of expert-verified gold standards. Arguably, the lack of gold standard reference standards reflects the biggest challenge in the development of phytotherapy knowledge discovery systems [73,74].

The lack of robust gold-standard reference standards that can be used for quantifying the quality of machine-derived imputations poses two significant challenges for phytotherapy knowledge discovery from biomedical literature: (1) determining plant toxicities; (2) separating therapeutic uses from folklore. The mere mention of a plant co-occurring with a disease or symptom, even with appropriate predicates that indicate treatment potential (e.g., "treats" or "cures") does not account for the full range of potential toxic effects that a plant may have. For this reason, phytotherapy recognition systems must, in their core design, also include the potential to identify possible toxic effects of plant therapies (e.g., associated with predicates like "kills" or "causes"). The separation of true therapeutic uses of a plant from folklore or traditional uses poses a more formidable challenge. For example, it may very well be stated in an article that an indigenous population uses a given plant to treat a particular disease. However, the evidence for this statement may not be further demonstrated or described through clinical trials, in vivo, or in vitro studies about the potential effect of the given plant. The repeated mention of the given plant being used for treatment of a particular disease may also provide a false relationship that is ranked of high importance by either probabilistic or model-based approaches. There may be other textual clues that can be incorporated into a phytotherapy knowledge discovery system, but these have not been described to date. Distinguishing between folklore-based therapies and those that may have real clinical utility is perhaps the single most daunting challenge in leveraging automated approaches for phytotherapy discovery.

17.5 CONCLUSION

Research on the potential of computational methods for phytotherapy knowledge discovery from biomedical literature is very much in its infancy. Recent advancements in computational methods for the identification of organisms and biomedical concepts, taken in combination with formal knowledge discovery processes, show great promise to usher in a new cadre of knowledge discovery systems. However, automated approaches cannot replace the in-depth analysis required by expert curators, and the lack of complete gold standards for evaluation of automated methodologies does challenge the development of robust systems. Nonetheless, there is much opportunity to leverage the sequestered phytotherapeutic knowledge that may be contained within the continually growing corpus of biomedical literature.

References

[1] Schmidt B, Ribnicky DM, Poulev A, Logendra S, Cefalu WT, Raskin I. A natural history of botanical therapeutics. Metabolism 2008;57(7 Suppl. 1):S3–9.
[2] Jones WP, Chin YW, Kinghorn AD. The role of pharmacognosy in modern medicine and pharmacy. Curr Drug Targets 2006;7(3):247–64.
[3] Baerheim Svendsen A, Scheffer JJ. Natural products in therapy. Prospects, goals and means in modern research. Pharm Weekbl Sci 1982;4(4):93–103.
[4] Miner J, Hoffhines A. The discovery of aspirin's antithrombotic effects. Tex Heart Inst J 2007;34(2):179–86.
[5] Mahdi JG, Mahdi AJ, Mahdi AJ, Bowen ID. The historical analysis of aspirin discovery, its relation to the willow tree and antiproliferative and anticancer potential. Cell Proliferation 2006;39(2):147–55.
[6] Wick JY. Aspirin: a history, a love story. Consult Pharm 2012;27(5):322–9.
[7] Jones R. Nonsteroidal anti-inflammatory drug prescribing: past, present, and future. Am J Med 2001;110(1A):4S–7S.
[8] Doyle D. Edinburgh doctors and their physic gardens. J R Coll Physicians Edinb 2008;38(4):361–7.
[9] Burnby J. Some early London physic gardens. Pharm Hist (Lond) 1994;24(4):2–8.
[10] A history of botanic gardens. JAMA 1915;LXV(2):170–1.

[11] Culpeper N, Sibly E. Culpeper's English physician, and complete herbal : to which are now first added upwards of one hundred additional herbs, with a display of their medicinal and occult properties, physically applied to the cure of all disorders incident to mankind. London: Printed for the proprietor, by Lewis and Roden; 1802. And sold at the British Directory Office, and by Champante and Whitrow.
[12] Hobbs C. The medical botany of John Bartram. Pharm Hist 1991; 33(4):181–9.
[13] The Elgin botanic garden, between 50th & 51st Sts. and 5th & 6th Aves., 1825. 1859.
[14] Brown A. The Elgin botanic garden. Lancaster, PA: Press of the New era printing company; 1908. iv, 57 p.
[15] Hosack D. A statement of facts relative to the establishment and progress of the Elgin botanic garden, and the subsequent disposal of the same to the state of New-York. New-York: Printed by C.: S. Van Winkle; 1811. 56 p.
[16] Flexner A. Medical education in the United States and Canada; a report to the Carnegie Foundation for the Advancement of teaching. Norwalk, CT: The Easton Press; 1994.
[17] Stahnisch FW, Verhoef M. The flexner report of 1910 and its impact on complementary and alternative medicine and psychiatry in North America in the 20th century. Evidence-based complementary and alternative medicine. eCAM 2012;2012:647896.
[18] Haller JS. A profile in alternative medicine : the Eclectic Medical College of Cincinnati, 1845–1942. Kent, Ohio: Kent State University Press; 1999. xii, 212 p.
[19] World Health Organization. WHO traditional medicine strategy: 2014–2023. Hong Kong: World Health Organization; 2013.
[20] Hollinger MA. Introduction to pharmacology. 3rd ed. Boca Raton, FL: CRC Press; 2008. 434 p.
[21] Akerele O. Nature's medicinal bounty: don't throw it away. World Health Forum 1993;14(4):390–5.
[22] Heywood VH. Ethnopharmacology, food production, nutrition and biodiversity conservation: towards a sustainable future for indigenous peoples. J Ethnopharmacol 2011;137(1):1–15.
[23] Tan G, Gyllenhaal C, Soejarto DD. Biodiversity as a source of anticancer drugs. Curr Drug Targets 2006;7(3):265–77.
[24] Basso LA, da Silva LH, Fett-Neto AG, de Azevedo Jr. WF, Moreira Ide S, Palma MS, et al. The use of biodiversity as source of new chemical entities against defined molecular targets for treatment of malaria, tuberculosis, and T-cell mediated diseases—a review. Mem Inst Oswaldo Cruz 2005;100(6):475–506.
[25] De Luca V, Salim V, Atsumi SM, Yu F. Mining the biodiversity of plants: a revolution in the making. Science 2012;336(6089): 1658–61.
[26] Hill S, Mao J, Ungar L, Hennessy S, Leonard CE, Holmes J. Natural supplements for H1N1 influenza: retrospective observational infodemiology study of information and search activity on the Internet. J Med Internet Res 2011;13(2):e36.
[27] Developmental Therapeutics Program NCI/NIH [Internet]. [cited 2014-11-05]; Available from: http://dtp.nci.nih.gov/.
[28] Drug Discovery at the National Cancer Institute [Internet]. [cited 2014-11-05]; Available from: http://www.cancer.gov/cancertopics/factsheet/NCI/drugdiscovery.
[29] Schepartz SA. History of the National Cancer Institute and the plant screening program. Cancer Treat Rep 1976;60(8):975–7.
[30] Collins JM. The NCI developmental therapeutics program. Clin Adv Hematol Oncol 2006;4(4):271–3.
[31] Cragg GM. Natural product drug discovery and development: the United States National Cancer Institute role. P R Health Sci J 2002; 21(2):97–111.
[32] Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT. Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J Am Chem Soc 1971;93(9):2325–7.
[33] Wall ME, Wani MC. Camptothecin and taxol: discovery to clinic—thirteenth Bruce F. Cain Memorial Award Lecture. Cancer Res 1995;55(4):753–60.
[34] Kone WM, Solange KN, Dosso M. Assessing sub-Saharan Erythrina for efficacy: traditional uses, biological activities and phytochemistry. Pak J Biol Sci 2011;14(10):560–71.
[35] Dhiman A, Nanda A, Ahmad S. A recent update in research on the antihepatotoxic potential of medicinal plants. Zhong xi yi jie he xue bao J Chin Integr Med 2012;10(2):117–27.
[36] Li JW, Vederas JC. Drug discovery and natural products: end of an era or an endless frontier? Science 2009;325(5937):161–5.
[37] Howes MJ, Perry E. The role of phytochemicals in the treatment and prevention of dementia. Drugs Aging 2011;28(6):439–68.
[38] Leteane MM, Ngwenya BN, Muzila M, Namushe A, Mwinga J, Musonda R, et al. Old plants newly discovered: Cassia sieberiana D.C. and Cassia abbreviata Oliv. Oliv. root extracts inhibit in vitro HIV-1c replication in peripheral blood mononuclear cells (PBMCs) by different modes of action. J Ethnopharmacol 2012; 141(1):48–56.
[39] Loub WD, Farnsworth NR, Soejarto DD, Quinn ML. NAPRALERT: computer handling of natural product research data. J Chem Inf Comput Sci 1985;25(2):99–103.
[40] Scalbert A, Andres-Lacueva C, Arita M, Kroon P, Manach C, Urpi-Sarda M, et al. Databases on food phytochemicals and their health-promoting effects. J Agric Food Chem 2011;59(9):4331–48.
[41] Buenz EJ, Schnepple DJ, Bauer BA, Elkin PL, Riddle JM, Motley TJ. Techniques: bioprospecting historical herbal texts by hunting for new leads in old tomes. Trends Pharmacol Sci 2004; 25(9):494–8.
[42] Sharma V, Sarkar IN. Bioinformatics opportunities for identification and study of medicinal plants. Brief Bioinform 2012;14(2): 238–50.
[43] Islamic Medical Manuscripts at the National Library of Medicine. [cited 2014-11-05]; Available from: http://www.nlm.nih.gov/hmd/arabic/arabichome.html.
[44] Medical Heritage Library. [cited 2014-11-05]; Available from: http://www.medicalheritage.org/
[45] Institute for the Preservation of Medical Traditions. [cited 2014-11-05]; Available from: http://medicaltraditions.org.
[46] Sharma V, Sarkar IN. Bioinformatics opportunities for identification and study of medicinal plants. Briefings Bioinf 2013;14(2): 238–50.
[47] Detailed indexing statistics: 1965–2013. 2014 [cited 2014 May 21, 2014]; Available from: http://www.nlm.nih.gov/bsd/index_stats_comp.html.
[48] Rogers FB. Medical subject headings. Bull Med Libr Assoc 1963; 51:114–6.
[49] Giedd JN, Smith KG. Online access to journal abstracts and articles. J Child Adolesc Psychopharmacol 1997;7(3):201–10.
[50] Sarkar IN. Methods in biomedial informatics: a pragmatic approach. Boston: Academic Press; 2013.
[51] Cohen KB, Demner-Fushman D. Biomedical natural language processing. Natural language processing. Philadelphia: John Benjamins Publishing Company; 2014.
[52] Sarkar IN. Biodiversity informatics: organizing and linking information across the spectrum of life. Briefing Bioinf 2007;8(5): 347–57.
[53] Gerner M, Nenadic G, Bergman CM. LINNAEUS: a species name identification system for biomedical literature. BMC Bioinformatics 2010;11:85.
[54] Pafilis E, Frankild SP, Fanini L, Faulwetter S, Pavloudi C, Vasileiadou A, et al. The species and organisms resources for fast and accurate identification of taxonomic names in text. PLoS One 2013;8(6):e65390.
[55] Koning D, Sarkar IN, Moritz T. TaxonGrab: extracting taxonomic names from text. Biodiversity Informatics 2005;2:79–82.

[56] Sautter G, Boehm K, Agosti D. A combining approach to final all taxon names (FAT) in legacy biosystematics literature. Biodiversity Inf 2006;3:46–58.
[57] Akella LM, Norton CN, Miller H. NetiNeti: discovery of scientific names from text using machine learning methods. BMC Bioinf 2012;13:211.
[58] Wei CH, Kao HY, Lu Z. SR4GN: a species recognition software tool for gene normalization. PLoS One 2012;7(6):e38460.
[59] Thessen AE, Cui H, Mozzherin D. Applications of natural language processing in biodiversity science. Adv Bioinf 2012;2012: 391574.
[60] Collen MF. Computer medical databases: the first six decades (1950–2010). Health informatics. London, New York: Springer; 2012. xix, 288 p.
[61] Aronson AR, Lang FM. An overview of MetaMap: historical perspective and recent advances. J Am Med Inform Assoc 2010; 17(3):229–36.
[62] Bodenreider O. The unified medical language system (UMLS): integrating biomedical terminology. Nucleic Acids Res 2004; 32(Database issue):D267–70.
[63] Aronson AR, Mork JG, Gay CW, Humphrey SM, Rogers WJ. The NLM indexing Initiative's medical text indexer. Stud Health Technol Inform 2004;107(Pt 1):268–72.
[64] Jonquet C, Shah NH, Musen MA. The open biomedical annotator. Summit Translat Bioinf 2009;2009:56–60.
[65] Shah NH, Bhatia N, Jonquet C, Rubin D, Chiang AP, Musen MA. Comparison of concept recognizers for building the Open Biomedical Annotator. BMC Bioinf 2009;10(Suppl. 9):S14.
[66] Federhen S. The NCBI taxonomy database. Nucleic Acids Res 2012;40(Database issue):D136–43.
[67] Nakamura Y, Cochrane G, Karsch-Mizrachi I. The international nucleotide sequence database collaboration. Nucleic Acids Res 2013;41(Database issue):D21–4.
[68] Swanson DR. Fish oil, Raynaud's syndrome, and undiscovered public knowledge. Perspect Biol Med 1986;30(1):7–18.
[69] Smalheiser NR, Swanson DR. Using ARROWSMITH: a computer-assisted approach to formulating and assessing scientific hypotheses. Comput Methods Programs Biomed 1998;57(3): 149–53.
[70] Hristovski D, Friedman C, Rindflesch TC, Peterlin B. Exploiting semantic relations for literature-based discovery. AMIA Annu Symp Proc 2006:349–53.
[71] Rindflesch TC, Fiszman M. The interaction of domain knowledge and linguistic structure in natural language processing: interpreting hypernymic propositions in biomedical text. J Biomed Inform 2003;36(6):462–77.
[72] Cairelli MJ, Miller CM, Fiszman M, Workman TE, Rindflesch TC. Semantic MEDLINE for discovery browsing: using semantic predications and the literature-based discovery paradigm to elucidate a mechanism for the obesity paradox. In: AMIA Annu Symp Proc, 2013; 2013. p. 164–73.
[73] Sharma V, Sarkar IN. Leveraging concept-based approaches to identify potential phyto-therapies. J Biomed Inform 2013;46(4): 602–14.
[74] Sharma V, Sarkar IN. Leveraging biodiversity knowledge for potential phyto-therapeutic applications. J Am Med Inform Assoc 2013;20(4):668–79.
[75] Taine SI. The medical literature analysis and retrieval system (MEDLARS) of the U. S. National library of medicine. Methods Inf Med 1963;2(2):65–9.
[76] Rogers FB. The development of Medlars. Bull Med Libr Assoc 1964;52:150–1.
[77] Moll W. MEDLINE evaluation study. Bull Med Libr Assoc 1974; 62(1):1–5.

LIST OF ABBREVIATIONS

MEDLINE Medical literature analysis and retrieval system online
MeSH Medical subject headings
MTI Medical text indexer
NCBO National center for biomedical ontology
NER Named entity recognition
NLG Natural language generation
NLM United states national library of medicine
NLP Natural language processing
NLU Natural language understanding
PMC PubMed central
TNR Taxonomic name recognition
UMLS Unified medical language system

This page intentionally left blank

CHAPTER

18

Botanicals as Medicinal Food and Their Effects against Obesity

Kakali Mukherjee, Rajarshi Biswas, Sushil K. Chaudhary, Pulok K. Mukherjee

School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

OUTLINE

18.1 INTRODUCTION

An excess deposition of body fat in adipose tissue may result in overweight and/or obesity and the proportionate increase in risks caused with increasing-degrees of obesity. According to World Health Organization (WHO) the definition of obesity is established on the body mass index (BMI), which is computed as weight in kilograms divided by height in meters squared (kg/m^2). Obesity is defined as a BMI greater than 30 kg/m^2, and overweight is determined as a BMI from 25 to 30 kg/m^2. The metabolic syndrome

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00018-0

is a collective term that refers to obesity-associated metabolic abnormalities/risk factors [1]. There are several health risks associated with obesity and according to WHO can be categorized into three groups: diseases that are greatly increased due to obesity (type 2 diabetes, gall bladder diseases, dyslipidemia); those which are moderately increased (coronary heart disease, hypertension, osteoarthritis); and finally diseases that are mildly increased (cancer especially breast cancer in postmenopausal women, endometrial and colon cancer, reproductive hormone abnormalities, polycystic ovary syndrome). On June 2013, the American Medical Association officially recognized obesity as a disease [2].

Utilization of plant components and its derived products has a prospective future for controlling the prevalence of metabolic syndrome. Several evidences are exploring to support the use of herbs as an alternative way of obesity control and weight management [3]. The pathogenesis of obesity is very complex and requires different intervention strategies to undertake this problem. Despite going for lifestyle modification or pharmacotherapy in terms of weight loss, there has always been disappointing results which indicated the need of other treatment modalities to produce better and long-lasting results. Diet-based therapies and herbal supplements are among the most common complementary and alternative medicine modalities for weight loss. The great ratio of the population depends on traditional practitioners and their prescription of medicinal plants in society to assemble health care needs [4]. Hence, it is really obvious that plants may offer an efficient option for the treatment of obesity.

18.2 PATHOGENESIS OF OBESITY AND MANAGEMENT STRATEGIES

18.2.1 Etiology

A hypothesis suggests that obesity is linked to genetic predisposition and environmental factors which leads to the accumulation of excess adipose tissue. Usually, both the environmental factors and genetic factor(s) should be present for the occurrence of obesity. This hypothesis is indubitably true for majority of obese people worldwide. There are two parts to the obesity equation:

(1) An excessive intake of food items with increased amounts of fat, salt, and sugars, but lesser amounts of minerals, vitamins, and other nutrients; and (2) decrease in physical activity because of sedentary lifestyles, comfortable modes of transportation, lack of maintenance of daily routines, and increasing urbanization. Thus, the energy discrepancy between calorie intake and those expended is the fundamental cause of obesity and overweight [5].

18.2.2 Pathophysiology

Although multiple candidate genes contribute to the pathogenesis of obesity, these findings are not consistent. The genes include the chromosome 10p, melanocortin-4 receptor gene, β_3-adrenergic receptor gene, peroxisome proliferators activated receptor gamma two gene, and other genetic polymorphisms. Hormones such as adipokines, gut-related hormones, and many others are involved in the regulation and pathophysiology of obesity. One of them is ghrelin, which is a circulating peptide hormone derived from the stomach. It is the only known peripherally acting orexigenic hormone that is responsible for stimulating appetite. But the gut-derived hormones act as anorectic agents that attenuate food intake to attain optimal digestion and absorption rather avoiding the cost of overconsumption, such as insulin resistance and hyperinsulinemia [6].

Peptide YY (PYY) is present in the intestine at increasingly higher levels, having maximum levels in colon and rectum. It is mainly secreted by the L cells of the distal small bowel and colon. PYY reduces gastric secretion by modulating signals to the hypothalamus, ensuing in delayed gastric emptying. Food consumption is decreased if PYY is administered before meal [7]. Cholecystokinin (CCK) is produced in the pancreas, stomach, and gallbladder. Dietary fat is responsible for the release of CCK and accumulated in the small intestine. The major functions of CCK involve pancreatic exocrine secretion, gastric emptying, gallbladder contraction, and gut motility. CCK increases satiety and simultaneously decreases appetite by acting centrally via subtype CCK-A receptors on the afferent brain vagal fibers, causing inhibition of appetite. Oxyntomodulin is released postprandially resulting in the limitation of food intake. Secretion of this peptide occurs from the intestinal cells that also are responsible for the secretion of PYY. Oxyntomodulin suppresses appetite and reduces food intake for a long period that is also associated with a decline in fasting ghrelin levels. Intravenous administration of glucagon-like peptide-1 in humans enhances satiety and also reduces food intake [6].

Adipokines are a group of hormones produced by the adipocytes. Adipokines secretion is regulated by tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), leptin, and adiponectin. The role of TNF-α in obesity has been associated with insulin resistance via the liberation of free fatty acids, decreased production of adiponectin, and modulate insulin signaling. Inflammatory mediators are recruited in vascular tissue through the activation of nuclear factor-kappa B(NF-κB) by TNF-α. Immune, endothelial, fibroblasts, and adipocytes cells are secreting one of the pleiotropic circulating cytokine, intreleukin-6 (IL-6) which causes

inflammation, impairment of host defenses, and tissue injury. It acts by inhibiting insulin receptor signal transduction in hepatocytes, increasing circulating free fatty acids from adipose tissue, and reducing adiponectin secretion [6]. Leptin is one type of adipokine that plays a key role in regulating energy intake and expenditure, including appetite and hunger, metabolism, and behavior. Leptin can cross the blood–brain barrier by binding to specific receptors in the hypothalamus resulting in the suppression of appetite. True leptin deficiency in humans is rare; however, obese humans are, in part, leptin-resistant [8]. Adiponectin is a 244 amino acid long polypeptide derived from plasma protein. The role of adiponectin is glucose regulation and fatty acid oxidation [6].

The level of inflammatory mediators such as IL-6, TNF-α, and CCK are increased by increasing the visceral fat, as a result proinflammatory mediators like adiponectin and interleukin-10 levels are decreased that leads to increases in the chances of metabolic dysfunction which is one of the prime causes of obesity. Neuroendocrine diseases are secondary causes of obesity.

18.2.3 Obesity Pharmacotherapy by Phytocostituents

A huge number of plants, phytochemicals, and plant derivatives possess antiobesity activity by their unique mode of action. Broadly, phytocostituents generally act through modulating physiological functions that may restore balance between energy intake and expenditure. Phytoconstituents encompass antiobesity activity and their mechanism of action is discussed. Schematic representation of major targets for antiobesity phytoconstituents is seen in Figure 18.1.

18.2.3.1 Pancreatic Lipase

A diet containing fat is neither digested nor absorbed in the intestine until it has been possessed by the action of pancreatic lipase enzyme. Hence, one of the most promising strategies to inhibit fat absorption from intestine can achieve by blocking the action of pancreatic lipase through phytochemicals. Phytochemicals covalently bond to the active serine site on pancreatic lipases enzyme in the gut lumen. By forming the covalent bond,

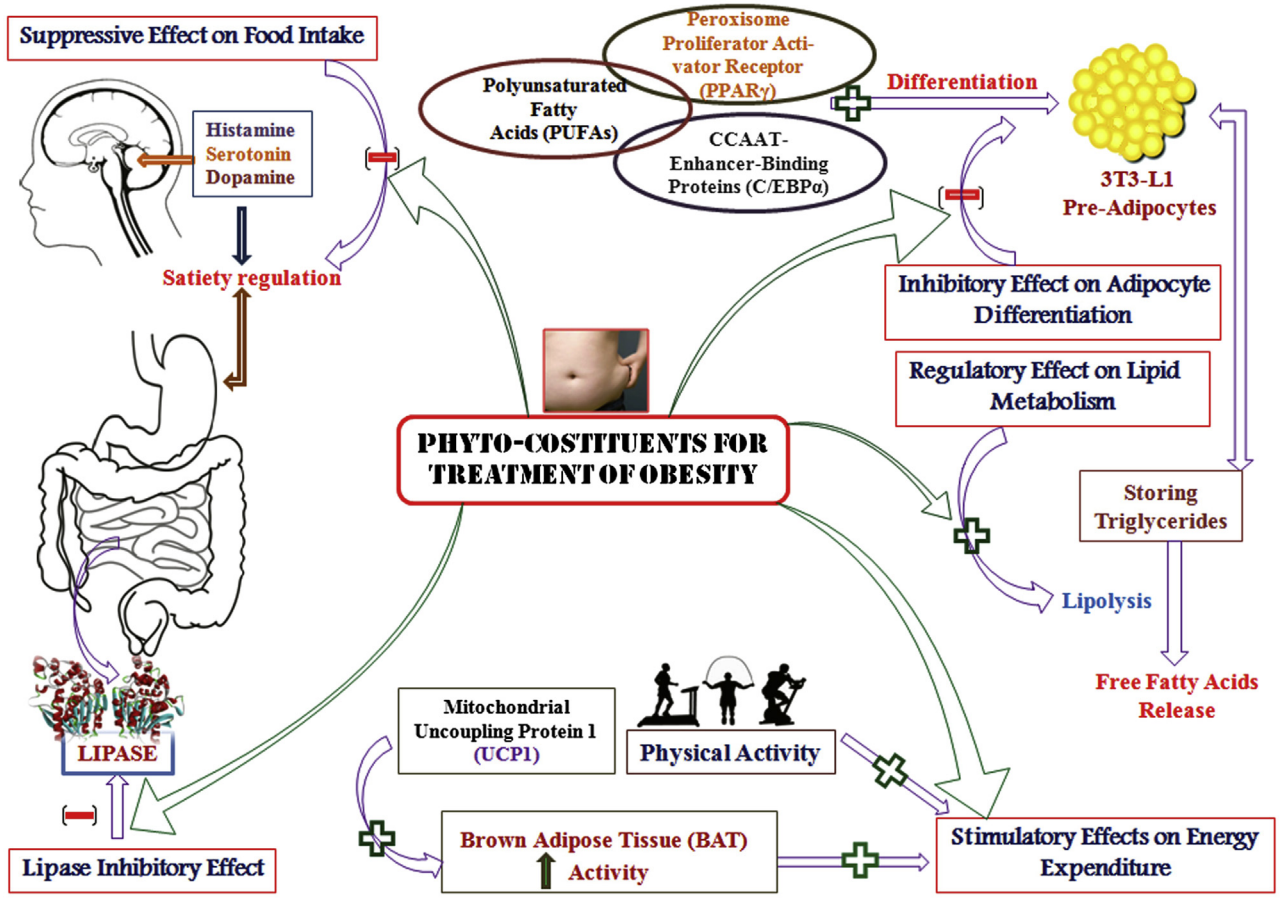


FIGURE 18.1 Major targets for anti-obesity phyto-constituents. (Five major target for anti-obesity activity possess phyto-constituents: satiety regulation; lipase inhibitory effect; stimulatory effect on energy expenditure; regulatory effect on lipid metabolism; inhibitory effect on adipocyte differentiation).

it inhibits lipases activity to hydrolysis of dietary fats into absorbable monoglycerides and fatty acids. Therefore, fats then tend to be excreted in feces rather than being absorbed to be used as a source of energy, in turn leading to weight loss in individuals [9].

18.2.3.2 *Adipocyte Differentiation and Proliferation*

Adipocytes, also known as lipocytes and fat cells, are the cells that primarily compose of adipose tissue, play a central role in the maintenance of lipid homeostasis and energy balance by storing triglycerides and releasing free fatty acids in response to changing energy demands. There are two types of adipose tissue, white adipose tissue and brown adipose tissue (BAT). Growth of the adipose tissue depends on hyperplasia and hypertrophy of adipocytes. Therefore, several studies are performed to focus on the processes of adipocyte proliferation and differentiation. In research, uses of 3T3-L1 preadipocyte cells are advantageous in vitro model for the study of obesity, due to its triglycerides accumulation ability during differentiation in cell culture [10]. This process is accomplished by expression of adipocyte specific genes, such as peroxisome proliferator-activated receptors-γ (PPARγ) and CCAAT/enhancer-binding protein-α (C/EBPα). Research is done to find the potential natural product which shows a promising inhibitory activity on adipogenesis, with regard to the potential treatment of obesity. However, current studies in this area suggest that inhibiting adipogenesis is unhealthy, leading to type 2 diabetes and other metabolic diseases, such as atherosclerosis [11]. Polyunsaturated fatty acids (PUFAs) are integral molecules of phospholipids of cell membrane and act as a signal transducer in adipocyte differentiation via regulating adipocyte-specific gene expression. Moreover, PUFA can withstand to the formation of triglycerides then other saturated and monounsaturated fatty acid. Therefore, PUFA plays an essential role in limiting fatty acid synthesis and regulating adipocyte differentiation through the suppression of late-phase adipocyte differentiation [9].

18.2.3.3 *Lipid Metabolism*

The lipolysis of fats can be achieved in two different ways. The first approach is stimulating triglyceride hydrolysis in order to diminish fat stores and another option is augmented fatty acid oxidation which is released from triglyceride store, thereby combating obesity. β_3-Adrenergic agonists played a pivotal role in this regard. However, excessive lipolysis causes high circulating fatty acid levels in the blood stream leading to dyslipidemia; a blockade of such a fatty acid release may be of therapeutic interest [9].

18.2.3.4 *Energy Intake and Energy Expenditure*

Appetite control can suppress body weight gain through a cascade of multifactorial events, which is typically interrelated with neurological and hormonal function of the body. Histamine, dopamine, and their closely associated receptor activities are responsible for satiety regulation. Appetite suppression can be achieved by modifying various hypothalamic neuropeptides' levels and/or via decrease in the function of monoamine neurotransmitters in the central nervous system (CNS). It may be suitable targets for appetite suppressant drug development [12]. Serotonin is a monoaminergic neurotransmitter of sensory and motor neurons that may modulate behavioral processes by acting through 5-hydroxy tryptamine (5-HT) receptor subtypes. These receptors played a crucial role in the energy intake reduction and may be useful for antiobesity drug development from natural product [13]. A potential appetite suppressant should be considered in terms of: (1) the psychological experience and behavioral expression of appetite, (2) metabolism and peripheral physiology, and (3) the CNS neural pathways' functioning [12].

Hunger is a sensation experienced when one feels the physiological need to eat food. In contrast, satiety is the absence of hunger. It is the sensation of feeling full. However, ghrelin secretion from stomach may increase the desire of food intake in animals and humans. Thus, ghrelin antagonism may decrease or blunt the desire for food; consequently decreased feeding, may be a possible adjunctive treatment for obesity [14]. Melanin-concentrating hormone receptor antagonism may also prove an important target for obesity treatment through appetite regulation. Increased adipose tissue concentration causes excessive food intake as a result of insufficient energy expenditure. To regulate body weight and energy expenditure, mammalian BAT plays an imperative role in energy homeostasis, BAT dissipates energy in the form of heat, a process called nonshivering thermogenesis. UCP-1 (mitochondrial uncoupling protein-1) is a key player in this process, which discharges the proton gradient generated in oxidative phosphorylation and thereby dissipating energy as heat. Thus, phytoconstituents work on the upregulation of UCP-1 gene expression may be considered as prospective agents for obesity control through increasing energy expenditure. UCP-3 is an analog of UCP-1; UCP-3 is also regulating thermogenesis by the thyroid hormone, β_3-adrenergic agonists and leptin in some organs may be recognized as a potent target for antiobesity drug development in future [9].

18.2.4 Antiobesity Drugs

The currently available antiobesity drugs can be divided into two classes: central acting and peripheral

acting. Orlistat is the sole representative of the group of peripheral-acting drugs. Drugs that act on the CNS (modulating monoamine levels in the synaptic cleft) do so by means of three mechanisms, namely catecholaminergic (noradrenaline and dopamine), serotoninergic (5-hydroxytriptamine), or both [15].

Orlistat is a Food and Drug Administration (FDA)-approved weight-loss drug that is available without a prescription. Orlistat inhibits gastrointestinal lipases, reducing fat absorption. Its most common side effect is steatorrhea [16]. Some cases of severe liver injury have been reported. It is advisable to stop the drug immediately if there are any symptoms of liver problems which may include dark urine, itching, light-colored stools, loss of appetite, and sometimes yellow coloration of eyes and skin [15]. Lorcaserin is another FDA-approved antiobesity drug, for long-term use. Lorcaserin is a selective agonist of 5-HT2C receptors (subtype of 5-HT receptor). A rare but serious side effect of this drug is serotonin syndrome (high fever, muscle rigidity, and confusion), which most commonly occurs if the drug is taken along with antidepressant drugs like selective serotonin reuptake inhibitors or monoamino oxidase inhibitors. Other serious side effects are psychiatric disorders with cognitive impairment, bradycardia, hematological changes, and prolactin elevation [16].

Numerous complex etiologies are involved in obesity in human. So, monotherapy will not be sufficient to reverse the disease condition. Therefore, combination therapies have been evaluated clinically and it shows promising results in the management of obesity. One such combination therapy that has recently been approved in the United States is phentermine/topiramate extended-release formulation for the treatment of obesity [17]. The role of phentermine is the downregulation of catecholamines concentration in the satiety centers of the hypothalamus; as a result appetite is suppressed. Topiramate exerts its effects through partial antagonism of α-amino-3-hydroxy-5-methyl-4-isoxazole propionate/kainite receptors, although induction of γ-aminobutyric acid receptor-mediated inhibitory currents and modification of voltage-gated calcium and sodium channels may also play a role [16]. It must not be used during pregnancy because it may cause harm to the baby. Rare side effects associated with topiramate include kidney stones and acute glaucoma.

18.3 PHYTOCHEMICALS USEFUL AGAINST METABOLIC DISORDER

Phytochemicals derived from vegetables, fruits, herbs, and spices have beneficial health effects such as antiobesity, lipid-lowering, and/or antidiabetic properties [18]. Considering the above-mentioned facts, several phytochemicals possessing antiobesity activity have been summarized in Table 18.1.

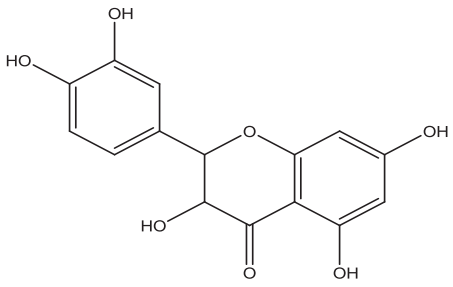
18.3.1 Phenolic Acids

Phenolic acids are composed of a basic phenol moiety with one carboxylic acid group. Chlorogenic and coumaric acids caused significant inhibition of cell growth as well as enhancing apoptosis on mouse preadipocytes. Gallic acid was not affecting the adipocyte cell cycle, but increased the number of apoptotic cells. A recent study explored that ferulic acid can suppress the high fat diet (HFD) induced weight gain by inhibiting fatty acid biosynthesis on lipid metabolism of mice [24].

18.3.2 Flavonoids

The most common expression of phenolic compounds is flavonoids abundantly present in plants, fruits, seeds, and vegetables. Flavonoids have the basic chemical structure of diphenylpropanes ($C_6{-}C_3{-}C_6$), and most often aglycones e.g., quercetin (**3**) or kaempferol (**4**), moieties are found attached to sugars (glycosides).

Quercetin [3]



Kaempferol [4]

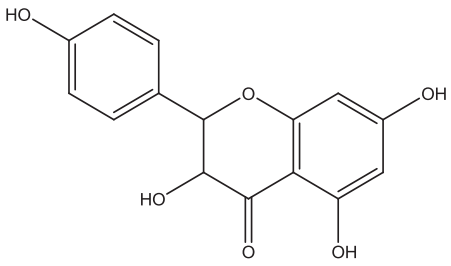
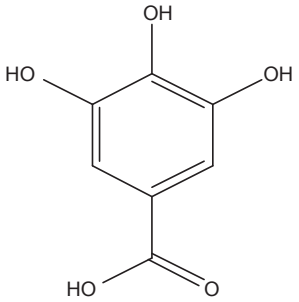
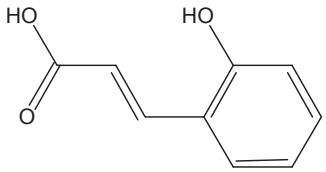
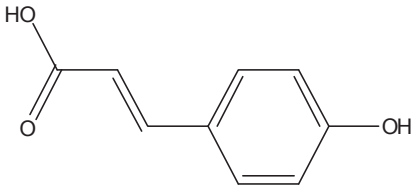
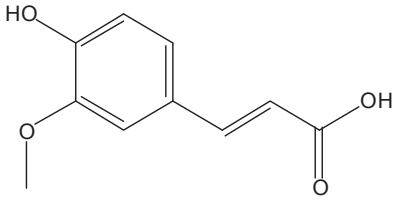
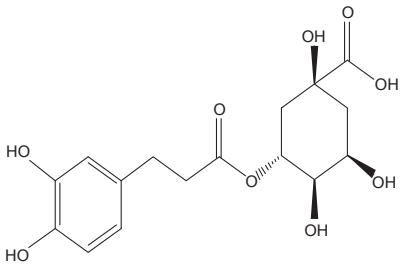
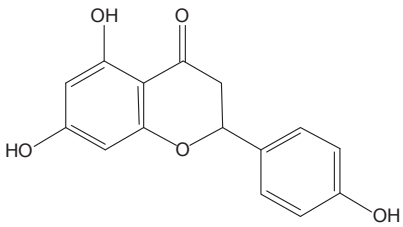
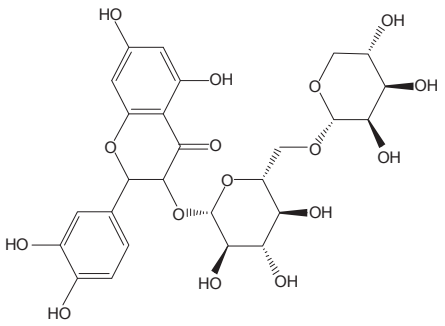
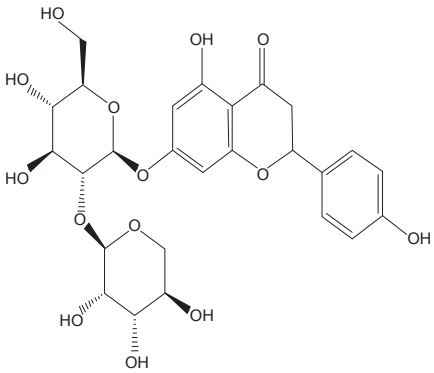


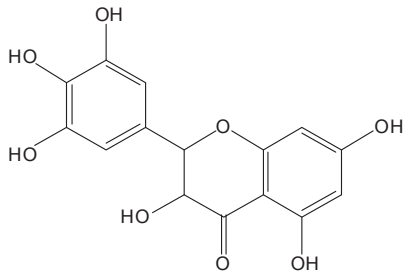
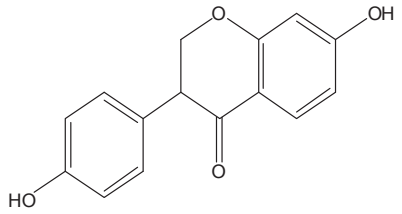
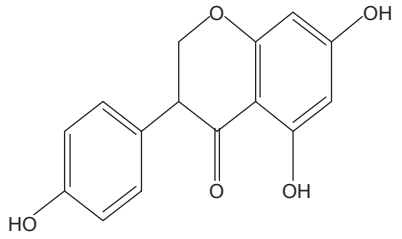
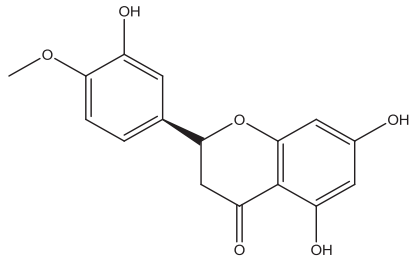
TABLE 18.1 Several Phytochemicals Possessing Antiobesity Potential

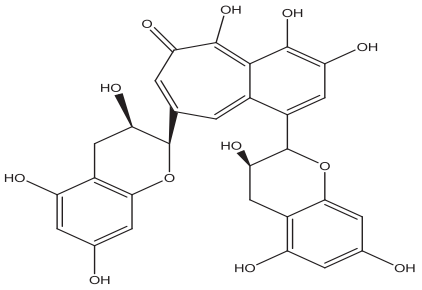
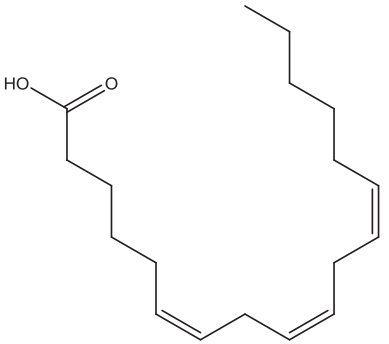
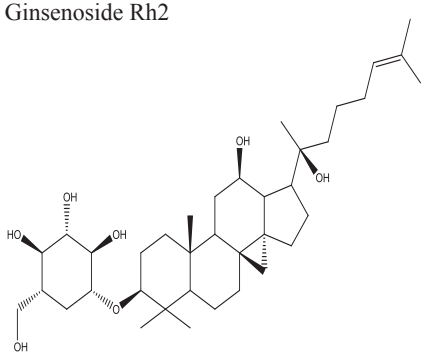
Name	Structure	Mode of action	Role in antiobesity	References
PHENOLIC ACID				
Gallic acid		↓ Triglyceride (TG), phospholipid, total cholesterol, low density lipoprotein-cholesterol (LDL-C), insulin and leptin levels	↓ Dyslipidemia, hepatosteatosis, and oxidative stress	[19]
		Inhibiting pancreatic lipase activity, ↓ TG	↓ Weight gain	[20]
		Upregulation of Peroxisome proliferator-activated receptors-γ (PPARγ) expression and Akt activation	Improves glucose tolerance and lipid metabolism	
Coumaric acid	O-Cumaric acid 	↓ Serum lipid profiles, insulin, and leptin ↓ TG and cholesterol levels ↓ Oxidative stress and glutathione disulfide(GSSG) content ↑ Glutathione (GSH), GSH peroxidase (GPx), GSH reductase (GRd), and GSH S-transferase (GST)	↓ Dyslipidemia, hepatosteatosis, and oxidative stress	[21]
	P-Cumaric acid 	↓ Expression of CCAAT/enhancer-binding protein α (C/EBPα), PPARγ, sterol regulatory element-binding protein-1c (SREBP-1c), and aP2 ↓ Fatty acid synthase and adiponectin mRNAs ↑ adenosine monophosphate-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) phosphorylation	Inhibited adipogenesis	[22]
Ferulic acid		Improved the hepatic steatosis	↓ Body weight gain ↓ Hyperglycemia ↓ Hypercholesterolemia	[23]
		↑ Fecal lipid excretion and antioxidant ↑ Lipogenic enzymes activities	Hypolipidemic	[24]
Chlorogenic acid		Inhibit fatty acid synthase, 3-hydroxy-3-methylglutaryl CoA reductase and acyl-CoA:cholesterol acyltransferase activities ↑ Fatty acid beta-oxidation activity and PPARα expression ↓ TG, leptin, and insulin ↑ Absorption and utilization of glucose	↓ Body weight gain improve lipid metabolism ↓ Body mass and body fat	[25]

FLAVONOIDS				
Naringenin		↑ Fatty acid oxidation ↓ Very-low-density lipoprotein (VLDL) overproduction ↓ Hepatic cholesterol and cholesterol ester synthesis Improved overall insulin sensitivity and glucose tolerance	↓ Hepatic steatosis ↓ Dyslipidemia	[26]
		Inhibits toll-like receptors expression during adipocyte differentiation ↓ Tumor necrosis factor-alpha (TNF-α) and monocyte chemotactic protein-1	Antihyperglycemia Anti-inflammatory	
		Inhibits adipogenesis and impairs mature fat cell function ↓ Insulin receptor substrate 1 tyrosine phosphorylation Inhibited adiponectin protein expression	Antihyperlipidemic	[27]
Rutin		In vivo ↓ body weight gain ↓ PPARγ and C/EBPα	Suppressing adipocyte differentiation	[21]
		↓ Serum lipid profiles, insulin, and leptin ↓ TG and cholesterol levels ↓ Oxidative stress and GSSG content ↑ GSH, GPx, GRd, and GST	↓ Dyslipidemia, hepatosteatosis and oxidative stress	
		↓ Endoplasmic reticulum (ER) stress and production of reactive oxygen species Protect fatty liver and insulin resistance ↑ Energy expenditure	Blocking macrophage mediated inflammation and inflammation induced obesity	[28]
Naringin		↓ Cholesterol and TG concentrations ↓ 3-Hydroxy-3-methylglutaryl-coenzyme A reductase activity ↓ Cholesterol acyltransferase activity	Hypolipidemic ↓ Hepatic cholesterol biosynthesis	[29]
		↓ Inflammatory cell infiltration, oxidative stress, plasma lipid concentrations ↑ Liver mitochondrial function	Cardioprotective	
		↓ Plasma acute-phase protein and haptoglobin concentrations—naringin (0.1%)	Improve the obesity-related inflammatory state	[30]

Continued

TABLE 18.1 Several Phytochemicals Possessing Antiobesity Potential—cont'd

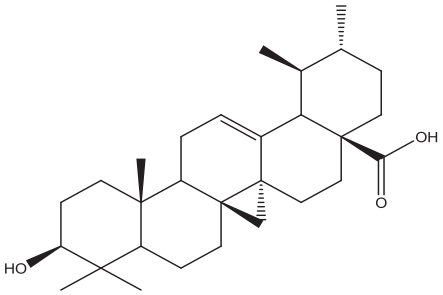
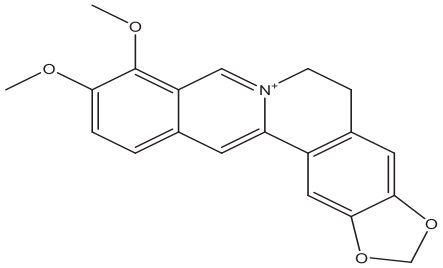
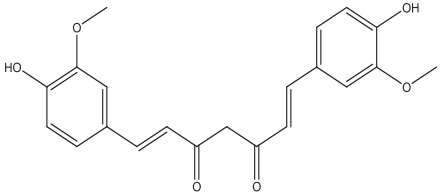
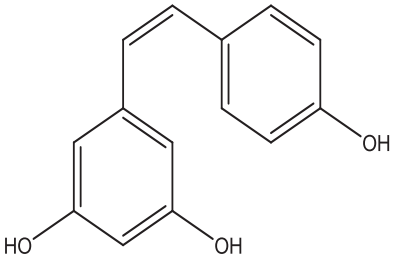
Name	Structure	Mode of action	Role in antiobesity	References
Myricetin		↓ PPARα ↓ Acyl-CoA oxidase and cytochrome P450 isoform 4A1 ↑ Expressions of hepatic SREBPs	↓ Body weight gain and body fat accumulation	[31]
Daidzein		↓ Glucose-6-phosphatase and phosphoenolpyruvate carboxykinase activities ↓ Fatty acid beta-oxidation and carnitine palmitoyltransferase ↑ Malic enzyme and glucose-6-phosphate dehydrogenase	Insulin-dependent diabetes mellitus	[32]
		↑ Leptin ↓ Adiponectin ↑ Uncoupling protein 1 ↓ Expression of stearoyl coenzyme A desaturase	Hepatic steatosis ↓ Weight gain and fat content	
Genistein		↑ Methylation of six cytosine–guanine sites in a retrotransposon upstream of the transcription start site of the agouti gene Permanently altering the epigenome	Alters susceptibility to obesity in adulthood	[33]
		↓ PPARγ and C/EBPα ↓ Glycerol-3-phosphate dehydrogenase ↓ Adipocyte fatty acid binding protein, fatty acid synthase, ↓ SREBP-1, leptin, lipoprotein lipase	Inhibited adipogenic differentiation	[34]
Hesperetin		↓ PPARγ2 ↓ TG and cholesterol levels ↓ Body weight	Inhibited the adipocyte differentiation	[35]
		↑ Release of cholecystokinin ↑ Intracellular Ca(2+) concentrations ([Ca(2+)]i)	Suppression of appetite	[36]

Theaflavin		Theaflavin digallate as potential plasminogen activator inhibitor type one inhibitor	↓ Body weight	[37]
		↓ Lipid accumulation, fatty acid synthesis Inhibited ACC activities	Prevention of fatty liver and obesity	[38]
		↓ Total Cholesterol (TC), TG, and LDL-C ↓ Atherogenic index, ↑ Insulin sensitive index Inhibited the hepatic lipase activity ↓ Leptin ↓ Serum alanine transaminase activity ↑ Serum superoxide dismutase activity	Reduce the risk of type 2 diabetes and cardiovascular disease in obesity	[39]
TERPINOIDS				
Gamma linolenic acid		↓ Body weight ↓ Adipose fatty acids	Weight loss in humans	[40]
		↑ Insulin-mediated glucose transport activity Reductions in the glucose-insulin index	Insulin-resistant obesity	
		Poly(ethylene glycol) + 1 conjugated linoleic acid ↓ PPARγ ↓ C/EBPα ↓ aP2	Antiadipogenic	[41]
Ginsenosides (Rh2, F2 and Rh3)	Ginsenoside Rh2 	↓ PPARγ activity ↑ AMPK signaling pathway	Antiadipogenesis	[42]
		Glucocorticoid receptor through ↑ Adipogenesis	↑ Lipogenesis in adipose tissue	

Continued

TABLE 18.1 Several Phytochemicals Possessing Antiobesity Potential—cont'd

Name	Structure	Mode of action	Role in antiobesity	References
	Ginsenoside F2	PPARγ and perilipin gene expression	Antiadipogenesis	[43]
	Ginsenoside Rg3	↓ Blood glucose ↑ Insulin secretion ↑ Fatty acid oxidation	Hyperglycemia	[44]
		↓ Fat accumulation ↓ PPARγ AMPK inhibition	Antiadipogenesis	[45]
Oleanolic acid		↓ PPARγ ↓ C/EBPα Visfatin (a proinflammatory and visceral fat-specific adipokine expressed in adipocytes) inhibition	Suppress obesity-associated inflammation	[46]
		↓ Body weights, visceral adiposity, plasma lipids ↑ Leptin ↓ Ghrelin	↑ Glucose tolerance ↑ Carbohydrate and fat metabolism	[47]

Ursolic acid		Translocating hormone-sensitive lipase ↓ Perilipin A expression by the protein kinase A pathway ↑ Adipose triglyceride lipase	↑ Lipolysis	[48]
		Ursolic acid stearoyl glucoside by ↓ Lipid parameters, TG ↓ Body weight, parametrial adipose tissue weight	Inhibiting pancreatic lipase activity	[49]
		↑ AMPK ↑ Liver kinase B1	Inhibit preadipocyte differentiation and adipogenesis	
OTHER GROUP OF PHYTO-CHEMICAL				
Berberine		↑ AMPK − in peripheral tissues ↓ Level of malonyl-CoA and stimulated the expression of fatty acid oxidation genes, centrally	Improve fatty liver	[50]
		↓ Weight gain and food intake ↓ Serum glucose, TG, and total cholesterol levels ↓ PPARγ expression ↑ GATA-binding protein 3 expression	Inhibited adipogenesis	[51]
		Modulation of the gut microbiota ↑ Levels of serum lipopolysaccharide-binding protein, monocyte chemoattractant protein-1 (MCP1), and leptin ↓ Level of adiponectin	Obesity-mediated insulin resistance	
Curcumin		Curcumin from *Curcuma longa* extract (0.09%) ↓ α1-acid glycoprotein	Improve the obesity-related inflammatory state	[30]
Resveratrol		↓ Body weight gain ↓ Levels of TG, free fatty acid, total cholesterol, glucose ↓ TNF-α and monocyte chemoattractant protein-1 ↓ Galanin-mediated adipogenesis signaling cascade	Antiadipogenic, anti-inflammatory	[52]
		Resveratrol-enriched rice DJ-526 rice ↓ Body weights and abdominal fat ↓ Lipid and glucose levels	Antiobesity	

↑ Increase the effect, ↓ decrease the effect.

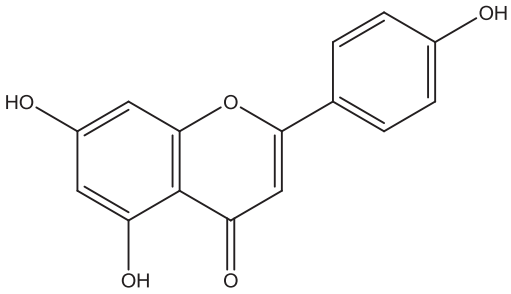
18.3.2.1 Flavonols

Quercetin and kaempferol are a common dietary flavonol found in plants and they have potential antiobesity effects. Quercetin has been shown to inhibit adipogenesis and induce apoptosis in mouse preadipocytes [19]. In a recent study, quercetin was seen to amplify the adenosine monophosphate-activated protein kinase (AMPK) signal pathway in 3T3-L1 preadipocytes cells that may be responsible for antiadipogenesis activity of the compound, while the quercetin-induced apoptosis of mature adipocytes was mediated by modulation of the extracellular signal-regulated kinases and c-Jun N-terminal kinase pathways, which play a key role during apoptosis [53]. Effect of quercetin with a combination of genistein and resveratrol was observed in human adipocytes (HAs). The combined treatments caused an enhanced inhibition of lipid accumulation in maturing HAs that was greater than the responses to individual compounds. Kaempferol has also possessed antiobesity effect to a lesser extent [54].

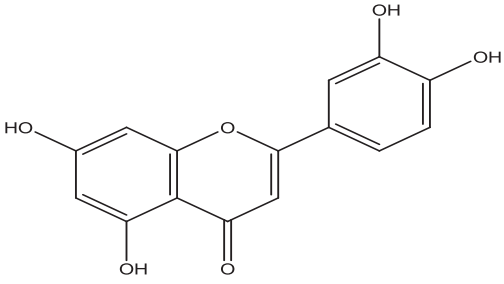
18.3.2.2 Flavones

Natural flavones are mainly apigenin (**5**), luteolin (**6**), chrysin (**7**), baicalein (**8**), scutellarein (**9**), wogonin (**10**), and their glycosides. Luteolin inhibited intracellular triglyceride (TG) accumulation of murine 3T3-L1 preadipocytes in a dose-dependent manner without producing cytotoxicity. Its antiadipogenic effects were exerted through suppressing adipogenic transcription factors and by inhibiting the *trans*-activation of PPARγ. An earlier study on apigenin suggests that it induced lipolysis in rat adipocytes [53].

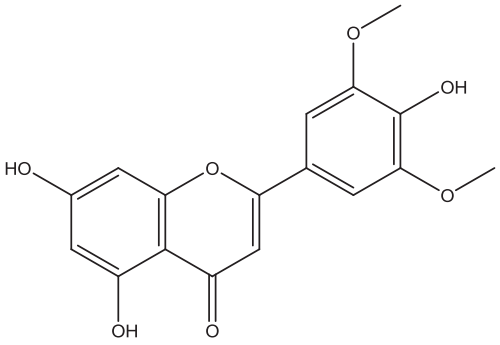
Apigenin [5]



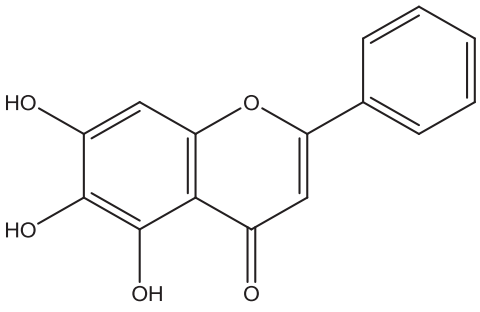
Luteolin [6]



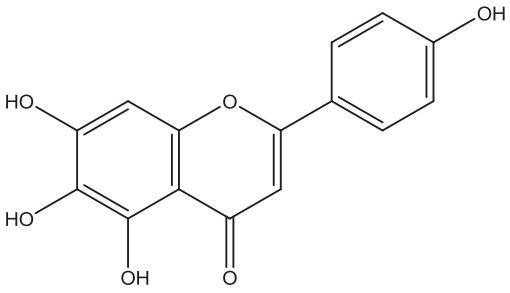
Crysin [7]



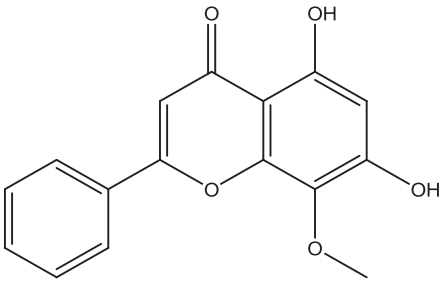
Baicalein [8]



Scutellarein [9]



Wogonin [10]



18.3.2.3 Anthocyanins

Anthocyanins are another class of flavonoids mostly water-soluble pigments that may appear red, purple, or blue depending on the pH and biosynthesized via the phenylpropanoid pathway. It has conquered the HFD-induced obesity in mice significantly. Cyanidins (**11**) are considered as the most widely spread anthocyanin in the plant kingdom. Cyanidin can reduce blood glucose levels as well as downregulating inflammatory protein cytokines such as monocyte chemoattractant protein-1 (MCP-1) in the adipose tissue of mice [53]. A study was revealed that cyanidin 3-glucoside stimulated in vitro insulin secretion from rodent pancreatic beta-cells. Among cyaniding glycosides, cyanidin 3-rutinoside and cyanidin 3-galactoside have been proposed as new noncompetitive α-glucosidase inhibitors [55]. These recent studies suggest that cyanidins have a unique therapeutic advantage and important implications in the prevention of obesity and diabetes.

18.3.3 Terpenoids

The terpenoids, sometimes called isoprenoids, were derived from five-carbon isoprene units (CH_2=$C(CH_3)$–CH=CH_2) assembled and modified in different ways. The daily eating of plant-derived terpenoids might be useful for the management for obesity and obesity associated syndrome, such as type 2 diabetes, hyperlipidemia, insulin resistance, cardiovascular disorder (CVD), and a lower prevalence of metabolic syndrome. Astaxanthin (**12**) belongs to the xanthophyll class of carotenoids was reduced the hepatic accumulation of TG and hyperlipidemia in HFD-induced mice [18]. Six closely related bicyclic diterpene was isolated from *Commiphora mukul* Gum possess lipid peroxidation and COX enzyme inhibitory activities. PPARs are dietary lipid sensors that control energy homeostasis. Researchers have observed abiotic acid, geranylgeraniol, bixin, geraniol, farnesol, phytol and auraptene are potential terpinoids activate PPARs significantly [56].

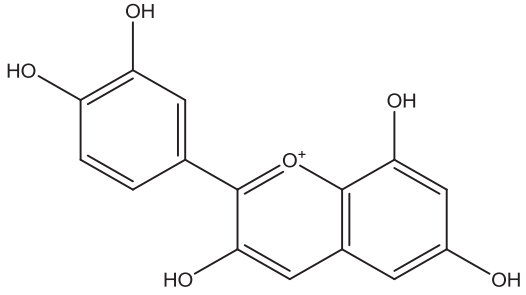
18.3.4 Carotenoids

Carotenoids belong to the category of tetraterpenoids (i.e., they contain 40 carbon atoms, being built from four terpene units each containing 10 carbon atoms). Hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls) are two main categories of carotenoids. β-carotene inhibits inflammatory gene expression in lipopolysaccharide-stimulated macrophages and has been suggested that its antioxidant activity contributes to beneficial effect on obesity and CVD. Possible pharmacological actions of α- and β-carotene have been postulated based on the finding lower level of plasma carotenoids among overweight and obese children compared to healthy weight children [53]. A similar result was also found when investigated the relationship between abdominal adiposity and serum levels of carotenoids in a healthy Japanese population [57].

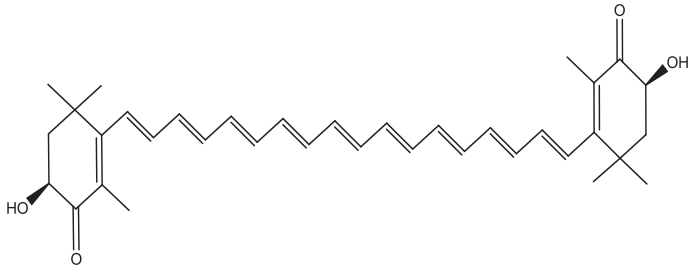
18.3.5 Organosulphurs

Organosulfur are sulfur containing organic compounds. Vegetables like garlic, onion, scallion, chive, shallot, and leek are the major source of bioactive organosulfur compounds such as allicin (**13**), allixin (**14**), and allyl sulfides. Allicin is the principal constituent of allium vegetables, which has induced apoptosis of human tumor cells [53]. Elkayam and coauthors [58] observed that pure allicin can lower blood pressure, insulin, and triglycerides levels in fructose-fed rats. In the same experiment, the control group was treated with fructose enriched diet that's shown continued to weight gain, whereas the groups fed allicin did not. Thus, allicin could be established as a useful therapeutic agent to combat obesity. In another study, antiobesity potential of ajoene was evaluated in 3T3-L1 adipocytes. The ajoene induced apoptosis in 3T3-L1 adipocytes was occurred mainly due to regulating fat cell numbers via generation of hydrogen peroxide, which leads to activation of mitogen-activated protein kinases, degradation

Cyanidins [11]



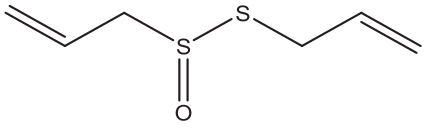
Astaxanthin [12]



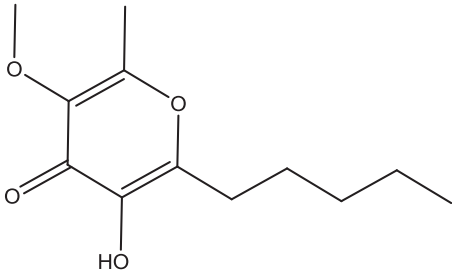
of PARP-1, translocation of apoptosis-inducing factor, and fragmentation of DNA [59].

The glucosinolates (**15**) are sulfur containing natural occurring organosulfurs of many pungent plants such as mustard, cabbage and horseradish. Breakdown products of glucosinolate are biologically active most notably the isothiocyanates and indoles, this byproduct has received much attention for its apparent anticarcinogenic activity and possible antiobesity effects. Sulforaphane (SFN), a member of the aliphatic isothiocyanate family, is a biologically active compound extracted from cruciferous vegetables such as broccoli, cauliflower, radishes and cabbage [53]. In a recent study, Choi and coworkers investigated the effect of sulforaphane on HFD induced obesity in C57BL/6N mice. Experimental results suggest that the role of antiadipogenesis activity of SFN possibly through downregulation of PPARγ and CCAAT/enhancer-binding protein α (C/EBPα) and by suppressing lipogenesis through activation of the AMPK pathway. Perhaps clinical trials are required to confirm the antiobesity effects of these phytochemicals [60].

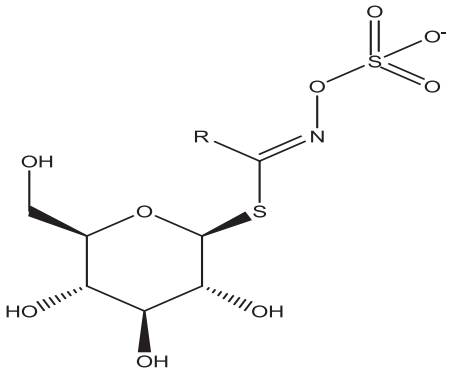
Allicin [13]



Allixin [14]



Glucosinolates [15]



18.3.6 Phytosterol

Phytosterols are natural compounds structurally similar to mammalian cell-derived cholesterol. The main sources of phytosterols are vegetable oils, nuts, grains, and grain derived products. The sterols are abundant in nature, they exist in both esterified and free alcohol forms. Phytosterols with potential effects of obesity are diosgenin (**16**), campesterol, brassicasterol, β- and γ-sitosterol (**19, 20**), stigmasterol, and guggulsterone E (**22**). High intakes of these compounds can also protect against atherosclerosis and decrease serum low density lipoprotein-cholesterol (LDL-C) levels [61]. Dietary plant sterols are reducing intestinal cholesterol absorption by increase fecal excretion of cholesterol as well as via regulating the expression of cholesterol homeostasis genes in the liver [62]. In another study explored that phytosterols can inhibit cholesterol absorption by competitive solubilization of mixed micelle formation of cholesterol in the intestinal lumen [63].

A bioactive phytochemical, protodioscin, isolated from the rhizomes of *Dioscorea nipponica*, was identified for its antihyperlipidemic effect. In hyperlipidemic rats, the administration of protodioscin significantly reduced the blood levels of TG, cholesterol, LDL and high-density lipoproteins (HDL) [64]. Dioscin and diosgenin the active components of *D. nipponica* are a potent porcine pancreatic lipase inhibitor. Diosgenin (5 and 10 μmol/L) inhibited the accumulation of TG and the expression of lipogenic genes in HepG2 cells. It is also ameliorates

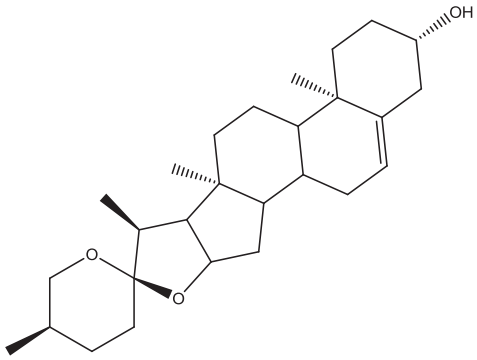
dyslipidemia by decreasing the hepatic lipid content in diabetic mice [65]. Furthermore, diosgenin is improving the lipid profile of rats feed with a high-cholesterol diet supplemented. Diosgenin showed significant therapeutic and preventive effect on hypercholesterolemia in mice. The serum total cholesterol level was decreased when rats were pretreated with diosgenin [66].

Guggulsterone (GS) is an active agent of the guggul plant (*Commiphora mukul*) which is used for treatment of obesity, arthritis, cancer, and CVD. GS and its isomers exert antiobesity effects by inhibiting differentiation of preadipocytes, and by inducing apoptosis and promoting lipolysis of mature adipocytes [67]. It is also potentiates antiadipogenic and proapoptotic effects in maturing 3T3-L1 preadipocytes considered as a potential antiobesity agent [68]. Researchers have discovered that GS can selectively decrease the expression of bile acid genes by act as an antagonist for farnesoid X receptor. It is hypoglycemic and hypolipidemic activity was tested on HFD induced rat [69]. Finally, studies indicate that GS can significantly lower lipid, cholesterol and TG and help in rising HDL in serum.

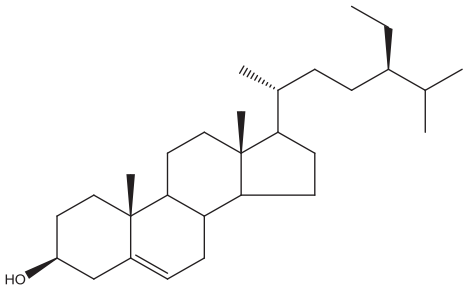
18.4 HERB AS FOOD USEFUL IN OBESITY MANAGEMENT

The best and most useful option for overweight and obese individual is calorie restriction and exercise. Obesity prevention through diets may be accomplished by bioactive constituents of herbal food supplements that could modulate molecular pathways and gene/protein expressions of the obese individual along with diet control and physical activity. Most recent researches on food were shown its ability to modulate some specific physiological functions in the organism through food intake [70]. Body weight control by food supplements requires knowledge of the process by which body gaining weight. Serrano and Sánchez-González [71] reported the main strategies for body weight control by the functional food ingredients: inhibition of food intake (by inhibiting orexigenic signals or enhancing anorexigenic signals), limiting the bioavailability of nutrients (by suppressing the digestive enzymes and/or interacting with them to physically prevent their absorption), stimulation of energy expenditure (thermogenesis), and

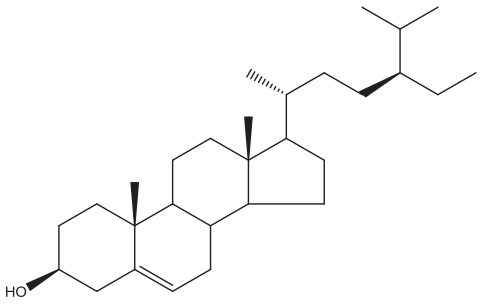
Diosgenin [16]



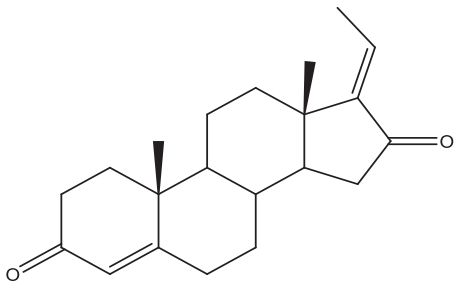
β-Sitosterol [19]



γ-sitosterol [20]



Guggulsterones E [22]



modifying the composition of the gut microbiota. The specific roles of gut microbiota are modulating metabolic energy storage by increase the capacity to harvest energy from the diet and modulate plasma lipopolysaccharides levels, which activate the inflammatory tone and the onset of obesity and type 2 diabetes [72]. This section is discussed the role of herbal food as medicine in alleviating obesity and associated complications. Table 18.2 summarized some important antiobesity activity possesses food plants.

18.4.1 Berries

The word berry is used for many dissimilar kinds of small fruits that bear many seeds and can be used as food. Some examples are raspberry, blueberry and lingonberry. In that respect are different species of berries which contains different types of ingredients most frequently polyphenols. Acai berries (*Euterpe oleracea* Mart.) are known as "superfood" with antiaging and weight loss properties. This fruit is small and reddish purple in color. This fruit pulp is rich in antioxidant component. It reduces fasting glucose, insulin levels, total cholesterol and LDL-cholesterol, in healthy overweight adults [112].

Blackberry is an edible fruit produced by many species in the *Rubus* genus in the Rosaceae family. It contains 87% cyanidin-3-O-β-d-glucoside (C_3G). C_3G is an anthocyanin compound which is having powerful antioxidative and anti-inflammatory activity. It has been sorted from the literature review that consumption of C_3G-rich blackberries are effective in prevention of

TABLE 18.2 Food Plants Possessing Antiobesity Potential

Plants	Parts used	Active principle	Mechanism of action	References
Aegle marmelos Correa. (Rutaceae)	Leaves	Coumarines, umbelliferone, and esculetin	↑ Lipolysis	[73]
Allium cepa L.(Amaryllidaceae)	Peels	Quercentin	Suppression of preadipocyte differentiation and inhibition of adipogenesis.	[74]
	Bulb	Cycloalliin, S-methyl-L-cysteine, S-propyl-L-cysteine sulfoxide and dimethyl trisulfide	↓ Serum TG and free fatty acid (FFA) levels on diabetes rats Inhibit formation of oil drop in the cells-suppressing obesity	
Brassica juncea L. Czern (Brassicaceae)	Oil	Crude extract	Regulate body weight gain, adipose tissue mass, lipid, and glucose metabolism	[75]
Brassica oleracea L. (Brassicaceae)	Sprouts	Crude extract	Cholesterol-lowering effect and potentially reduce lipid storage	[76]
Carica papaya L. (Caricaceae)	Fruit	Crude extract	↓ Triglyceride (TG), Total cholesterol (TC), low density level (LDL), and Very-low-density lipoprotein (VLDL) while high density lipoprotein -cholesterol (HDL-C) elevated	[77]
Cinnamomum zeylenicum Nees (Lauraceae)	Fruit	Crude extract	↓ TG, TC, and LDL-cholesterol	[78]
Citrus sinensis L. Osbeck (Rutaceae)	Peels	Pectin, synephrine	Pectin reduce blood cholesterol levels by decreasing its reabsorption in the colon and synephrine, reduces the production of cholesterol in the liver	[79]
Coffea canephora robusta (Rubiaceae)	Seeds and leaves	Caffeine, chlorogenic acid, Neochlorogenic acid, Feruloyquinic acids	Decrease the body weight Gain, ↓ hepatic TG level, and inhibit the fact accumulation in liver	[80]
Coriandrum sativum L. (Apiaceae)	Seed	Crude extract	↑ Hepatic bile acid synthesis and the degradation of cholesterol to fecal bile acids and neutral sterols	[81]

TABLE 18.2 Food Plants Possessing Antiobesity Potential—cont'd

Plants	Parts used	Active principle	Mechanism of action	References
Crocus sativus L. (Iridaceae)	Stigma	Crocin	Inhibit the absorption of dietary fat and cholesterol by hydrolysis of fat, pancreatic lipase inhibitor	[82]
Cuminum cyminum L. (Apiaceae)	Fruits	Crude extract	Reduction of macro vesicular steatosis in hepatic tissues and a significantly decreased number of lipid droplets and size of adipocytes	[83]
Curcuma longa L (Zingiberaceae)	Rhizome	Curcumin	Fatty acid oxidation, adipocyte apoptosis, AMPK activation, decrease expression of Peroxisome proliferator-activated receptors-γ (PPARγ) and CCAAT/enhancer-binding protein α (C/EBPα)	[84]
			Inhibited pancreatic lipase activity	
			Anti-inflammatory and improved metabolic conditions in obesity and improves glycemic control of type 2 diabetes in mouse models	
Emblica officinalis Gaertn. (Phyllanthaceae)	Fruit	Crude extract	Normalize adipose mRNA expression of nuclear transcription factor, peroxisome and inhibit lipid accumulation in mouse adipocytes	[85]
Ferula asafetida L. (Umbelliferae)	Oleo gum resin	Rhizome and root	↓ Body weights, abdominal fat, and size of epididymal adipocyte, serum leptin	[86]
Foeniculum vulgare Mill. (Apiaceae)	Fruit	Phenolics and flavonoids	Restricts the increase in body weight, fat pad weights, and disturbance of Total cholesterol (TC), HDL, LDL, and TGs	[87]
Garcinia cambogia Desr. (Guttiferae)	Fruit	Hydroxyl citric acid	Lipogenesis inhibition, lower body weight and reduce fat mass in humans	[9]
Glycine max (L.) Merr (Fabaceae)	Seeds	Protein	Pancreatic lipase inhibitor	[88]
Gymnema sylvestre R. Br (Asclepiadaceae)	Leaves	Gymnemic acids	↑ Fecal cholesterol and cholic acid-derived bile acid excretion	[89]
			↓ Serum leptin, insulin, LDH, LDL-C, total cholesterol, TG, and apolipoprotein-B levels	
Lagenaria siceraria (Molina) Standl (Cucurbitaceae)	Fruit	Crude extract	Fat amassment, ↓ TG and TC	[90]
Litchi chinensis Sonn. (Sapindaceae)	Litchi water extract	Crude extract	↓ TG and FFA	[91]
Malus domestica Borkh. (Rosaceae)	Fruit	Polyphenols	↓ Plasma and LDL cholesterol and triglyceride accumulation in heart and liver	[92]
Mangifera indica L. (Anacardiaceae)	Seed kernel	Crude extract	↓ The activity of glycerol 2-phosphate dehydrogenase in 3T3-	[93]

Continued

TABLE 18.2 Food Plants Possessing Antiobesity Potential—cont'd

Plants	Parts used	Active principle	Mechanism of action	References
			L1 adipocytes, and inhibit cellular lipid accumulation through downregulation of transcription factors such as PPARγ and C/EBPα	
Momordica charantia L. (Cucurbitaceae)	Fruit juice	Crude extract	Reduced adiposity mass with increased in lipid oxidative enzyme activities and uncoupling of protein expression	[94]
			Anti-inflammation and reduced oxidative stress, modulates mitochondrial activity, suppresses apoptosis activation, and inhibits lipid accumulation in liver	
			Reduce insulin resistance antidiabetic Downregulation of expressions of lipogenic genes inhibit visceral fat accumulation and adipocyte hypertrophy	[95]
			Reduced adipose tissue inflammation in diet-induced obese mice. ↓ Mast cell recruitment and proinflammatory cytokine monocyte chemotactic protein-1 (MCP-1) expression recruitments in epididymal adipose tissues (EAT), ↓ interleukin-6 (IL-6) and TNF-α expression in EAT	
Moringa oleifera Lam (Moringaceae)	Leaves	Polyphenolic, flavonoids	↓ TG, LDL, Very-low-density lipoprotein (VLDL), Total cholesterol (TC)	[96]
Murraya koenigii L. Spreng (Rutaceae)	Leaves	Carbazole alkaloids, mahanimbine	Pancreatic lipase inhibitor	[97]
Myristica fragrans Houtt. (Myristicaceae)		2,5-Bis-aryl-3, 4-dimethyltetrahydrofuran lignans, tetrahydrofuroguaiacin B, saucernetindio, verrucosin, nectandrin B, nectandrin A and galbacin	Activators of AMP-activated protein kinase	[98]
Phaleous vulgaris L. (Fabaceae)	Whole	α-amylase inhibitor, starch blocker	α-Amylase inhibitor	[99]
Psidium guajava L. (Myrtaceae)	Fruit peel	Crude extract	↓ TG, LDL, Very-low-density lipoprotein (VLDL), Total cholesterol (TC)	[100]
Spinacia oleracea (Amaranthaceae)	Leaves	Crude form	Improve abnormal postprandial hyperglycemic or hyperlipidemic responses	[101]
Solanum tuberosum L. (Solanaceae)	Tubers	Crude extract	Inhibition of lipid metabolism	[102]
Syzygium aromaticum L. Merr. et Perry. (Myrtaceae)	Dried flower buds	Crude extract	Regulation of genes related to lipid metabolism in the liver and white adipose tissue, ↓ lipid accumulation	[103]
Tamarindus indica L. (Fabaceae)	Pulp	Crude extract	Significant reduction in adipose tissue weights, as well as lowers the degree of hepatic steatosis	[104]

TABLE 18.2 Food Plants Possessing Antiobesity Potential—cont'd

Plants	Parts used	Active principle	Mechanism of action	References
Terminalia chebula Retz (Combretaceae)	Fruit, leaves	Myrobalan	Prevent cholesterol absorption, cholesterol excretion, enhanced lecithin: Cholesterol acyl transferase activity, lowers TG and TC	[105]
Trichosanthes dioica Roxb. (Cucurbitaceae)	Fruit	Flavonoids, alkaloids, glycosides, terpenes, sterols, lectins	↓ TG, LDL and Very-low-density lipoprotein (VLDL)	[106]
Trigonella foenum-graecum L., (Fabaceae)	Seed	Crude extract	↓ Very-low-density lipoprotein (VLDL), TGs, lactate dehydrogenase, and ↑ serum HDL-C ↓ Body weight gain	[107]
Vitis vinifera L. (Vitaceae)	Seeds	Cyanidol chloride (7%), Monomer (30%)	↓ Weight gain ↓ Blood lipid concentration ↑ Serum HDL-C concentration ↑ mRNA levels of lipolytic genes ↓ mRNA levels of lipogenic genes	[108]
	Grape skin	Resveratrol	↓ Adipogenic transcription factors (PPAR, C/EBPα, and their target genes (fatty acid synthase, aP2, SCD-1, and lipoprotein lipase)	[109]
	Seeds	Phenolic content	Inhibited lipid accumulation of C3H10T1/2 and 3T3-L1 adipose cells ↓ Expression of PPARγ	
Zingiber officinale Roscoe. (Zingiberaceae)	Rhizome	Crude extract, Gingerols and shogaol	Inhibition of dietary fat absorption ↓ Body weight, glucose, insulin, and lipid levels Inhibition of carbohydrate metabolism enzymes, ↑ insulin release ↓ Lipid content	[110]
Ziziphus jujuba Mill. (Rhamnaceae)	Fruit	Crude extract	Suppression of lipid accumulation and glycerol-3-phosphate dehydrogenase	[111]

↑ Increase the effect, ↓ decrease the effect.

weight gain and inflammation associated with ovariectomy-induced menopause in a rat model. The study was modeled for 100 days and after 100 days treatment revealed that a diet containing 10% blackberry (w/w) decreased hepatic NF-κB, and cyclooxygenase-2 expression levels in female Sprague—Dawley rats [113].

Blackcurrant (*Ribes nigrum*) berries are a woody shrub rich source of anthocyanin content. The concentration of anthocyanin in this type of berries are fourfold greater than those of other common fruits. 80% of the total anthocyanin content contains four major anthocyanins such as cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-glucoside and delphinidin-3-rutinoside. Maximum anthocyanins are found in fully ripe stage. In vitro or in vivo evidence suggested that anthocyanins are effective as natural antioxidants, anticarcinogenic, anti-inflammatory, vasoprotective, and antiobese agent [114]. Blueberries (vaccinium) are the perennial flowering plants with indigo-colored berries containing several bioactive compounds like anthocyanins (anthocyanidins, or phenolic aglycone conjugated with sugar), chlorogenic acid, flavonoids, α-linolenic acid, pterostilbene, resveratrol, and vitamins. The mechanism behind its obesity management involves the suppression of adipocyte differentiation, adipogenesis, and cell proliferation [115].

Bilberry is low-growing shrubs of the Ericaceae family. The obesity management potential of bilberries has been studied by Lehtonen, in 2011. It was found that bilberries decrease weight, waist limits, vascular cell adhesion molecule and intercellular adhesion molecule of obese women [116]. The extract of bilberry is enriched

with anthocyanidins which inhibited adipocyte differentiation by affecting the genes expressions of the insulin pathway; decreased PPAR, sterol regulatory element-binding protein 1c and tyrosine residues of insulin receptor substrate 1 phosphorylation [117]. Black chokeberry (*Aronia melanocarpa*) is native to eastern North America belonging to the family of Rosaceae found to contain high concentrations of anthocyanins and procyanidins. Chokeberry reduces weight gain and modulates insulin, adipogenic and inflammatory signaling pathways in epididymal adipose tissue of rats on a fructose-rich diet [118].

Indian gooseberry (amla) has been traditionally used in Ayurvedic herbal preparation or rejuvenating medicine. In an HFD induced mice model it significantly inhibited body weight gain as well as adipose tissue weight. Amla normalized adipose mRNA expression of nuclear transcription factor, PPARγ. Its aqueous extract was more effective in inhibiting lipid accumulation in 3T3-L1 mouse adipocytes treated during differentiation [85]. Mulberry is a long multiple fruit of Moraceae family. Peng and coauthors have investigated the potential of mulberry in obesity management. Mulberry water extracts (MWE) contain polyphenolic components like gallic acid, chlorogenic acid, rutin, and anthocyanins may responsible for hypolipidemic action by reducing serum triacylglycerol, cholesterol, free fatty acid, LDL/HDL ratio in 6-week-old male hamsters. MWE protects livers from impairment by decreasing hepatic lipids through regulating lipogenesis and lipolysis [119].

Rubus idaeus is a type of berries known to be effective in obesity management. The major aromatic component responsible for the obesity management is raspberry ketone (RK), 4-(4-hydroxyphenyl) butan-2-one; which is the main compound of red raspberry. It has structural similarity with capsaicin and synephrine. RK decreases the hepatic triacylglycerol content in HFD-induced mice. It translocate hormone sensitive lipase from the cytosol to lipid droplets in rat epididymal fat cells, thereby significantly increases norepinephrine-induced lipolysis. Specifically RK alters the lipid metabolism and increases norepinephrine-induced lipolysis in white adipocytes. In these ways RK prevented elevations in HFD-induced body weight and the weights of the liver and visceral adipose tissues [120].

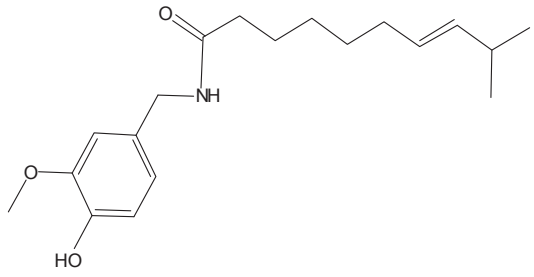
The *Solanum lycopersicum* is the edible red fruit/berry. It is commonly known as a tomato plant. In a recent investigation the effect of red and green tomato extract on has been studied in high-fat-diet-induced C57BL/6 mice. The investigation indicated that the green tomato extract attenuates obesity, which may be associated with activation of the AMPK pathway [60]. In another investigation into the effect of tomato vinegar (TV) containing phytochemicals has been evaluated in vitro and in vivo. In HFD-induced rats TV inhibited adipocyte differentiation of 3T3-L1 preadipocyte and lipid accumulation during differentiation. TV supplementation markedly decreased visceral fat weights, hepatic TG and cholesterol level without changing the food and calorie intakes. Furthermore, plasma LDL-cholesterol and antherogenic index has been lowered. It also elevates HDL-cholesterol to total cholesterol ratio. Thereby this study suggested that TV can be used as an antiobesity therapeutic agent [121].

18.4.2 Capsicum

Capsicum is the fruit of different species of capsicum plants. Capsicum is also known as red pepper or chili pepper or bell pepper, variety of names depending on place and type. Capsaicin (8-methyl-N-vanillyl-6-nonenamide) (**23**) is the most common naturally occurring capsaicinoids which is present all varieties of Capsicum plants. Other capsaicinoids are capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin. Antiobesity effects of water extracts of *Capsicum annuum* L. were examined through the evaluation of lipoprotein lipase (LPL) mRNA expression level in mouse preadipocytes. In another study of antiadipogenic effect of *C. annuum* L. seeds in 3T3-L1 adipocytes cells were examined [122]. From the experimental outcome, it has observed significant decrease in the expression of LPL mRNA level, adipogenic transcription factors C/EBPβ, C/EBPα, and PPARγ, may possible mechanism of antiobesity activity of capsicum. Red chili pepper consumption can be augmented satiety and reduced energy and fat intake, the stronger reduction with oral exposure suggests a sensory effect of capsaicin [123]. In another possible mechanism of antiobesity effects of capsaicin may be thermogenesis caused by primary sensory neurons of the "pain" pathway to stimulate the transient receptor potential vanilloid receptor 1 [124]. Capsaicin can also increase catecholamine (epinephrine, norepinephrine (NE) and dopamine) secretion from sympathetic nervous system and as a result, increases blood pressure. The explored evidence may be another postulate of thermogenesis [125]. Furthermore, prospective antiobesity effects of capsicum were examined in diet-induced obese rats. The result suggests that capsaicin may diminish body weight and fat accumulation in obese rats significantly. These effects may be arbitrated by the up regulation of uncoupling protein 2 (UCP2) gene expressions and its ability to inhibit glycerol-3-phosphate dehydrogenase activity [126]. Clinical studies have shown that diet-induced thermogenesis has amplified by capsaicin. The most recent study reveals capsaicin to the diet has been shown to increase energy expenditure by negative energy balance and promotes fat oxidation [127]. In

addition, nanoemulsion oleoresin capsicum was explored as a potential antiobesity agent in HFD induced rats. Very few studies have been executed to measure safety issues of capsinoids. One of the study indicate 6 mg/day capsinoids consumption is safe which will improve the obesity condition by decrease body weight (abdominal fat loss) and changes in metabolism specially increase oxidation of fat [128]. Several studies indicate Capsicum fruit may consider as a potential antiobesity food.

Capsaicin [23]



18.4.3 Citrus

Citrus is one of the most popular food stuff in the world and is a rich source of nutrients and bioactive compounds. Citrus fruits contain vitamins, minerals, dietary fibers, and pectins along with abundant of bioactive compounds, including coumarins, flavonoids, carotenoids, and limonoids. Antiobesity and cardiovascular toxic effects of *Citrus aurantium* extracts in the rat were observed by Calapai et al. [129]. In this study citrus fruit extract was standardized by synephrine. Repeat dose administration had shown significantly reduced the food intake and body weight gain in rats. The immature peel extract of *Citrus sunki* Hort. was tested on HFD induced obese C57BL/6 mice and mature 3T3-L1 adipocytes. In vitro results suggest that *C. sunki* extract (CSE) had an antiobesity effect via elevated β-oxidation and lipolysis in adipose tissue. *In vivo* study of animal was explored *Citrus* peel extract causes decrease body weight gain, adipose tissue weight, reduce serum total cholesterol, and TG in the CSE-administered group significantly compared to the HFD group [130]. In another study, nomilin from citrus fruit and seeds causes lower body weight, serum glucose, serum insulin, and enhanced glucose tolerance in male HFD-induced C57BL/6J mice [131]. Limonin (Lim) is a white crystalline substance, usually found in orange and lemon seeds. Halder et al. [35] report the antiobesity effects of cyclodextrin (CD)-treated Lim along with naringenin (Ng) and hesperetin (Hes). The results were indicated that Lim, Ng, and Hes decreased cell viability in 3T3-L1 preadipocyte cells. Lim, Ng, and Hes inhibited the adipocyte differentiation in response to adipogenic inducers. The evidence for this inhibition included fewer Oil Red O positive droplets and a decreased expression of the adipocyte-specific gene PPARγ2. In animal studies, Lim-, Hes-, and combination-treated mice gained less body weight than control mice without treatment. The plasma TG and cholesterol levels were significantly reduced by Lim and the other substances. Furthermore, Lim increased the mRNA expression on lipid metabolism-related genes, including Acox1, UCP2. and carnitine palmitoyltransferase1 in the liver. In another experiment on Lim and its glycoside isolated from *Citrus reticulate*, was shown induction of mitochondria mediated intrinsic apoptosis in colon adenocarcinoma (SW480) cells [132]. In conclusion, we found that citrus fruits can prevent the development of obesity induced by an HF diet and lowers hyperlipidemia.

18.4.4 Garlic

Allium sativum (garlic) is a well known food plant gaining popularity as hyperlipidemic as well as a hypoglycemic agent. It is the member of Liliacae family got attractiveness both as food and medicine for many years. It is reported to contain a variety of effective compounds such as sulfur containing compounds, trace minerals etc. [133]. The sulfur compounds found in garlic cloves are mainly two types present in equal amount S-alkylcysteine sulfoxides and the γ-glutamyl-S-alkylcysteines. The most abundant sulfur compound is allicin (detail in Section 18.3.5). A thorough literature study has revealed that it lowers cholesterol level and decrease lipid peroxidation. In an in vitro experimental evidence showing garlic components suppress LDL oxidation and short-term supplementation of garlic in human has exhibited inhibition of LDL oxidation [134]. Three hundred and sixty days randomized, single-blind, placebo controlled study of garlic supplementation was conducted on Type 2 diabetic patients. The results of the garlic treated group significantly decrease total cholesterol, LDL-C and increase HDL cholesterol compared to placebo treated group. This study suggests garlic possess cardioprotective activity [135]. In addition, garlic can prevent the aortic plaque formation on cholesterol-fed rabbits. Moreover, vascular calcification is inhibited in human patients with high blood cholesterol by supplementation with garlic extract [136]. Garlic is also used for its hypoglycemic activity. The probable mechanism of garlic's hypoglycemic effects is increased insulin oozing and sensitivity. Evidence suggests that garlic possess antioxidative, anti-inflammatory, and antiglycative properties [137]. Garlic supplementation has been shown to boost testosterone levels and decrease plasma

corticosterone, hormones associated with protein anabolism, in rats fed a high protein diet [138]. Thus, garlic may emerge as an effective medicine for diabetic obese patient.

18.4.5 Grains

Whole grain contains a number of bioactive constituents' e.g., dietary fiber, polyphenolic compounds, carotenoids, tocotrienols, tocopherols, phytoestrogens, and vitamins. Its consumption was declining body mass index in school children, even after taking high calorie diet [139]. It also possesses protective action against stroke and metabolic syndrome. Due to poor digestibility rice protein reduced mammals' body weight and lipid level by increasing lipolysis and decreasing lipogenesis [140]. Barley Flakes: of all the grains, ceryain forms of barley have among the lowest glycemic indexes. Pearled barley (glycemic index (GI) = 36) and cracked barley (GI = 72) have lower GI than sweet corn (GI = 78), rolled barley (GI = 94), and instant white rice (GI = 128). Barley is a low glycemic source of carbohydrates and a great source of fiber (1.5%), both of which are advantageous in maintaining good glucose levels and weight control [141]. Nuts (tree nuts and peanuts) are rich sources of nutrient such as minerals, protein, unsaturated fats, fiber, phytosterols, phenolics, and other bioactive compounds and they do not contribute to weight gain rather its consumption showed reduced coronary heart disease and gallstones incidences in both genders and diabetes in women [142]. Early life soya intake produces higher leptin and MCP-1 levels, which contribute to the prevention of obesity. The polyphenol-rich black soybean seed coat extract containing cyanidin 3-glucoside, catechins, and procyanidins, suppresses abdominal fat accumulation, plasma glucose level and enhances insulin sensitivity, UCP-1 and UCP-2 expression in HFD mice, to deter obesity and diabetes by enhancing energy expenditure and suppressing inflammation [143].

18.4.6 Punica

Punica granatum L. (pomegranate) is a fruit bearing plant is probably originated in Iran and now cultivated throughout India. Till date several researches have been performed for investigating of its antitumor, antibacterial, astringent, antidiarrheal, and antiobesity activities. The presence of a wide range of bioactive components in leaf, flower, seed, and juice of pomegranate may attribute to its antiobesity effects [144].

The fruit of *P. granatum* could be considered as a functional food because it has contained a number of bioactive compounds that display functional and medicinal effects. Gallic acid, ursolic acid, and oleanolic acid are the major metabolites found in pomegranate fruit extract (PFE) posses' antihyperlipidemic properties. Along with the secondary metabolites the peels are also a rich in complex polysaccharides, and minerals, including potassium, nitrogen, calcium, magnesium, phosphorus, and sodium [144]. The antihyperlipidemic potential of PFE has been investigated by many researchers and it has been observed that PFE consumption decrease hepatic triacylglycerol and fatty droplets content without altering total cholesterol and it lowers serum lipids and glucose levels by 18−25% [145]. In another study, endothelial NO synthase expression by pomegranate juice (PJ) and seed oil was studied in obese Zucker rats, a model of metabolic syndrome. Results indicated that PJ significantly decreased the expression of vascular inflammation markers, thrombospondin (TSP), and cytokine TGFβ1, whereas prominent downregulation of TSP-1 expression occurred by seed oil. Plasma nitrate and nitrite (NO (x)) levels were significantly increased by PJ [146]. These data emphasize promising clinical applications of PJ in metabolic disorder. The pomegranate seeds represent about 3% of the fruit weight. The oil constitutes 12−20% of the total seed weight contains a high concentration of fatty acids such as linoleic acid and linolenic acid, as well as other lipids, including punicic, oleic, other than lipids, decent amounts of proteins, fibers, vitamins and minerals, polyphenols, and isoflavones are present. Investigation on seed hypolipidemic profile Vroegrijk and coworker speculated that the administration of 1 g seed oil had decreased body weight and fat mass in male C57Bl/J6 mice for 12 weeks [147]. Vroegrijk et al. also reported that supplementation with pomegranate seed oil improved insulin sensitivity in HFD-fed mice. Hontecillas, studied the effect of catalpic acid in C57Bl/67 obese mice and observed the reduction in the white adipose tissue accumulation, triacylglycerol content as well as augmented HDL in plasma [148]. Numerous in vitro, animal, and human experiments have demonstrated the enormous potential benefits of pomegranate. Therefore, it is necessary to establish the therapeutic profile of all the constituents in the diet and commercialized forms to understand about the potential benefits of the pomegranate for the prevention of obesity and related disorders.

18.4.7 Tea

Tea is the most widely consumed aromatic beverage in worldwide. The variety of chemical compounds within this plant plays an important role in management of human health. The three categories of teas are black, green, and oolong-tea. Among these three types, black tea is mostly consumed. The antiobesity potential of black tea (*Camellia sinensis*) is due to the presence of

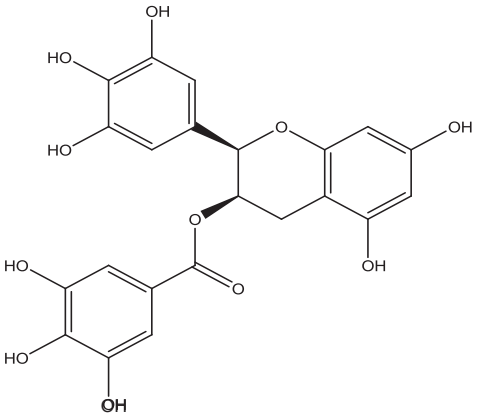
variety of polyphenolic compounds such as theaflavins, theaflavin 3-O-gallate, theaflavin 30-O-gallate, theaflavin 3, 30-O-gallate, epigallocatechin gallate (**24**), epicatechin gallate, catechins (**25**), quercetin glycosides, quinic acid, gallic acid and caffeine (**26**). These polyphenols inhibits pancreatic lipase thereby produces the antiobesity effect [149].

Green tea is another category of tea produced by the unfermented dried leaves of *C. sinensis*. Diverse mechanisms are involved behind the antiobesity effect of this tea. The main constituents of the green tea are the polyphenols like flavonols, flavones and flavon-3-ols, out of which flavan-3-ols also known as the catechins are the most widely found accounting to 60–80% of the polyphenols. This catechin helps in reducing metabolic syndrome and decrease the body weight of overweight/obese men, without affecting blood pressure or metabolic function biomarkers [150]. Catechin gallate is the strongest inhibitor of fatty acid synthase found in green tea. Green tea helps in increasing the energy utilization by increasing the potency of NE which is responsible for increasing energy utilization and fat oxidation. It also increases the lipolytic pathway, reduces adipose tissue and low-grade inflammation in HFD animal model. The other health beneficial role of green tea may be partly because of the caffeine present in it which increases energy utilization [151].

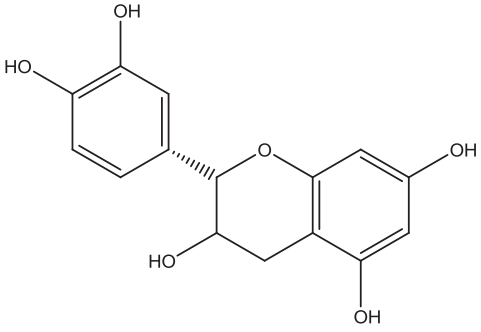
White tea is the category of tea produced from the unfermented young shoots of *C. sinensis*. The catechins level in this plant is more than green tea. It reduces blood triacylglycerols by increasing fecal lipids and reduces oxidative stress in the liver and adipose tissue without reducing food intake, body weight, visceral adiposity, and cholesterol lipoprotein profile [152].

Oolong tea is partially fermented tea. It may have some impact on increasing energy expenditure due to its catechin content. It also treats obesity, diabetes, atherosclerosis, high cholesterol, and skin allergies such as eczema; and boosts the immune system which is due to the presence of caffeine. Literature survey has revealed that three days of oolong tea consumption at five cups per day increases the resting metabolic rate 3–4%. This attributed to the increased fat oxidation, thereby decreasing the body fat stores [141].

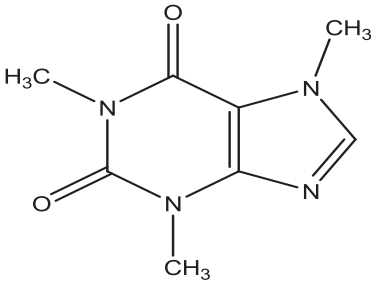
Epigallocatechin Gallate [24]



Catechin [25]



Caffeine [26]



18.5 MEDICINAL PLANT FOR TREATMENT OF OBESITY

Miscellaneous plant products such as plant protein, dietary fiber, prebiotic having the highest diversity in their properties which can reduce body weight and prevent diet-induced obesity. Therefore, in this section, we are discussing about antiobesity activity possesses plants and plant-derived products.

18.5.1 Plant Extract and Herbal Supplement

Several evidences suggested that not only isolated compounds from the plant, but crude plant extracts also possess desired therapeutic effects. Occasionally synergistic interaction of a plant's different groups of chemicals may improve the therapeutic activity in testing animals and also in humans. In Unani and Ayurvedic system of medicine, *Nigella sativa* (NS) seed extracts are used in the treatment of metabolic disorders, including dyslipidemia and obesity. The antiobesity activity of NS was clinically studied, which showed that the activity of different treatment group was not statistically significant but produced better activity than the control group. Results suggested that larger dose and longer duration of NS consumption will give better results [153]. In another clinical study, antihypertensive and hypolipidemic activity of *Hibiscus sabdariffa* extract was explored [154]. Furthermore, an acute and chronic effect of *Opuntia ficus-indica* extract was investigated on obese prediabetic patients. The result shows acute blood glucose lowering effects among the treatment group [155]. *Carum carvi* L. (caraway), an effective medicinal plant, is conventionally recommended for treating obesity. A randomized, triple-blind, placebo-controlled clinical trial on obese women suggests that caraway can successfully be used for obesity control in women without having any side effects [156]. Snacking is an uncontrolled eating habit, resulting in weight gain and obesity. Satiereal (Inoreal Ltd, Plerin, France) is an oral dietary supplement containing *Crocus sativus* L. (saffron) extract that may reduce snacking and enhance satiety through its suggested mood-improving effect. In addition, researchers are postulating that antioxidant rich compounds of saffron, e.g., crocins, picrocrocins, and safranal, may exert potential antiobesity activity but sufficient research has not yet been done [82].

Herbal supplements are nonfood substances intended to supplement the diet, contain one or more dietary ingredients (including vitamins, minerals, herbs, or other botanicals, amino acids, and certain other substances) or their constituents, and are generally available in forms such as tablet, capsule, powder, softgel, gelcap, or liquid. Herbal supplements and diet-based therapies for weight loss are among the most common in complementary and alternative medicine modalities. Additionally, traditional system of medicine used polyherbal preparation (combination of two or more plants) for the purpose of enhancement of effects. In this context, researchers have been examining the synergistic interaction of therapeutically active plant extracts in combinations; positive results are obtained which shows combination can increase or decrease the individual therapeutic activity or toxicity. While the exact mechanism of action of a combination of herbal preparation is not yet explored, a number of published data indicate that the herbal extract in combination being more efficacious than a single dose of one of its components alone. A combination of *Citrus pinnatifida* fruit and *Citrus unshiu* peel extracts shows superior antiobesity effects of HFD-induced obese rats [157]. A randomized, double blind, placebo-controlled clinical trial of herbal supplement (combination of *Asparagus officinalis* (*C. sinensis*), black tea, *Guarana Paullinia* cupana (guarana), *Phaseolus vulgaris* (kidney bean), *Garcinia cambogia* and chromium yeast) shows significant changes of the Body Composition Improvement Index and decrease in body fat in herbal supplement subjects compared to placebo [158]. In a recent study, Chinese herbal supplement (RCM-104) was examined for the management of simple obesity. A double-blind, randomized, placebo-controlled trial result studied RCM-104 and it was seen to be well tolerated and beneficial in reducing body weight and BMI in obese subjects [159].

18.5.2 Plant-derived Proteins

Some plants are good sources of protein, e.g., whole grains, soy, legumes, nuts, fruits, and seeds. We can get sufficient essential amino acids by eating a variety of plant proteins. Dietary proteins are considered to increase thermogenesis and satiety that may help in the prevention of obesity. Stem bromelain is a proteolytic enzyme obtained from *Ananas comosus* (pineapple) and this showed inhibitory effects on 3T3-L1 adipocyte differentiation. It may be attributed to antiobesity activity by suppressing the PPARγ-regulated adipogenesis pathway and by augmenting TNF-α-induced lipolysis and apoptosis in mature 3T3-L1 adipocytes [70]. Soy protein (SP) is an important component of soybeans and provides an abundant source of dietary protein. The antiobesity activity of dietary SP has been investigated in Wistar fatty rats. Results suggested that SP efficiently reduced the body weight of fatty rats by suppressing the lipogenic enzyme gene. In addition, this effect of SP has been examined on genetically modified obese rodents by Aoyama and coauthors [160]. Experimental results indicate that SP significantly

decreased body weight and plasma glucose level. Consequent research on SP recognized that it is a suitable protein source in energy-restricted diets for the treatment of obesity. Sixty days randomized single-blind study compared the effects of soy protein and pork-meat protein and carbohydrate diet on 24-h energy expenditure. The 24-h energy expenditure was higher with the pork followed by soy and then carbohydrate diet. The result suggests that soy has a greater thermogenic effect, than a carbohydrate diet which may be relevant for the prevention and treatment of obesity. Allison and coworkers [161] performed a 12-week randomized controlled trial of a low calorie soy-based meal replacement program in 100 obese subjects. Soy-based dietary formula was effective in lowering body weight, fat mass, and decreasing LDL cholesterol level in serum. However, long-term effects of dietary soy protein on obesity have not yet revealed. Further research is required to identify bioactive protein from plant sources that may play an important role against metabolic disorders.

18.5.3 Dietary Fiber and Prebiotics

Regulation of energy intake can be controlled by increased intake of dietary fiber (DF). DFs are nondigestible and nonstarch polysaccharides derived from plants. An increased intake of DF is useful in the management of obesity and diabetes. The obesity management potential is related to its unique physical and chemical properties, which aid in the early signals of satiation and/or prolonged signals of satiety. Sufficient amount of fiber in diet controls satiety via diverse mechanisms which includes cut-off in excessive food intake and deposits of fat accumulation, lowering the energy density of the diet, increasing sensory exposure time to a food in the oral cavity, slowing down gastric emptying, modifying the postprandial glucose response, promoting intestinal satiety, and changing neural and humoral signals in the gut [162]. Pectin, β-glucan, xylan, arabinoxylan, insulin, resistant starch, and guar gum are some of the examples of DFs which are beneficial in obesity. Guar gum slows down the gastric emptying and highly viscous fibers like oat reduce fasting glucose levels and elevated LDL-cholesterol level without changing the HDL fraction [163]. Whole-grain cereals significantly lower the risk of obesity, diabetes, coronary heart disease, stroke, hypertension, gastrointestinal diseases, and boosts the immune system and stimulates the growth of beneficial microbes in the colon. High amylose maize resistant starch (RS) is effective in treating obesity. RS supplementation (15–30 g/day) improved insulin sensitivity in overweight and obese subjects, thereby helping in the alleviation of complications associated with insulin resistance. Wheat arabinoxylan supplementation decreased adiposity, body weight gain, serum, and hepatic cholesterol and insulin resistance to HFD-induced obese mice [70].

Prebiotics are nondigestible oligosaccharides that pass undigested through the upper gastrointestinal tract and stimulate the growth and/or activity of advantageous bacteria. They are produced by enzymatic hydrolysis of polysaccharides or by transglycosylations. Gut microbiota (namely prebiotics) plays a nutritional role in the management of obesity by inducing a host response, controlling the gut's barrier, and endocrine functions [164]. Prebiotics like fructooligosaccharides, galactooligosaccharides, and lactulose have already been approved by the European Union. The effectiveness of some prebiotics is yet to be established. Supplementation of inulin (a type of fructans found in onion, banana, chicory, and artichokes) in the diet reduces liver and abdominal fat weight, enhances satiety, decreases energy intake, and regulates body weight in human and animal studies [70]. Therefore, proper and careful dietary manipulations can help in the prevention or management of obesity and other degenerative disorders.

18.6 PROSPECT OF PHYTOCHEMICALS, FOODS AND BOTANICALS IN OBESITY MANAGEMENT

Published data indicate that phytochemicals play a promising role for the treatment of obesity and its associated metabolic diseases. Several in vivo studies have repeatedly indicated that the intake of some phytochemicals could inhibit HFD-induced obesity in mice, hamsters, rats, or even humans [53]. The adipose tissue mass can be scaled down by inhibiting adipogenesis and/or inducing apoptosis in adipocytes. The pathway is important for the investigation of mode of action that natural products exert on antiobesity activity as well as for defining the strategies for future investigation.

Ghrelin is the only known circulating orexigenic hormone. Its effect on obesity is associated with increased appetite and food intake while reducing energy expenditure. Blocking ghrelin's action via a decrease in ghrelin secretion in the stomach may provide a promising target for antiobesity drug development programs [16]. A randomized, double-blind, placebo-controlled clinical trial on obese women was done and it was found that green tea extract significantly reduced the obesity-related hormone peptides such as adiponectin and ghrelin [165].

An effective weight-management product should provide improvements in blood pressure, lipids, glycemia, or other beneficial outcomes that are commensurate with the degree of weight loss [166]. Body fat

homeostasis typically balances body's energy regulation. Thus, drugs which are acting on either energy intake or expenditure fail to make the desired outcome after long-term treatment because compensatory mechanism balances the body weight. In the time to come, it is possible that plant derivatives, herbal supplement, and phytochemicals, combat the problem by their multiple mechanisms and emerge as effective antiobesity drugs.

18.7 CONCLUSION

Herbal food and plant-derived phytoconstituents along with regular exercise may provide efficient control over weight gain. However, there remains a heavy heap of research and understanding about the herb–drug interaction, pharmacokinetic measures, toxicological limitation, and beneficial effect of combination therapy. Progressive research on the recognition of new phytochemicals, functional food, or botanicals could be relevant for future advanced therapies and preventive steps to develop safe and effective therapeutics for obesity.

Acknowledgment

The authors wish to express their gratitude to Indian Council of Medical Research (File-45/43/2010/BMS/TRM) Govt. of India, New Delhi, for providing financial support through Research Associateship to Kakali Mukherjee.

References

[1] Gummesson A. Pathogenesis of obesity and effects of treatment. Västra Frölunda (Sweden): Intellecta Infolog AB; 2009. p. 4–42.
[2] Kim GW, Lin JE, Blomain ES, Waldman SA. Antiobesity pharmacotherapy: new drugs and emerging targets. Clin Pharmacol Ther 2014;95:53–66.
[3] Chandrasekaran CV, Vijayalakshmi MA, Prakash K, Bansal VS, Meenakshi J, Amit A. Review article: herbal approach for obesity management. Am J Plant Sci 2012;3:1003–14.
[4] Mukherjee PK. Plant products with hypocholesterolemic potentials. In: Taylor SL, editor. Advances in food and nutrition research, 47. Elsevier Academic Press; 2003. p. 175–324.
[5] Verma RK, Paraidathathu T. Herbal medicines used in the traditional indian medicinal system as a therapeutic treatment option for overweight and obesity management: a review. Int J Pharm Pharm Sci 2014;6:40–7.
[6] Kaila B, Raman M. Obesity: a review of pathogenesis and management strategies. Can J Gastroenterol 2008;22:61–8.
[7] Degen L, Oesch S, Casanova M, Graf S, Ketterer S, Drewe J, et al. Effect of peptide YY3-36 on food intake in humans. Gastroenterology 2005;129:1430–6.
[8] Mizuno TM, Kelley KA, Pasinetti GM, Roberts JL, Mobbs CV. Transgenic neuronal expression of proopiomelanocortin attenuates hyperphagic response to fasting and reverses metabolic impairments in leptin-deficient obese mice. Diabetes 2003;52:2675–83.
[9] Yun JW. Possible anti-obesity therapeutics from nature - a review. Phytochemistry 2010;71:1625–41.
[10] Cowherd RM, Lyle RE, McGehee Jr RE. Molecular regulation of adipocyte differentiation. Semin Cell Dev Biol 1999;10:3–10.
[11] Lefterova MI, Lazar MA. New developments in adipogenesis. Trends Endocrin Met 2009;20:107–14.
[12] Halford JC, Blundell JE. Pharmacology of appetite suppression. Prog Drug Res 2000;54:25–58.
[13] Chantre P, Lairon D. Recent findings of green tea extract AR25 (exolise) and its activity for the treatment of obesity. Phytomedicine 2002;9:3–8.
[14] Bays HE. Current and investigational antiobesity agents and obesity therapeutic treatment targets. Obes Res 2004;12:1197–211.
[15] Dietrich MO, Horvath TL. Limitations in anti-obesity drug development: the critical role of hunger-promoting neurons. Nat Rev Drug Discovery 2012;11:675–91.
[16] Colon-Gonzalez F, Kim GW, Lin JE, Valentino MA, Waldman SA. Obesity pharmacotherapy: what is next? Mol Aspects Med 2013;34:71–83.
[17] Vivus. FDA issues complete response letter to vivus regarding new drug application for Qnexa(R). 2010. http://ir.vivus.com/releasedetail.cfm?ReleaseID=524576. July 1, 2011.
[18] Park T, Kim Y. Phytochemicals as potential agents for prevention and treatment of obesity and metabolic diseases. Anti Obes Drug Discovery Dev 2011;1:1–48.
[19] Hsu CL, Yen GC. Introduction of cell apoptosis in 3T3-L1 preadipocytes by flavonoids is associated with antioxidant activity. Mol Nutr Food Res 2006;50:1072–9.
[20] Bak EJ, Kim J, Jang S, Woo GH, Yoon HG, Yoo YJ, et al. Gallic acid improves glucose tolerance and triglyceride concentration in diet-induced obesity mice. Scand J Clin Lab Invest 2013;73:607–14.
[21] Hsu CL, Wu CH, Huang SL, Yen GC. Phenolic compounds rutin and o-coumaric acid ameliorate obesity induced by high-fat diet in rats. J Agric Food Chem 2009;57:425–31.
[22] Kang SW, Kang SI, Shin HS, Yoon SA, Kim JH, Ko HC, et al. *Sasa quelpaertensis* Nakai extract and its constituent *p*-coumaric acid inhibit adipogenesis in 3T3-L1 cells through activation of the AMPK pathway. Food Chem Toxicol 2013;59:380–5.
[23] Bocco BMLC, Lorena FB, Fernandes GW, Lancelloti CLP, Cysneiros RM, Ribeiro MO. Caffeic acid and ferulic acid improves diet induced metabolic syndrome on mice but induces inflammatory process in white adipose tissue. FASEB J 2013;27.630.9.
[24] Son MJ, Rico CW, Nam SH, Kang MY. Influence of oryzanol and ferulic acid on the lipid metabolism and antioxidative status in high fat-fed mice. J Clin Biochem Nutr 2010;46:150–6.
[25] Cho AS, Jeon SM, Kim MJ, Yeo J, Seo KI, Choi MS, et al. Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. Food Chem Toxicol 2010;48:937–43.
[26] Yoshida H, Watanabe W, Oomagari H, Tsuruta E, Shida M, Kurokawa M. Citrus flavonoid naringenin inhibits TLR2 expression in adipocytes. J Nutr Biochem 2013;24:1276–84.
[27] Richard AJ, Amini-Vaughan Z, Ribnicky DM, Stephens JM. Naringenin inhibits adipogenesis and reduces insulin sensitivity and adiponectin expression in adipocytes. Evid Based Complement Alternat Med 2013;2013:10. Article ID 549750.
[28] Gao M, Ma Y, Liu D. Rutin suppresses palmitic acids-triggered inflammation in macrophages and blocks high fat diet-induced obesity and fatty liver in mice. Pharm Res 2013;30:2940–50.
[29] Alam MA, Kauter K, Brown L. Naringin improves diet-induced cardiovascular dysfunction and obesity in high carbohydrate, high fat diet-fed rats. Nutrients 2013;5:637–50.

[30] Leray V, Freuchet B, Le Bloc'h J, Jeusette I, Torre C, Nguyen P. Effect of citrus polyphenol- and curcumin-supplemented diet on inflammatory state in obese cats. Br J Nutr 2011;1: S198–201.
[31] Chang CJ, Tzeng TF, Liou SS, Chang YS, Liu IM. Myricetin increases hepatic peroxisome proliferator-activated receptor α protein expression and decreases plasma lipids and adiposity in rats. Evid Based Complement Alternat Med 2012;2012:11. Article ID 787152.
[32] Crespillo A, Alonso M, Vida M, Pavón FJ, Serrano A, Rivera P, et al. Reduction of body weight, liver steatosis and expression of stearoyl-CoA desaturase 1 by the isoflavone daidzein in diet-induced obesity. Br J Pharmacol 2011;164:1899–915.
[33] Dolinoy DC, Weidman JR, Waterland RA, Jirtle RL. Maternal genistein alters coat color and protects A^{vy} mouse offspring from obesity by modifying the fetal epigenome. Environ Health Perspect 2006;114:567–72.
[34] Kim MH, Park JS, Seo MS, Jung JW, Lee YS, Kang KS. Genistein and daidzein repress adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells via Wnt/β-catenin signalling or lipolysis. Cell Prolif 2010;43:594–605.
[35] Halder D, Das ND, Jung KH, Choi MR, Kim MS, Lee SR, et al. Cyclodextrin-clathrated limonin suppresses diet-induced obesity in mice. J Food Biochem 2013;38:216–26.
[36] Kim HY, Park M, Kim K, Lee YM, Rhyu MR. Hesperetin stimulates cholecystokinin secretion in enteroendocrine STC-1 cells. Biomol Ther (Seoul) 2013;21:121–5.
[37] Skrzypczak-Jankun E, Jankun J. Theaflavin digallate inactivates plasminogen activator inhibitor: could tea help in alzheimer's disease and obesity? Int J Mol Med 2010;26:45–50.
[38] Lin CL, Huang HC, Lin JK. Theaflavins attenuate hepatic lipid accumulation through activating AMPK in human HepG2 cells. J Lipid Res 2007;48:2334–43.
[39] Jin D, Xua Y, Meia X, Menga Q, Gaoa Y, Lia B, et al. Antiobesity and lipid lowering effects of theaflavins on high-fat diet induced obese rats. J Funct Foods 2013;5:1142–50.
[40] Schirmer MA, Phinney SD. Gamma-linolenate reduces weight regain in formerly obese humans. J Nutr 2007;137:1430–5.
[41] Moon HS, Lee HG, Seo JH, Chung CS, Kim TG, Kim IY, et al. Down-regulation of PPARγ2-induced adipogenesis by PEGylated conjugated linoleic acid as the pro-drug: attenuation of lipid accumulation and reduction of apoptosis. Arch Biochem Biophys 2006;456:19–29.
[42] Niu CS, Yeh CH, Yeh MF, Cheng JT. Increase of adipogenesis by ginsenoside (Rh2) in 3T3-L1 cell via an activation of glucocorticoid receptor. Horm Metab Res 2009;41:271–6.
[43] Siraj FM, Sathishkumar N, Kim YJ, Kim SY, Yang DC. Ginsenoside F2 possesses anti-obesity activity via binding with PPARγ and inhibiting adipocyte differentiation in the 3T3-L1 cell line. J Enzyme Inhib Med Chem 2014. http://dx.doi.org/10.3109/14756366.2013.871006.
[44] Park MW, Ha J, Chung SH. 20(S)-ginsenoside Rg3 enhances glucose-stimulated insulin secretion and activates AMPK. Biol Pharm Bull 2008;31:748–51.
[45] Hwang JT, Lee MS, Kim HJ, Sung MJ, Kim HY, Kim MS, et al. Antiobesity effect of ginsenoside Rg3 involves the AMPK and PPARγ signal pathways. Phytother Res 2009;23:262–6.
[46] Sung HY, Kang SW, Kim JL, Li J, Lee ES, Gong JH, et al. Oleanolic acid reduces markers of differentiation in 3T3-L1 adipocytes. Nutr Res 2010;30:831–9.
[47] de Melo CL, Queiroz MG, Fonseca SG, Bizerra AM, Lemos TL, Melo TS, et al. Oleanolic acid, a natural triterpenoid improves blood glucose tolerance in normal mice and ameliorates visceral obesity in mice fed a high-fat diet. Chem Biol Interact 2010;185: 59–65.
[48] Kazmi I, Afzal M, Rahman S, Iqbal M, Imam F, Anwar F. Antiobesity potential of ursolic acid stearoyl glucoside by inhibiting pancreatic lipase. Eur J Pharmacol 2013;709:28–36.
[49] He Y, Li Y, Zhao T, Wang Y, Sun C. Ursolic acid inhibits adipogenesis in 3T3-L1 adipocytes through LKB1/AMPK pathway. PLoS One 2013;8:1–12.
[50] Hu Y, Davies GE. Berberine inhibits adipogenesis in high-fat diet-induced obesity mice. Fitoterapia 2010;81:358–66.
[51] Zhang X, Zhao Y, Zhang M, Pang X, Xu J, Kang C,M, et al. Structural changes of gut microbiota during berberine-mediated prevention of obesity and insulin resistance in high-fat diet-fed rats. PLoS One 2012;7:1–12.
[52] Baek S, Chung H, Lee H, D'Souza R, Jeon Y, Kim H, et al. Treatment of obesity with the resveratrol-enriched rice DJ-526. Sci Reports 2014;4:1–5.
[53] Williams DJ, Edwards D, Hamernig I, Jian L, James AP, Johnson SK, et al. Vegetables containing phytochemicals with potential anti-obesity properties: a review. Food Res Int 2013;52:323–33.
[54] Park HJ, Yang JY, Amabati S. Combined effects of genistein, quercetin and resveratrol in human and 3T3-L1 adipocytes. J Med Food 2008;11:773–83.
[55] Jayaprakasam B, Vareed SK, Olson,. LK, Nair MG. Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. J Agric Food Chem 2005;53:28–31.
[56] Goto T, Takahashi N, Hirai S, Kawada T. Various terpenoids. Derived from herbal and dietary plants function as PPAR modulators and regulate carbohydrate and lipid metabolism. PPAR Res 2010;10:1–9.
[57] Suzuki K, Inoue T, Hioki R, Ochiai J, Kusuhara Y, Ichino N, et al. Association of abdominal obesity with decreased serum levels of carotenoids in a healthy Japanese population. Clin Nutr 2006;25: 780–9.
[58] Elkayam A, Mirelman D, Peleg E, Wilchek M, Miron T, Rabinkov A, et al. The effects of allicin on weight in fructose-induced hyperinsulinemic, hyperlipidemic, hypertensive rats. Am J Hypertens 2003;16:1053–6.
[59] Yang JY, Della-Fera MA, Nelson-Dooley C, Baile CA. Molecular mechanisms of apoptosis induced by ajoene in 3T3-L1 adipocytes. Obesity 2006;14:388–97.
[60] Choi KM, Lee YS, Kim W, Kim SJ, Shin KO, Yu JY, et al. Sulforaphane attenuates obesity by inhibiting adipogenesis and activating the AMPK pathway in obese mice. J Nutr Biochem 2014; 25:201–7.
[61] Marangoni F, Poli A. Phytosterols and cardiovascular health. Pharmacol Res 2010;61:193–9.
[62] Jesch ED, Lee JY, Carr TP. Dietary plant sterols regulate genes involved in cholesterol metabolism in mouse liver but not intestine. FASEB J 2008;22:700–35.
[63] Izar MC, Tegani DM, Kasmas SH, Fonseca FA. Phytosterols and phytosterolemia: gene–diet interactions. Genes Nutr 2011;6:17–26.
[64] Wang T, Choi RC, Li J, Li J, Bi CW, Zang L, et al. Anti-hyperlipidemic effect of protodioscin, an active ingredient isolated from the rhizomes of *Dioscorea nipponica*. Planta Med 2010;76:1642–6.
[65] Uemura T, Goto T, Kang MS, Mizoguchi N, Hirai S, Lee JY, et al. Diosgenin, the main aglycon of fenugreek, inhibits LXRα activity in HepG2 cells and decreases plasma and hepatic triglycerides in obese diabetic mice. J Nutr 2011;141:17–23.
[66] Son IS, Kim JH, Sohn HY, Son KH, Kim JS, Kwon CS. Antioxidative and hypolipidemic effects of diosgenin, a steroidal saponin of yam (*Dioscorea* spp.), on high-cholesterol fed rats. Biosci Biotechnol Biochem 2007;71:3063–71.
[67] Yang JY, Della-Fera MA, Baile CA. Guggulsterone inhibits adipocyte differentiation and induces apoptosis in 3T3-L1 cells. Obesity 2008;16:16–22.

[68] Rayalam S, Della-Fera MA, Ambati S, Boyan B, Baile CA. Enhanced effects of guggulsterone plus 1,25(OH)2D3 on 3T3-L1 adipocytes. Biochem Biophys Res Commun 2007;364:450–6.
[69] Sharma B, Salunke R, Srivastava S, Majumder C, Roy P. Effects of guggulsterone isolated from *Commiphora mukul* in high fat diet induced diabetic rats. Food Chem Toxicol 2009;47:2631–9.
[70] Baboota KR, Bishnoi M, Ambalam P, Kondepudia KK, Sarma MS, Boparaic KR, et al. Functional food ingredients for the management of obesity and associated co-morbidities – a review. J Funct Foods 2013;5:997–1012.
[71] Serrano J, Sánchez González I. Trends in functional foods against obesity: functional ingredients, technologically modified foods and full diets. Rev Esp Nutr Comunitaria 2008;14: 193–200.
[72] Trigueros L, Peña S, Ugidos AV, Sayas-Barberá E, Pérez-Álvarez JA, Sendra E. Food ingredients as anti-obesity agents: a review. Crit Rev Food Sci Nutr 2014;53:929–42.
[73] Karmase A, Birari R, Bhutani KK. Evaluation of anti-obesity effect of *Aegle marmelos* leaves. Phytomedicine 2013;20:805–12.
[74] Moon J, Do HJ, Kim OY, Shin MJ. Antiobesity effects of quercetin-rich onion peel extract on the differentiation of 3T3-L1 preadipocytes and the adipogenesis in high fat-fed rats. Food Chem Toxicol 2013;58:347–54.
[75] Malik ZA, Goyal A, Sharma PL. Mustard oil based high fat diet is associated with decreased body weight gain, less adiposity and improved glucose and lipid homeostasis in wistar rats. Asian J Clin Nutr 2011;3:43–52.
[76] Shin HD, Kim AR, Lee YM, Lee MY, Lee JJ. Effect of broccoli sprout on cholesterol-lowering and anti-obesity effects in rats fed high fat diet. J Korean Soc Food Sci Nutr 2009;38:309–18.
[77] Athesh K, Karthiga D, Brindha P. Anti-obesity effect of aqueous fruit extract of *Carica papaya* L. in rats fed on high fat cafeteria diet. Int J Pharm Pharm Sci 2012;4:327–30.
[78] Khan A, Safdar M, Khan MMA. Effect of various doses of cinnamon on lipid profile in diabetic individuals. Pak J Nutr 2003;2: 312–9.
[79] Etebu E, Nwauzoma AB. A review on sweet orange (*Citrus sinensis* L Osbeck): health, diseases and management. Am J Res Commun 2014;2:33–70.
[80] Shimoda H, Seki E, Aitani M. Inhibitory effect of green coffee bean extract on fat accumulation and body weight gain in mice. BMC Complement Altern Med 2006;6:9.
[81] Chithra V, Leelamma S. Hypolipidemic effect of coriander seeds (*Coriandrum sativum*): mechanism of action. Plant Foods Hum Nutr 1997;51:167–72.
[82] Mashmoul M, Azlan A, Khaza'ai H, Yusof BNM, Noor SM. Saffron: a natural potent antioxidant as a promising anti-obesity drug. Antioxidants 2013;2:293–308.
[83] Haque RM, Ansari HS, Najmi KA. *Cuminum cyminum* L. fruits distillate ameliorates the high fat diet-induced obesity. Pharmacogn Commun 2013;3:49–57.
[84] Lee YK, Lee WS, Hwang JT, Kwon DY, Surh YJ, Park OJ. Curcumin exerts anti-differentiation effect through AMP Kα-PPAR-γ in 3T3-L1 adipocytes and antiproliferatory effect through AMPKα-COX-2 in cancer cells. J Agric Food Chem 2009;57: 305–10.
[85] Sato R, Buesa LM, Nerurkar PV. Anti-obesity effects of *Emblica officinalis* (Amla) are associated with inhibition of nuclear transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ). FASEB J 2010;24:661–4.
[86] Azizian H, Rezvani ME, Esmaeilidehaj M, Bagheri SM. Anti-obesity, fat lowering and liver steatosis protective effects of Ferula asafoetida gum in type 2 diabetic rats: possible involvement of leptin. Iran J Diabetes Obes 2012;4:120–6.
[87] Garg C, Ansari SH, Khan SA, Garg M. Effect of *Foeniculum vulgare* Mill. Fruits in obesity and associated cardiovascular disorders demonstrated in high fat diet fed albino rats. J Pharm Biomed Sci. 2011;8:1–5.
[88] Gargouri Y, Julien R, Pieroni G, Verger R, Sarda L. Studies on the inhibition of pancreatic and microbial lipases by soybean proteins. J Lipid Res 1984;25:1214–21.
[89] Kumar V, Bhandari U, Tripathi CD, Khanna G. Evaluation of antiobesity and cardioprotective effect of *Gymnema sylvestre* extract in murine model. Indian J Pharmacol 2012;44:607–13.
[90] Nadeem S, Dhore P, Quazi M, Pawar S, Raj N. *Lagenaria siceraria* fruit extract ameliorate fat amassment and serum TNF-α in high-fat diet-induced obese rats. Asian Pac J Trop Med 2012;5: 698–702.
[91] Guo J, Li L, Pan J, Qiu G, Li A, Huang G, et al. Pharmacological mechanism of Semen Litchi on antagonizing insulin resistance in rats with type 2 diabetes. Zhong Yao Cai 2004;27:435–8.
[92] Aprikian O, Busserolles J, Manach C, Mazur A, Morand C, Davicco MJ, et al. Lyophilized apple counteracts the development of hypercholesterolemia, oxidative stress, and renal dysfunction in obese zucker rats. J Nutr 2002;132:1969–76.
[93] Kobayashi M, Matsui-Yuasa I, Fukuda-Shimizu M, Mandai Y, Tabuchi M, Munakata H, et al. Effect of mango seed kernel extract on the adipogenesis in 3T3-L1 adipocytes and in rats fed a high fat diet. Health 2013;5:9–15.
[94] Xu J, Cao K, Li Y, Zou X, Chen C, Szeto IM, et al. Bitter gourd inhibits the development of obesity-associated fatty liver in C57BL/6 mice fed a high-fat diet. J Nutr 2014;144:475–83.
[95] Bao B, Chen Y, Zhang L, Xu YLN, Wang X, Liu J, et al. *Momordica charantia* (bitter melon) reduces obesity-associated macrophage and mast cell infiltration as well as inflammatory cytokine expression in adipose tissues. 2013;8:1–12.
[96] Mbikay M. Therapeutic potential of *Moringa oleifera* leaves in chronic hyperglycemia and dyslipidemia: a review. Front Pharmacol 2012;3:1–12.
[97] Birari R, Javia V, Bhutani KK. Antiobesity and lipid lowering effects of *Murraya koenigii* (L.) spreng leaves extracts and mahanimbine on high fat diet induced obese rats. Fitoterapia 2010; 81:1129–33.
[98] Nguyen PH, Le TV, Kang HW, Chae J, Kim SK, Kwon KI, et al. AMP-activated protein kinase (AMPK) activators from *Myristica fragrans* (nutmeg) and their anti-obesity effect. Bioorg Med Chem Lett 2010;20:4128–31.
[99] Obiro WC, Zhang T, Jiang B. The nutraceutical role of the *Phaseolus vulgaris* a-amylase inhibitor. Br J Nutr 2008;100:1–12.
[100] Rai PK, Mehta S, Watal G. Hypolipidaemic and hepatoprotective effects of *Psidium guajava* raw fruit peel in experimental diabetes. Indian J Med Res 2010;131:820–4.
[101] Maruyama C, Kikuchi N, Masuya Y, Hirota S, Araki R, Maruyama T. Effects of green-leafy vegetable intake on postprandial glycemic and lipidemic responses and α-tocopherol concentration in normal weight and obese men. J Nutr Sci Vitaminol (Tokyo) 2013;59:264–71.
[102] Yoon SS, Rhee YH, Lee HJ, Lee EO, Lee MH, Ahna KS, et al. Uncoupled protein 3 and p38 signal pathways are involved in anti-obesity activity of *Solanum tuberosum* L. cv. Bora Valley. J Ethnopharmacol 2008;118:396–404.
[103] Jung CH, Ahn J, Jeon T, Kim WT, Ha TY. *Syzygium aromaticum* ethanol extract reduces high-fat diet-induced obesity in mice through downregulation of adipogenic and lipogenic gene expression. Exp Ther Med 2012;4:409–14.
[104] Azman KF, Amom Z, Azlan A, Esa NM, Ali RM, Shah ZM, et al. Antiobesity effect of *Tamarindus indica* L. pulp aqueous extract in high-fat diet-induced obese rats. J Nat Med 2012;66: 333–42.
[105] Rathore HS, Soni S, Bhatnagar D. Hypocholesterolemic effect of *Terminalia chebula* fruit (Myrobalan) in mice. Anc Sci Life 2004;23: 11–5.

[106] Rai PK, Gupta SK, Srivastava AK, Gupta RK, Watal G. A scientific validation of anti-hyperglycemic and anti-hyperlipidemic attributes of *Trichosanthes dioica*. ISRN Pharmacol; 2013:1–7. http://dx.doi.org/10.1155/2013/473059.
[107] Kumar P, Bhandari U. Protective effect of *Trigonella foenum-graecum* Linn. on monosodium glutamate-induced dyslipidemia and oxidative stress in rats. Indian J Pharmacol 2013;45:136–40.
[108] OH J, Jung S, Lee Y, Park KW, Kim SY, Han J. Antioxidant and antiobesity activities of seed extract from campbell early grape as a functional ingredient. J Food Process Preserv 2013;37: 291–8.
[109] Zhang XH, Huang B, Choi SK, Seo JS. Anti-obesity effect of resveratrol-amplified grape skin extracts on 3T3-L1 adipocytes differentiation. Nutr Res Pract 2012;6:286–93.
[110] Li Y, Tran VH, Duke CC, Roufogalis BD. Preventive and protective properties of *Zingiber officinale*(ginger) in diabetes mellitus, diabetic complications, and associated lipid and other metabolic disorders: a brief review. Evid Based Complement Alternat Med 2012;2012. Article ID 516870.
[111] Kubota H, Morii R, Kojima-Yuasa A, Huang X, Yano Y, Matsui-Yuasa I. Effect of *Zizyphus jujuba* extract on the inhibition of adipogenesis in 3T3-L1 preadipocytes. Am J Chin Med 2009;37: 597–608.
[112] Udani JK, Singh BB, Singh VJ, Barrett ML. Effects of acai (*Euterpe oleracea* Mart.) berry preparation on metabolic parameters in a healthy overweight population: a pilot study. Nutr J 2011;10: 45–50.
[113] Kaume L, Gilbert WC, Brownmiller C, Howard LR, Devareddy L. Cyanidin 3-O-β-d-glucoside-rich blackberries modulate hepatic gene expression, and anti-obesity effects in ovariectomized rats. J Funct Foods 2012;4:480–8.
[114] Bordonaba JG, Chope GA, Terry LA. Terry maximising blackcurrant anthocyanins: temporal changes during ripening and storage in different genotypes. J Berry Res 2010;1:73–80.
[115] Moghe SS, Juma S, Imrhan V, Vijayagopal P. Effect of blueberry polyphenols on 3T3-F442A preadipocyte differentiation. J Med Food 2012;15:448–52.
[116] Lehtonen HM, Suomela JP, Tahvonen R, Yang B, Venojärvi M, Viikari J, et al. Different berries and berry fractions have various but slightly positive effects on the associated variables of metabolic diseases on overweight and obese women. Eur J Clin Nutr 2011;65:394–401.
[117] Suzuki R, Tanaka M, Takanashi M, Hussain A, Yuan B, Toyoda H, et al. Anthocyanidins-enriched bilberry extracts inhibit 3T3-L1 adipocyte differentiation via the insulin pathway. Nutr Metab 2011;8:14–9.
[118] Qin B, Anderson RA. An extract of chokeberry attenuates weight gain and modulates insulin, adipogenic and inflammatory signalling pathways in epididymal adipose tissue of rats fed a fructose-rich diet. Br J Nutr 2012;108:581–7.
[119] Peng CH, Liu LK, Chuang CM, Chyau CC, Huang CN, Wang CJ. Mulberry water extracts possess an antiobesity effect and ability to inhibit hepatic lipogenesis and promote lipolysis. J Agric Food Chem 2011;59:2663–71.
[120] Morimoto C, Satoh Y, Hara M, Inoue S, Tsujita T, Okuda H. Anti-obese action of raspberry ketone. Life Sci 2005;77:194–204.
[121] Lee JH, Cho HD, Jeong JH, Lee MK, Jeong YK, Shim KH, et al. New vinegar produced by tomato suppresses adipocyte differentiation and fat accumulation in 3T3-L1 cells and obese rat model. Food Chem 2013;141:3241–9.
[122] Baek J, Lee J, Kim K, Kim T, Kim D, Kim C, et al. Inhibitory effects of *Capsicum annuum* L. water extracts on lipoprotein lipase activity in 3T3-L1 cells. Nutr Res Pract 2013;7:96–102.
[123] Westerterp-Plantenga MS, Smeets A, Lejeune MP. Sensory and gastrointestinal satiety effects of capsaicin on food intake. Int J Obes (Lond) 2005;29:682–8.
[124] Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, et al. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 2000;288: 306–13.
[125] Watanabe T, Kawada T, Kurosawa M, Sato A, Iwai K. Adrenal sympathetic efferent nerve and catecholamine secretion excitation caused by capsaicin in rats. Am J Physiol 1988;255:E23–7.
[126] Ann JY, Lee MS, Joo H, Kim CT, Kim Y. Reduction of body weight by capsaicin is associated with inhibition of glycerol-3-phosphate dehydrogenase activity and stimulation of uncoupling protein 2 mRNA expression in diet-induced obese rats. J Food Sci Nutr 2011;16:210–6.
[127] Janssens PLHR, Hursel R, Martens EAP, Westerterp-Plantenga MS. Acute effects of capsaicin on energy expenditure and fat oxidation in negative energy balance. PLoS One 2013;8: 1–7.
[128] Kim JY, Lee MS, Jung S, Joo H, Kim CT, Kim IH, et al. Anti-obesity efficacy of nanoemulsion oleoresin capsicum in obese rats fed a high-fat diet. Int J Nanomed 2014;9:301–10.
[129] Calapai G, Firenzuoli F, Saitta A, Squadrito F, Arlotta MR, Costantino G, et al. Antiobesity and cardiovascular toxic effects of *Citrus aurantium* extracts in the rat: a preliminary report. Fitoterapia 1999;70:586–92.
[130] Kang SI, Shin HS, Kim HM, Hong YS, Yoon SA, Kang SW, et al. Immature *Citrus sunki* peel extract exhibits antiobesity effects by β-oxidation and lipolysis in high-fat diet-induced obese mice. Biol Pharm Bull 2012;35:223–30.
[131] Ono E, Inoue J, Hashidume T, Shimizu M, Sato R. Anti-obesity and anti-hyperglycemic effects of the dietary *Citrus limonoid* nomilin in mice fed a high-fat diet. Biochem Biophys Res Commun 2011;410:677–81.
[132] Murthy CKN, Jayaprakasha GK, Kumar V, Rathore KS, Patil BS. *Citrus limonin* and its glucoside inhibit colon adenocarcinoma cell proliferation through apoptosis. J Agric Food Chem 2011; 59:2314–23.
[133] Pratt DA. Garlic and other alliums. The lore and the science. By Eric Block. Angew Chem Int Ed 2010;49:7162.
[134] Lau BH. Suppression of LDL oxidation by garlic compounds is a possible mechanism of cardiovascular health benefit. J Nutr 2006;136:765S–8S.
[135] Ashraf R, Aamir K, Shaikh AR, Ahmed T. Effects of garlic on dyslipidemia in patients with type 2 diabetes mellitus. J Ayub Med Coll Abbottabad 2005;17:60–4.
[136] Durak I, Kavutcu M, Aytaç B, Avci A, Devrim E, Ozbek H, et al. Effects of garlic extract consumption on blood lipid and oxidant/antioxidant parameters in humans with high blood cholesterol. J Nutr Biochem 2004;15:373–7.
[137] Liu CT, Sheen LY, Lii CK. Does garlic have a role as an antidiabetic agent? Mol Nutr Food Res 2007;51:1353–64.
[138] Oi Y, Imafuku M, Shishido C, Kominato Y, Nishimura S, Iwai K. Garlic supplementation increases testicular testosterone and decreases plasma corticosterone in rats fed a high protein diet. J Nutr 2001;131:2150–6.
[139] Choumenkovitch SF, McKeown NM, Tovar A, Hyatt RR, Kraak VI, Hastings AV, et al. Whole grain consumption is inversely associated with BMI *Z*-score in rural school-aged children. Public Health Nutr 2013;16:212–8.
[140] Yang L, Chen JH, Lv J, Wu Q, Xu T, Zhang H, et al. Rice protein improves adiposity, body weight and reduces lipids level in rats through modification of triglyceride metabolism. Lipids Health Dis 2012;11:24–9.
[141] Choudhary M, Grover K. Development of functional food products in relation to obesity. Funct Foods Health Dis 2012;2:188–97.
[142] Mohamed S. Functional foods against metabolic syndrome (obesity, diabetes, hypertension and dyslipidemia) and cardiovasular disease. Trends Food Sci Technol 2013;35:114–28.

[143] Kanamoto Y, Yamashita Y, Nanba F, Yoshida T, Tsuda T, Fukuda I, et al. Black soybean seed coat extract prevents obesity and glucose intolerance by up-regulating uncoupling proteins and down-regulating inflammatory cytokines in high-fat diet-fed mice. J Agric Food Chem 2011;59:8985–93.
[144] Viuda-Martos M, Fernandez-Lopez J, Perez-Alvarez JA. Pomegranate and its many functional components as related to human health: a review. Compr Rev Food Sci Food Saf 2010;9: 635–54.
[145] Lei F, Zhang XN, Wang W, Xing DM, Xie WD, Su H, et al. Evidence of antiobesity effects of the pomegranate leaf extract in high-fat diet induced obese mice. Int J Obes (Lond) 2007;31:1023–9.
[146] de Nigris F, Balestrieri ML, Williams-Ignarro S, D'Armiento FP, Fiorito C, Ignarro LJ, et al. The influence of pomegranate fruit extract in comparison to regular pomegranate juice and seed oil on nitric oxide and arterial function in obese Zucker rats. Nitric Oxide 2007;17:50–4.
[147] Vroegrijk IO, van Diepen JA, van den Berg S, Westbroek I, Keizer H, Gambelli L, et al. Pomegranate seed oil, a rich source of punicic acid, prevents diet-induced obesity and insulin resistance in mice. Food Chem Toxicol. 2011;49:1426–30.
[148] Hontecillas R, O'Shea M, Einerhand A, Diguardo M, Bassaganya-Riera J. Activation of PPAR gamma and alpha by punicic acid ameliorates glucose tolerance and suppresses obesity-related inflammation. J Am Coll Nutr 2009;28:184–95.
[149] Yuda N, Tanaka M, Suzuki M, Asano Y, Ochi H, Iwatsuki K. Polyphenols extracted from black tea (*Camellia sinensis*) residue by hot-compressed water and their inhibitory effect on pancreatic lipase in vitro. J Food Sci 2012;77:H254–61.
[150] Brown AL, Lane J, Holyoak C, Nicol B, Mayes AE, Dadd T. Health effects of green tea catechins in overweight and obese men: a randomised controlled cross-over trial. Br J Nutr 2011; 106:1880–9.
[151] Cunha CA, Lira FS, Rosa Neto JC, Pimentel GD, Souza GIH. Green tea extract supplementation induces the lipolytic pathway, attenuates obesity, and reduces low grade inflammation in mice fed a high-fat diet. Mediators Inflammation 2013; 2013:1–18.
[152] Teixeira GL, Lages CP, Jascolka LT, Aguilar EC, Soares PLF, Pereira SS, et al. White tea (*Camellia sinensis*) extract reduces oxidative stress and triacylglycerols in obese mice. Ciencia e Tecnolia de Alimentos 2012;32:733–41.
[153] Datau EA, Wardhana, Surachmanto EE, Pandelaki K, Langi JA, Fias. Efficacy of *Nigella sativa* on serum free testosterone and metabolic disturbances in central obese male. Acta Med Indones 2010;42:130–4.
[154] Gurrola-Díaz CM, García-López PM, Sánchez-Enríquez S, Troyo-Sanromán R, Andrade-González I, Gómez-Leyva JF. Effects of *Hibiscus sabdariffa* extract powder and preventive treatment (diet) on the lipid profiles of patients with metabolic syndrome (MeSy). Phytomedicine 2010;17:500–5.
[155] Godard MP, Ewing BA, Pischel I, Ziegler A, Benedek B, Feistel B. Acute blood glucose lowering effects and long-term safety of OpunDia supplementation in pre-diabetic males and females. J Ethnopharmacol 2010;130:631–4.
[156] Kazemipoor M, Radzi WJBMC, Hajifaraji M, Haerian BS, Mosaddegh MH, Cordel GA. Antiobesity effect of caraway extract on overweight and obese women: a randomized, triple-blind, placebo-controlled clinical trial. Evid Based Complementary Alternat Med 2013;2013. Article ID 928582.
[157] Lim DW, Song M, Park J, Park SW, Kim NH, Gaire BP, et al. Anti-obesity effect of HT048, a herbal combination, in high fat diet-induced obese rats. Molecules 2012;17:14765–77.
[158] Opala T, Rzymski P, Pischel I, Wilczak M, Wozniak J. Efficacy of 12 weeks supplementation of a botanical extract-based weight loss formula on body weight, body composition and blood chemistry in healthy, overweight subjects—a randomised double-blind placebo-controlled clinical trial. Eur J Med Res 2006;11:343–50.
[159] Lenon GB, Li KX, Chang YH, Yang AW, Da Costa C, Li CG, et al. Efficacy and safety of a chinese herbal medicine formula (RCM-104) in the management of simple obesity: a randomized, placebo-controlled clinical trial. Evid Based Complement Alternat Med 2012; 2012:1–11.
[160] Aoyama T, Fukui K, Takamatsu K, Hashimoto Y, Yamamoto T. Soy protein isolate and its hydrolysate reduce body fat of dietary obese rats and genetically obese mice (yellow KK). Nutrition 2000;16:349–54.
[161] Allison DB, Gadbury G, Schwartz LG, Murugesan R, Kraker JL, Heshka S, et al. A novel soy-based meal replacement formula for weight loss among obese individuals: a randomized controlled clinical trial. Eur J Clin Nutr 2003;57:514–22.
[162] Slavin JL. Dietary fiber and body weight. Nutrition 2005;21: 411–8.
[163] Smith U. Dietary fibre, diabetes and obesity. Int J Obes 1987;11: 27–31.
[164] Delzenne NM, Neyrinck AM, Backhed F, Cani PD. Targeting gut microbiota in obesity: effects of prebiotics and probiotics. Nat Rev Endocrinol 2011;7:639–46.
[165] Hsu CH, Tsai TH, Kao YH, Hwang KC, Tseng TY, Chou P. Effect of green tea extract on obese women: a randomized, double-blind, placebo-controlled clinical trial. Clin Nutr 2008;27:363–70.
[166] Food and drug administration (FDA), Center for drug evaluation and research (CDER). Draft. Guidance for industry. Developing products for weight management. FDA, USA 2007.

LIST OF ABBREVIATIONS

5-HT Hydroxy tryptamine
AIF Translocation of apoptosis-inducing factor
AMPK Adenosine monophosphate-activated protein kinase
BAT Brown adipose tissue
BCI Body composition improvement
BMI Body mass index
C/EBPα CCAAT/enhancer-binding protein-α
CAM Complementary and alternative medicine
CAPs Capsaicins
CCK Cholecystokinin
CDSCO Central Drug Standard Control Organization
COX-2 Cyclooxygenase-2
CVD Cardio vascular disorder
EBPα Enhancer-binding protein α
EMA European Medicines Agency
eNOS Endothelial NO synthase
ERK Extracellular signal-regulated kinases
FDA Food and Drug Administration
FXR Farnesoid X receptor
GABA-γ Aminobutyric acid
GI Glycemic index
GPDH Glycerol-3-phosphate dehydrogenase
HAs Human adipocytes
HDL High density lipoprotein
IL-6 Interleukin-6
JNK- c Jun N-terminal kinase-c
LDL Low density lipoprotein
LPL Lipoprotein lipase
MAOi Monoamino oxidase inhibitors
MAPK Mitogen-activated protein kinase
MCH Melanin-concentrating hormone
MCP-1 Monocyte chemoattractant protein-1
NE Norepinephrine

NF-κB Nuclear factor-kappa B
NO Nitric oxide
PGE2 Prostaglandin E2
PPARγ Peroxisome proliferator-activated receptors-γ
PUFAs Polyunsaturated fatty acids
PYY Peptide YY
mRNA Messenger RNA
SNS Sympathetic nervous system
SSRIs Selective serotonin reuptake inhibitors
TG Triglyceride
TNF-α Tumor necrosis factor-alpha
TRPV1 Transient receptor potential vanilloid receptor-1
TV Tomato vinegar
UCP-1 Uncoupling protein 1
UCP-2 Uncoupling protein 2
WAT White adipose tissue
WHO World Health Organization

This page intentionally left blank

CHAPTER

19

Applications of High Performance Liquid Chromatography in the Analysis of Herbal Products

Satyajit D. Sarker, Lutfun Nahar

Medicinal Chemistry and Natural Products Research Group, School of Pharmacy and Biomolecular Sciences, Faculty of Science, Liverpool John Moores University, Liverpool, United Kingdom

OUTLINE

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00019-2

19.1 INTRODUCTION

High performance liquid chromatography (HPLC), also known as high pressure liquid chromatography, is one of the most popular, modern, powerful, and versatile chromatographic separation techniques that have been routinely used to separate the components in a mixture (e.g., an herbal extract or product), to identify each components (or at least as many components as possible), to quantify separated components, and to obtain the chemical profile or fingerprint of a crude mixture. Any HPLC system fundamentally comprises a pump (or pumps) to pass a pressurized (50–400 bar) mobile phase (solvent) through a compact column (typically 2.1–4.6 mm diameter and 30–250 mm length) filled with a stationary phase, e.g., reversed-phase C_{18} silica (typically 2–5 μm particle size), a sample injection system (manual or autosampler), at least one suitable detector, e.g., photo-diode-array (PDA) detector, to monitor the separation of any sample mixture, and nowadays, a PC-based software to control the whole operation and data processing (Figure 19.1). Depending on the demand of any particular experiment, a fraction collector, a column chamber (thermostat or column oven), an online degasser, or a few other additional modules can also be added to a standard HPLC system. While this chapter is not really intended to provide the details of the composition and function of any HPLC system, it will certainly focus on the applications of HPLC techniques in the analysis of various herbal products and will present several specific examples of protocols of such analysis. However, a brief overview of available HPLC techniques and methods will be presented. The examples of analyses of herbal products will primarily be limited to commercially available formulated products, not necessarily any crude herbs or herbal materials.

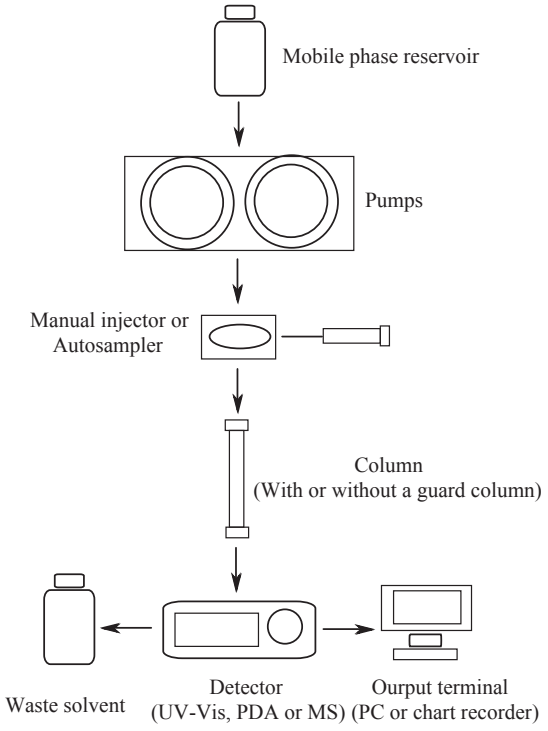


FIGURE 19.1 HPLC schematic.

19.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is the most widely used analytical separation technique for qualitative and quantitative determination of compounds in natural products extracts or fractions as well as finished products. For any analysis of herbal products, HPLC, coupled with various modern detection techniques, has now become the method of choice and is often recommended by various regulatory authorities working towards ensuring the quality, efficacy, and safety of herbal products. However, an HPLC operation requires careful consideration of a number of important factors, starting from sample preparation to the choice of appropriate detection technique and data processing modes.

19.2.1 Sample Preparation Techniques

The fundamental steps in sample preparation for HPLC analysis are complete dissolution of sample in the eluent and filtration of the sample solution using microfilters (usually 0.45 μm). The quality of the outcome from any HPLC analysis often depends on the sample preparation technique used [1–5].The choice of a sample preparation technique for HPLC analysis depends largely on the nature of samples to be analyzed, and the HPLC mode to be employed. For example, if the sample matrix is soluble in the mobile phase, a simple preparation of sample solution is suitable for HPLC analysis. However, if the sample is not readily soluble in the mobile phase, for example, powder of an herb, a suitable extraction protocol will have to be employed before it can be analyzed by HPLC. The chosen extraction procedures should be rapid but efficient, and they should include the total entity of the low molecular constituents of an herbal product. This is usually achieved, in most cases, using methanol (MeOH) or ethanol (EtOH). Additional fingerprints can be obtained by extraction with petroleum ether/*n*-hexane or chloroform (for lipophilic compounds) or water/water-acetone mixtures (for tannins, high polymeric procyanidines, and amino acids) as solvents. Polysaccharides and proteins can be characterized using their

sugar- or amino acid-fingerprints after enrichment and acidic or enzymatic hydrolysis.

Depending on the nature of the active ingredient(s) used in any formulations or products, e.g., tablets and capsules, either a simple dissolution or an extraction step followed by filtration must be introduced prior to HPLC analysis. A suitable sample preparation step is particularly important for the analysis of herbs and herbal products, because it is necessary to extract the desired chemical components from the herbal materials for further separation and characterization. Modern and classical sample preparation techniques [4–6] may include one or more of the following:

1. Solvent immersion.
2. Solvent partitioning.
3. Refluxing.
4. Ultrasonic extraction.
5. Solid-phase microextraction.
6. Supercritical-fluid extraction.
7. Pressurized-liquid extraction.
8. Microwave-assisted extraction.
9. Solid-phase extraction.
10. Surfactant-mediated extraction.

An excellent overview on various modern sample preparation methods for the extraction, cleanup, and concentration of analytes from medicinal plants or herbal products for HPLC analysis has been presented by Huie [7]. Generally, an ideal sample preparation technique should be safe, cost-effective, reproducible, not too time-consuming, able to extract maximally the target compounds without changing their structures, and able to exclude other compounds to minimize any unwanted interferences.

19.2.2 Modes of Separation: Stationary and Mobile Phases

An HPLC analysis of any crude mixture (e.g., herbal products) generally requires a normal-phase, reversed-phase gel permeation (size exclusion) or an ion exchange chromatographic mode [8,9]. The use of any particular mode relies on the compatibility of the stationary phase with the extract or mixture to be analyzed and also the chemical nature of the crude mixture. Commonly used stationary phases and their associated modes are presented in Table 19.1.

TABLE 19.1 Commonly Used Stationary Phases and Their Associated Modes in HPLC

Stationary phases	Modes
C_6 silica	Reversed-phase
C_8 silica	Reversed-phase
C_{18} silica	Reversed-phase
Silica	Normal phase
Diol	Normal and reversed-phase
Cyano (CN)	Normal and reversed-phase
Benzene sulphonic acid	Strong cation exchange
Polystyrene	Size exclusion

19.2.2.1 Normal-Phase HPLC (NP-HPLC)

Normal-phase HPLC (NP-HPLC), which is not the most popular form of HPLC nowadays, utilizes a polar stationary phase (usually silica) and less polar (nonaqueous) eluting solvents, e.g., *n*-hexane and ethyl acetate (mobile phase). The separation is based on the analyte's ability to engage in polar interactions, e.g., hydrogen bonding or dipole–dipole type interactions, with the sorbent surface. Generally, polar compounds in the mixture being passed through the column will be adsorbed more strongly to the polar silica than nonpolar compounds, which will pass more quickly through the column and will be eluted quicker than the polar ones. Increasing the polarity of the eluting solvent will decrease the elution time, i.e., shorter retention time.

NP-HPLC is suitable for separating lipophilic compounds, long-chain alkanes, or compounds that are sparingly soluble in aqueous conditions. As the interaction of various compounds with the stationary phase not only depends on the functional groups present in the structure of the analyte molecule, but also on stereochemical features, the effect of steric hindrance on interaction strength allows NP-HPLC methods to resolve geometric and positional isomers [9]. Since the introduction of reversed-phase HPLC (RP-HPLC) during 1970, NP-HPLC has been superseded by RP-HPLC. The NP-HPLC mobile phase consists of a nonpolar solvent such as *n*-hexane or *n*-heptane mixed with a slightly more polar solvent such as isopropanol, chloroform, or ethyl acetate [10]. Table 19.2 presents a list of commonly used eluents in NP-HPLC. There are four main factors, i.e., solvent strength, localization, basicity, and UV cutoff, involved in the choice of solvents for NP-HPLC.

19.2.2.2 Reversed-Phase HPLC (RP-HPLC)

Reversed-phase HPLC (RP-HPLC) is the most commonly used mode of HPLC and, as the name implies, this mode is just the reverse of NP-HPLC, whereby the stationary phase is more nonpolar than the eluting solvent. Generally, RP-HPLC has a nonpolar stationary phase, e.g., C_{18} silica (Table 19.1), and a moderately polar aqueous mobile phase [8,10]. One common RP-HPLC stationary phase is surface-modified silica, RMe_2SiCl, where R is a straight chain alkyl group

TABLE 19.2 Commonly Used Mobile Phases in NP-HPLC

Mobile phases	Strength (ε^0)	Localization	Basicity	UV-cut-off (nm)
n-Hexane	0.0	No	Not relevant	195
n-Heptane	0.0	No	Not relevant	200
Chloroform	0.26	No	Not relevant	245
Dichloromethane	0.30	No	Not relevant	233
Diethylether	0.38	Yes	Yes	218
Ethylacetate	0.48	Yes	No	256
Tetrahydrofuran	0.53	Yes	Yes	212
Isopropanol	0.60	Yes	Proton donor	210
Methanol	0.70	Yes	Proton donor	205

such as $C_{18}H_{37}$ or C_8H_{17}. Silica-based reversed-phase sorbents are also known as "bonded-phase" materials [8,11]. The eluent used in RP-HPLC is usually composed of a mixture of water and miscible organic solvents, usually acetonitrile (ACN), MeOH, or tetrahydrofuran (THF) [9,10] (Table 19.3). Sometimes, buffers, acids, or bases are also added to suppress compound ionization or to control the degree of ionization of free unreacted silanol groups to reduce peak tailing and improve chromatography [9].

In RP-HPLC there is strong attraction between the polar solvent and polar molecules in the mixture being passed through the column, but there is not much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Therefore, polar molecules in the mixture spend most of their time moving with the solvent. Nonpolar compounds in the mixture tend to form attractions with the hydrocarbon groups because of van der Waals dispersion forces. They are less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules. They spend less time in solution in the solvent, and this slows them down on their way through the column, which means longer retention time. In RP-HPLC the polar molecules travel through the column more quickly.

TABLE 19.3 Commonly Used Mobile Phases in RP-HPLC

Mobile phases	Polarity index (Snyder)	UV-cutoff (nm)
Acetonitrile	6.2	190
Isopropanol	4.3	210
Methanol	6.6	205
Tetrahydrofuran	4.2	212–230
Water	9.0	180

RP-HPLC allows purification of most classes of compounds, including compounds present in various herbal products, and is often the most preferable choice when analyzing and attempting to separate and identify compounds from a complex mixture [12].

19.2.2.3 Other HPLC Modes

Gel permeation chromatographic HPLC mode (also called size exclusion chromatography) is generally used for separation of large molecules, e.g., proteins and oligosaccharides. The stationary phase is made of inherently hydrophobic and chemically and physically inert polystyrene/divinylbenzene copolymers (Table 19.1). The pore size in the particles is strictly controlled, and compounds are separated by their ability to enter the pores; the smaller molecules are "trapped" temporarily in the pores, while larger molecules are not held up and pass through the column relatively unhindered [9].

Ion exchange HPLC utilizes an anionic or cationic stationary phase for the separation of acids and amines. Compounds with a net charge bind reversibly to the ionizable groups on the stationary phase and are eluted through displacement of a stronger ionized species in the eluent [9].

19.2.3 Isocratic versus Gradient Elution

Isocratic analysis is the analysis where the mobile phase composition, as well as the flow rate, is kept constant throughout the total HPLC run, and any re-equilibration of the column is not required. On the other hand, in gradient elution, the mobile phase composition changes over time, either gradually with a steady change in composition (linear gradient), or a stepwise change in composition (step gradient) [13–15]. While an isocratic elution is suitable for separating a mixture of a few compounds, a gradient elution can handle a mixture of several compounds of wide-ranging polarities [15]. In a gradient operation, the sample is injected onto the column in high aqueous conditions, and the organic proportion is increased over time to elute all the compounds off the column. For a typical analytical column (4.6 mm i.d. × 150 mm), the typical flow rate is 1.0 mL/min with the gradient time taking 30 min with a hold at the end of the gradient to ensure all compounds have been eluted [9]. However, the run time can be significantly reduced (five minutes as opposed to 30 min) in the recently introduced ultra performance liquid chromatography (UPLC), where the diameter of the column and the particle size of the stationary phase are much smaller

than conventional analytical HPLC columns [16,17]. In UPLC, the separation efficiency and resolution can be improved because of the use of much-reduced particle size in the UPLC columns [18,19].

19.2.4 Detectors

A suitable detection device (detector) is used with an HPLC to detect or monitor compounds separating out while eluting from a column. The most common detector is an Ultraviolet–Visible (UV–Vis) detector. However, hyphenation between an HPLC and more sophisticated detection techniques, e.g., mass spectrometer (MS) or nuclear magnetic resonance (NMR) spectrometer, has increased the capability of separating and solving structural problems of complex natural products [20]. Sometimes, multiple detection techniques are employed, e.g., LC-UV-Vis-MS, LC-MS-MS, and LC-NMR-MS.

A UV–Vis spectroscopic detector is a universal detector for any HPLC system. The photo-diode-array (PDA) detector is another form of UV–Vis detector that allows analysis of compounds containing chromophores, coumarins, flavonoids, isoflavonoids, etc. A PDA detector facilitates the analysis of individual HPLC peaks after completion of a run and helps to obtain complete UV–Vis spectrum of individual compounds. Independent chromatograms can also be constructed at various wavelengths to enhance selectivity of the data. PDA is one of the most popular detectors, but compounds with no chromophores are not UV–Vis sensitive, and cannot be detected.

Nowadays, an MS detector, generally in combination with a UV–Vis or PDA detector, is probably the most sought-after detection method employed for HPLC, especially when analyzing complex mixtures of compounds like natural product extracts or herbal products. When an MS detector is used, separated compounds emerging from the column can be identified on the basis of their mass spectral data. The ionization techniques used in HPLC-MS are generally soft ionization techniques, e.g., electrospray ionization mass spectrometry (ESI-MS) that display mainly the molecular ion species with only a few fragment ions are generally used in HPLC-MS. Sometimes, tandem mass spectrometry (MS–MS), which provides fragments through collision-induced dissociation of the molecular ions, is also employed [20].

An HPLC-MS system does not necessarily allow a complete and unambiguous online identification of a component, unless it is a well-known natural product and complementary online spectroscopic information is available in databases for comparison [20–22]. The quality of MS response invariably depends on a number of factors, e.g., nature of the compounds to be analyzed, the solvent and buffer used as the mobile phase, the flow rate, and, of course, the type of interface used, and thus, it often creates difficulties in relation to reproducibility of information [23].

NMR, albeit probably the least sensitive of all detection techniques, is also used as a detector for HPLC as it offers the most useful structural information towards the structure elucidation of natural products [20]. Often, a UV–Vis detector is also used as a primary detector when an NMR detector is used. NMR has not quite become a widely accepted detector for any HPLC operation, mainly because of its lower level of sensitivity, need for deuterated solvents, and higher cost compared to other available detectors.

Among the other types of detectors, an evaporative light scattering detector (ELSD), an infrared (IR) detector, an electrochemical detector, and a fluorescent detector are also used in special cases. For example, saponins with no chromophores may not be detected by conventional UV–Vis or PDA, but can be successfully detected by the ELSD. As one-way separation, single detection method, or data processing cannot fulfill the needs of the comprehensive quality control of herbal products, multidimensional information-based HPLC technologies (e.g., two dimensional HPLC), use of multidetectors, and HPLC fingerprinting coupled with multicomponent quantification can fulfill this demand [24].

19.2.5 Data Analysis

Most of the data analyses are now performed through various PC-based software that normally come with HPLC systems, e.g., Chromeleon for Dionex HPLC systems. However, for any HPLC analysis, the retention time, peak area, peak height, UV–Vis absorption maxima, MS data, and, in some cases, NMR data are generally analyzed for identification and/or quantification of separated components from a complex mixture. Data obtained from HPLC runs can also be analyzed for chemical fingerprinting analysis of various natural product extracts, including herbal products, especially in relation to quality control and authentication purposes. With the tremendous progress in electronics and computational techniques, the capabilities of available HPLC software have enhanced significantly in recent years, and various types of data analysis can now be easily performed.

19.2.6 Chemometrics and Principal Component Analysis

Chemometrics is not a single tool but a range of methods including basic statistics, signal processing, factorial design, calibration, curve fitting, factor

analysis, detection, pattern recognition, and neural network. Chemometrics, first introduced by the Swedish scientist Svante Wold in 1971, is simply the application of mathematical and statistical techniques to retrieve more information from the chromatographic data. This term has been officially defined as the science of relating measurements made on a chemical system or process to the state of the system via application of mathematical or statistical methods [25–30]. Various areas and principles that contribute to chemometrics are shown in Figure 19.2.

Chemometrics is the tool for extracting information from multivariate chemical data using tools of statistics and mathematics. With the advance of computational techniques, chemometrics has become a leading tool for faster analysis of results/data and shorter product development time. It is generally applied for one or more of three primary purposes to

1. Explore patterns of association in data.
2. Track properties of materials on a continuous basis.
3. Prepare and use multivariate classification models.

This tool has the capacity for analyzing and modeling a wide variety of data types for an even more diverse set of applications. Chemometrics are basically classified into two main categories [26]:

1. Pattern recognition methods (unsupervised and supervised) when a qualitative evaluation is considered.
2. Multivariate calibration for quantitative purposes.

The design of the experiment, data preprocessing, classification, and calibration are the main practical steps involved in any chemometrics analysis. Experimental design primarily screens factors that are important for the success of a process. Selection and implementation of the optimized conditions under which the process will be carried out come next.

Principal component analysis (PCA) is an unsupervised pattern recognition technique used for handling multivariate data without prior knowledge about the samples under investigation [31]. The supervised classification procedure using soft independent modeling of class analogy (SIMCA), based on making a PCA model to assign unknown samples into the predefined class model, is also used in chemometrics analysis [30]. The central idea of PCA is to reduce the dimensionality of a data set consisting of large amounts of interrelated variables, yet keeping maximum variation in the data set. PCA is generally used to evaluate the discrimination ability of common components using relative peak areas of common peaks as input data instead of the full fingerprint.

In the analysis of herbal products, especially by HPLC, and chemometrics follow-up analysis,

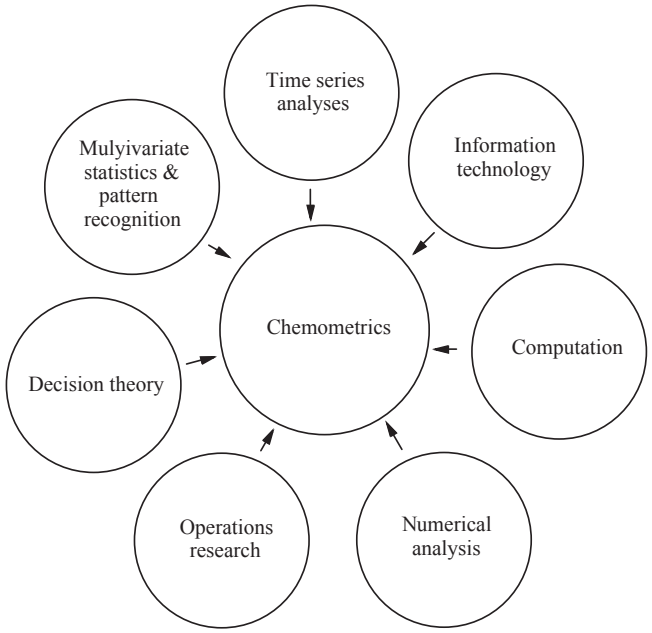


FIGURE 19.2 A flow diagram showing the generic steps involved in the HPLC-based analysis of herbal products.

pretreatment of data is required because of unknown components, overlapped peaks, and drifted baselines on the HPLC chromatogram. For herbal products data analysis for quality control and standardization, in addition to PCA as mentioned above, a number of other chemometrics techniques may also be applied, such as linear discriminate analysis (LDA), spectral correlative chromatography (SCC), information theory (IT), local least square (LLS), heuristic evolving latent projections (HELP), and orthogonal projection analysis (OPA) [30]. The commonly used pretreatment of data obtained from herbal products analysis are LLS and normalization. Details on the chemometrics techniques for the analysis of herbal products are available in a couple of excellent review articles published recently [30,32].

19.3 HERBAL PRODUCTS

Herbal medicine refers to medicine that utilizes various plant parts, e.g., seeds, berries, roots, leaves, bark, flower buds, or flowers for treating human ailments. It is also known as botanical medicine or phytomedicine. Herbal products are commercially available nutrition (dietary supplements), cosmetics, health, or health-related products, including medicines that contain one or more herbal components. An herbal product may be simply dried total plant parts, dried and ground plant parts, crude extracts of any plant parts, or in a more contemporary form of medicinal formulation, e.g., teas, tablets, capsules, solutions, or suspensions.

The use of herbs for human consumptions, particularly for medicinal purposes, goes back thousands of years, as far as 60,000 years ago when the Neanderthal men appeared to have used herbs for treating ailments. Various forms of major healthcare systems, based on the use of herbs and herbal constituents, were developed several thousand years ago; for example, the Traditional Chinese Medicine (TCM) and the Ayurvedic Medicine systems both are about 5000 years old. It is obvious that herbal medicine or herbal products have a long tradition of use outside of conventional medicine, and they have always enjoyed enviable popularity, especially in the developing world. It is well-known that, according to the estimation by the World Health Organization (WHO), over 80% of people worldwide rely on herbal medicines for some part of their primary health care.

Over the last few decades, the steady growth of the herbal products market, especially in the developed western countries, has been quite remarkable, and in some cases, it has superseded the growth of the so-called "modern medicine" market. In Germany, about 600–700 plant-based medicinal products are available and are prescribed by a majority (over 70%) of German physicians. In the past 20 years, in the USA, public dissatisfaction with the cost of prescription medications, combined with an interest in returning to natural or organic remedies, has prompted an increase in demands for herbal medicinal products. However, quality-related problems (lack of consistency, safety, and efficacy) seem to be overshadowing the potential genuine health benefits of various herbal products; thus, the quality herbal products can only be made effective and safe if proper standardization methods are followed right from the cultivation/collection of the herb to packaging and storage of the finished product.

All herbal products can broadly be divided into two classes: (1) monoherbal products and (2) polyherbal products.

19.3.1 Monoherbal Products

Monoherbal products or preparations, as the name indicates, contain only one herb or herbal component. For example, ginseng tea contains the powder of only ginseng roots, chamomile tea contains dried leaves of *Matricaria chamomilla*, Curcuma capsules contain only the powder of rhizomes of *Curcuma longa*, Milk Thistle capsules contain standardized extract of only Milk Thistle (*Silybum marianum*), and Ginkgo biloba standardized extract contains the extract of only *Ginkgo biloba*.

19.3.2 Polyherbal Products

Again, as the name implies, a polyherbal product or preparation, e.g., decoction, tincture, tablets, and capsules, is composed of two or more herbs or herbals components. Most of the TCM products or Ayurvedic preparations often contain more than two herbal components. For example, a polyherbal formulation based on *Euphorbia fusiformis* (common name: Dhudmul) to increase the flow of breast milk in women utilizes 500 g of roots of *E. fusiformis* made into a paste and 20–25 grains of *Piper nigrum* in the form of conventional tablets [33]. Further examples of some polyherbal formulations are presented in Table 19.4.

19.4 TYPES OF ANALYSIS OF HERBAL PRODUCTS

Most of the herbal products analysis can be categorized into two major categories: (1) chemical profiling or chemical fingerprinting and (2) authentication and quality control of herbal products to ensure efficacy and safety of the products. One of the characteristics of herbal preparations or products is that, most often, herbal products, either presenting as a single herb (monoherbal) or as collections of herbs in composite formula (polyherbal), are extracted with boiling water during the decoction process, with the exception of more modern products (e.g., tablets or capsules of herbal medicines). This sometimes poses the difficulties in quality control or analysis of herbal products.

Despite the common perception that herbal products are generally safe because of their long-standing use in various cultures, several serious adverse effects resulting from uses of herbal products have been well-documented in the literature. Many of these adverse effects can be implicated to the presence of toxic contaminants, e.g., heavy metals, microorganisms, microbial toxins, pesticides, and radioactive materials, and various adulterations. Some of the herbal components used in the herbal products may also be toxic themselves, especially when their presence in the product is well above the recommended safety levels. Therefore, appropriate analysis of herbal products to ensure quality, and thus ensuring safety, is paramount. This may require comprehensive phytochemical analysis incorporating identification and quantification of all or at least as many compounds as possible present in any herbal products.

The major objective of any herbal product analysis is generally aimed at overall standardization of the product, which is the first step for the establishment of any consistent biological activity, a consistent chemical profile, or simply a quality assurance program for production and manufacturing [45]. Standardization can be defined as the means of adjusting the herbal products to a defined content of a constituent or group of substances with defined therapeutic activity. According to the European Medicines Agency (EMEA), there is a

TABLE 19.4 Examples of Some Polyherbal Products

Polyherbal products	Compositions	Uses	References
Arjunarishta	Fermented decoction of the bark of *Terminalia arjuna* (2040 g), fruits of *Vitis vinifera* (1020 g), flowers of *Madhuca indica* (408 g), and flowers of *Woodfordia fruticosa* (408 g).	Cardioprotective	[34]
Ayurved Siriraj Prasachandaeng	*Citrus aurantifolia*, *Bouea macrophylla*, *Knema globularia*, *Dracaena loureiri*, *Myristica fragrans*, *Caesalpinia sappan*, *Conioselinum univitatum*, *Kaempferia galanga*, *Mesua ferrea*, *Jasminum sambac*, *Mammea siamensis*, and *Nelumbo nucifera*	Antipyretic drug	[35]
Contudol® capsule	(1) *Boswella serrata* (Shallaki) extract (standardized for boswellic acid 60%), 200 mg, (2) *Curcuma longa* (Haldi) extract (standardized for curcumin 20%), 100 mg, (3) *Moringa oleifera* (Sahjan) extract (standardized for tannins 1%, 50 mg, and (4) *Zingiber officinale* (Sunthi or Ada) extract (standardized for gingerol), 35 mg	Improves mobility and reduces pain in the case of arthritis and other musculoskeletal disorders.	[36]
Hongdoushan capsule	*Panax ginseng*, *Glycyrrhiza uralensis*, and *Taxus Chinensis*	To treat ovarian and breast cancers	[37]
Immunol	Extracts of *Asparagus racemosus* (7.0 mg), *Boerhaavaia diffusa* (7.0 mg), *Tinospora cordifolia* (14.5 mg), *Terminalia chebula* (3.5 mg), *Trigonella foebum-graecum* (4.8 mg), *Tylophora asthamatica* (4.8 mg), and *Withania somnifera* (7.0 mg)	To stimulate immune system	[38]
Jessica	Each 100 mg contains *Emblica offocinalis* (7.6 mg), *Nardostachys jatamansi* (77.0 mg), and *Rauwolfia serpentine* (15.4 mg)	Anxiolytic property: to help with sleep and relaxation	[39]
Maha Vartikawa Watee pills	Composed of 29 herbal ingredients of equal quantities: aerial parts of *Myristica fragrans*, bulbs of *Allium satium*, exocarps of *Acacia chundra* and *Ferula assa-foetida*, flower buds of *Syzygium aromaticum*, fruits of *Carum cavi*, *Cuminum cyminum*, *Piper nigrum*, *Piper longum*, and *Trigonella foenum-graecum*, heart-wood of *Santalum album*, leaves of *Piper betel*, *Trachyspermum ammi*, and *Vitex nigundo*, pollens of *Fumaria parviflora*, rhizomes of *Acorus calamus* and *Zingiber officinale*, roots of *Solanum virginianum*, seeds of *Brassica jancea*, *Caesalpinia bonduc*, *Embelica ribes*, *Myristica fragrans*, *Nigella sativa*, *Seasamus indicum*, stamen of *Mesua ferrea*, stem barks of *Cinnamomum verum*, and total plant of *Terminalia chebula*, *Terminalia bellarica*, and *Phyllanthus emblica*	To treat gastrointestinal disorders	[40]
Rasayana churna	(1) Powder of dried stem of *Tinospora cordifolia* (Guduchi), (2) powder of dried fruits of *Tribulus terrestris* (Gokshur), and (3) powder of dried pericarps of *Emblica officinalis* (Amlaki) in equal proportion.	To enhance general body resistance, promote longevity, and relieve stress.	[41]
Shrishadi	*Albezzia lebbeck*, *Cyprus rotan*, and *Solanum xanthocarpum*	To treat infectious respiratory disorders.	[42]
Tisane A	Roots of *Cocos nucifera* and *Coix lacryma-jobi*, leaves of *Aphloia theiformis*, *Antidesma madagascariense*, and *Bidens pilosa*, and bark of *Erythroxylum laurifolium*	To treat rheumatoid arthritis	[43]
Triphala powder or tablets	Ground dried pericarps (in equal proportion) of (1) *Terminalia chebula* (Haritaki), (2) *Terminalia bellerica* (Bibhitaki), and (3) *Emblica officinalis* (Amlaki)	For internal cleansing, purgation, rejuvenation, and detoxification	[44]

clear distinction between constituents with known therapeutic activity (which can be used to standardize any biological effect) and marker compounds, which allow standardization on a set amount of the chosen compound.

A marker compound can be defined as the chemically defined constituent of an herbal product for quality control purposes, irrespective of its direct link or not, to overall therapeutic property of the product. Valerenic acids in *Valeriana officinalis*, gingkolides and flavonoids in *G. biloba*, and hypericin and hyperforin in *Hypericum perfoliatum* are classic examples of some marker compounds.

According to the EU definition, there are three categories of herbal products: (1) those containing constituents (single or group of compounds) with known and experienced therapeutic activity that is solely responsible for clinical efficacy; (2) those containing chemically defined constituents possessing relevant pharmacological properties, which are likely to contribute to the clinical efficacy; and (3) those in which no constituents have been identified as being responsible for the therapeutic activity. All these three categories need to be covered or considered when analyzing any herbal product. A flow diagram to show the generic HPLC-based analysis of herbal products is shown in Figure 19.3.

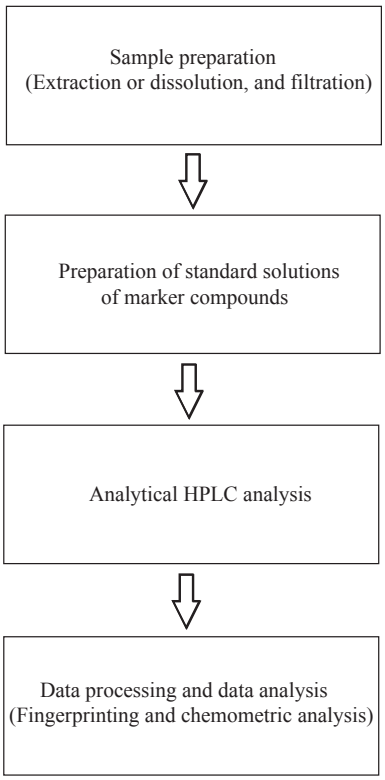


FIGURE 19.3 Areas and principles that contribute to chemometrics.

19.4.1 Marker-Based Authentication and Quality Control of Herbal Products

Authentication of the herbal products is the first step towards its quality control to ascertain efficacy and safety [46]. An herbal product, whether it is a monoherbal or polyherbal, contains several chemicals, which provide them with specific chemical identities or characteristics. So chemical constituents and their respective quantities can be analyzed to authenticate, i.e., to identify, an herbal product and differentiate the real product from a fake one [32,47,48].

Generally, one or two markers or pharmacologically active components in herbs or herbal products are employed for evaluating the quality and authenticity of herbal products, both in the identification and quantification of single herb and in multicomponent preparations [30,45]. According to the definition provided by the EMEA, chemical markers are the chemically defined constituents or groups of constituents of an herbal medicinal product, which are of interest for quality control purposes regardless whether they possess any therapeutic activity.

The presence and the quantity of a chemical marker are taken as primary indicators of the quality of any herbal product. However, marker-based determination may not necessarily offer a detailed picture of an herbal product, as multiple constituents are usually responsible synergistically for its therapeutic efficacy. Also, the chemical constituents in components herbal products may vary depending on harvest seasons, plant origins, drying processes, and other factors. This is why, nowadays, fingerprinting analysis of herbal products has become popular to ascertain authenticity and quality of an herbal product.

19.4.2 Chemical Profiling or Fingerprinting of Herbal Products

One of the main modes of analysis of herbal products is chemical profiling or chemical fingerprinting. Fingerprinting is a quality control tool that builds upon spectroscopic and chromatographic technology. An HPLC-based chemical fingerprint refers to the common peaks of any HPLC chromatogram, which can be used to characterize or authenticate any herbal product. A fingerprint of herbal medicinal products is the profile, which can illustrate the specific properties of the finished product after appropriate processing and be obtained by certain analytical techniques, e.g., HPLC.

A chemical fingerprint usually reflects the overall nature or "complete information" of the product. It elevates the quality control target from analysis of a single component to analysis of all the material in the product and thus may ensure consistency in the therapeutic efficacy of the products. Simply,

fingerprinting can be defined as a type of comprehensive and quantifiable method for authentication; it is one of the quality control models that is suitable for evaluating the authenticity and stability of herbal products. It has now become a globally accepted quality evaluation model for herbal medicinal products. Figure 19.4 presents an overview of the fingerprinting process of herbal products using HPLC.

The idea of "phytoequivalence" was first introduced in Germany in order to ensure consistency of herbal products [32]. As a result, a chemical profile, such as a chromatographic fingerprint, for an herbal product under evaluation was constructed and compared with the profile of a clinically proven reference product. A chemical fingerprint can be defined as a characteristic chemical profile of a sample, which reflects the unique complex chemical composition of the sample. The characteristics can be obtained by spectroscopic, chromatographic, electrophoretic techniques, or a combination of all of them. An HPLC coupled with a suitable detector can provide the required characteristic data set, e.g., retention time, peak area, spectral data, etc., to be used for fingerprinting analysis or profiling. It is often expected that this profile is featured by the fundamental attributions of "integrity or comprehensiveness" and "fuzziness," which are the two basic traits of any fingerprinting approach.

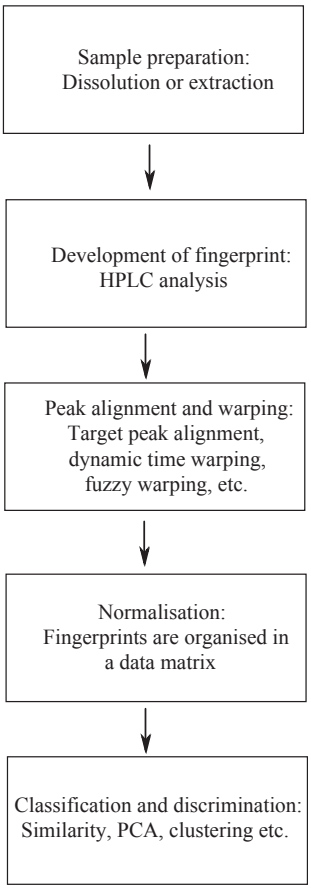


FIGURE 19.4 An overview of the fingerprinting process of herbal products using HPLC.

It has been shown that chemical fingerprinting or chemical profiling can help with authentication and identification of herbal products [49]. In this, generally an herbal product is viewed holistically, and the model of using only one or two marker components may not necessarily be the best means of evaluating the quality of herbal products. It can be noted that any fingerprinting protocol must be practical, specific, reproducible, and fully validated for its use in the quality assurance or authentication of any herbal products.

19.5 ANALYSIS OF HERBAL PRODUCTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Herbal products usually contain a large number of compounds, many of which may be present at low concentrations, yet are quite significant in terms of the overall quality, safety, and efficacy of the product. To assess the complete pattern of any herbal product, several chromatographic techniques, particularly HPLC, offers the desired separation of individual components and thus constructs a characteristic profile of the sample, which is generally known as "chemical fingerprinting." HPLC is undoubtedly the most popular separation technique used for the analysis of herbal products, especially in relation to authentication, quantification, quality control, fingerprinting, and standardization because of its reliability, reproducibility, precision, resolution, selectivity and sensitivity, and ease of hyphenation.

HPLC emerged as a powerful analytical tool during the 1970s and, since then, it has advanced to be the analytical separation method of choice for clinical, forensic, and pharmaceutical fields including herbal products. Increasingly, HPLC analytical techniques have been included in many of the latest monographs on the identification and determination of the plant constituents as well as the adulterants in herbal products. Applications of HPLC methods in the analysis of herbal products have been reviewed by various authors [19,21,50], and also, a good body of literature has become available in recent years. In the following sections, some specific examples with step-by-step protocols of such analysis are presented.

19.5.1 Specific Examples of Step-by-step Protocols: Single and Multiple Components Analysis Using Markers

It is quite common to use a single or multiple marker compounds (bioactive or nonbioactive) as an attempt

to assure quality of any herbal product using an HPLC-based method [51,52]. Most often, especially for a monoherbal preparation, a single marker compound is used. However, for polyherbal preparations, because of the complexity of the mixture, it is necessary to use more than one marker compound to assess the quality. For example, 13 marker compounds have been used to assess the quality of a Chinese polyherbal product, Gwakhyangjeonggi-san [53]. There are numerous publications describing various HPLC-based validated quality control protocols incorporating single or multiple marker compounds [41,53–60]. The following are just a few simple examples of protocols for HPLC analysis of herbal products using marker compounds.

19.5.1.1 *Vidanga* (Embelia ribes)

Vidanga (dried ripe fruits of *E. ribes*) is a popular Ayurvedic herbal medicinal product used traditionally in India as an anthelmintic, carminative, and stimulant. An HPLC-based method using a single marker compound, embelin, for the quality assurance of Vidanga was proposed by Sudani et al. [61]. The protocol is summarized below.

1. Three commercial samples of Vidanga were obtained from a local Indian market.
2. Samples were macerated with chloroform ($CHCl_3$) at room temperature for 48 h in the dark. The extracts were filtered and concentrated over a water bath until dry.
3. Embelin (1 mg/mL) in MeOH was used as the reference sample (marker).
4. Dried $CHCl_3$ extracts of test samples (10 mg equivalent of embelin) were resuspended in MeOH and sonicated for 10 min, filtered, and the volume was made to 10 mL with MeOH, and filtered again for HPLC analysis.
5. Each sample (20 µL) was analyzed by an optimized and validated HPLC-PDA method using a reversed-phase Chromatopak Peerless basic C_{18} column (4.6 × 250 mm, 5 µm), setting the detection/monitoring wavelength at 291 nm and column temperature at 25 °C, employing solvent A: MeOH and solvent B: phosphate buffer pH 3.0 (adjusted with 5% v/v acetic acid), using an isocratic elution with 10% B in A and at a flow rate of 1.4 mL/min. A Perkin–Elmer Series 200 HPLC system with PDA was used.
6. Presence of embelin in commercial samples was determined by direct comparison of the relative retention time and the UV–Vis absorption maxima of corresponding peak. Relative peak area was used to quantify embelin in the samples.

19.5.1.2 *Liquorice* (Glycyrrhiza glabra)

Liquorice (roots of *G. glabra*) is a popular herbal medicinal product, where glycyrrhizin is the main active component (2–15% w/w). It is also used as a natural sweetener because of the presence of glycyrrhizin, which is 200 times sweeter than sucrose. Dried roots of liquorice are commercially sold as an herbal product as well as a food additive. A semi-preparative HPLC-PDA-based method using glycyrrhizin as the marker compound has recently been published [62]. A summary of the protocol is presented below.

1. Nine samples of liquorice were obtained from various global markets.
2. Ground samples (15 g each) were Soxhlet-extracted, sequentially, with *n*-hexane and MeOH (400 mL each). The extracts were filtered and evaporated under a vacuum to dryness. The residue from the extracts of each sample was redissolved in MeOH (6.05 mL) and filtered before HPLC analysis (or TLC in the case of *n*-hexane extracts). As the *n*-hexane extract did not contain any glycyrrhizin (as checked by the TLC), the MeOH extracts of all nine samples were subjected to HPLC analysis.
3. Glycyrrhizin (20 mg/mL) in MeOH was used as the reference marker, and further dilutions (15, 10, 5, and 1 mg/mL) were made with MeOH. Each dilution of stock was injected into HPLC in triplicates to construct the calibration curve.
4. Each MeOH extract (100 µL) was analyzed by an optimized and validated semi-preparative HPLC-PDA method using a reversed-phase ACE 10C18-HL (150 × 10 mm, 10 µm) with a C_{18} guard column ACE3310110GD (10 × 10 mm, 10 µm), setting the detection/monitoring wavelength at 254 nm and column temperature at 25 °C, and using a binary gradient solvent system (30–100% B in A over 30 min) with a flow rate of 3.00 mL/min, where solvent A consisted of 0.1% v/v TFA in water and solvent B was 0.1% v/v of TFA in MeOH. A Dionex 3000 LC System coupled with Dionex 3000 semi-preparative pumps, a Dionex 3000 autosampler, a Dionex 3000 column chamber, and a Dionex PDA-3000 detector was used. Data were analyzed using the Chromeleon® Chromatography Data System.
5. While the retention time and the UV spectra were used to identify glycyrrhizin, peak area was used to quantify the amounts of glycyrrhizin present in each sample. The glycyrrhizin percentage level in the samples was in the range of 0.177–0.688% w/w of dry materials.

19.5.1.3 *Valerian* (Valeriana officinalis)

Valerian (*V. officinalis*) is a well-known herbal medicinal product, traditionally used to promote sleep and as

an anxiolytic agent for nervous unrest, to treat neuralgia, epilepsy, and to relieve digestive and other spasms of smooth muscle [63]. An HPLC-based method using a single bioactive marker compound, valerinic acid, for qualitative and quantitative analysis of some commercial brands of Valeriana products, including tablets, caplets, capsules, and drops, aiming at their quality control was reported by Ghafari et al. [63]. The protocol is summarized below.

1. Commercial samples (Neurogol tablets, Valerian capsules, antimigraine herbal drops, etc.) of Valerian products were obtained from local pharmacies in Tehran, Iran.
2. Samples (solid dosage forms) were macerated with 80% aqueous MeOH (20−60 mL) at room temperature. For analysis, four to six tablets, two caplets, and three capsules were used. The supernatants were filtered through a 0.45 μm filter. The extraction process was repeated twice. A portion (5 mL) of each extract was diluted with 20 mL of MeOH and again filtered through a 0.45 μm filter.
3. Valerenic acid in MeOH was used as the reference sample, and a calibration curve of the standard was made using various dilutions. Valerenic acid was identified within the samples by comparing its retention time with the internal standard of valerenic acid.
4. Each sample (10 μL) was analyzed by an optimized and validated HPLC-UV method using a reversed-phase VP-ODS C_{18} column (4.6 × 250 mm, 5 μm), setting the detection/monitoring wavelength at 220 nm and at a flow rate of 0.8 mL/min. A Shimadzu LC-10ADVP HPLC system with UV−Vis detector was used.

19.5.1.4 Compound Hongdoushan Capsule

This is a classic example of using HPLC for the analysis of a polyherbal product utilizing multiple marker compounds. Zhu et al. [37] used 10 bioactive markers, e.g., glycyrrhetinic acid, liquiritin, glycyrrhizin, baccatin III, 10-deacetylbaccatin III, cephalomannine, taxol, ginsenoside Rg1, ginsenoside Re, and ginsenoside Rb1, to analyze the compound Hongdoushan capsule, a widely known compound herbal preparation, which is often used for the treatment of ovarian and breast cancers and to stimulate the body immunity. A Hongdoushan capsule contains more than one herbal ingredient (*Panax ginseng*, *Glycyrrhiza uralensis*, and *Taxus Chinensis*) and thus can be quite difficult to control or assure the quality of the product. A summary of the protocol is presented below.

1. All samples were obtained from local markets in China.
2. Stock solutions of the marker compounds were prepared in MeOH at concentrations of 362.36 μg/mL for ginsenoside Rg1, 488.92 μg/mL for ginsenoside Re, 429.74 μg/mL for ginsenoside Rb1, 556.93 μg/mL for glycyrrhizin, 340.47 μg/mL for liquiritin, 100.52 μg/mL for glycyrrhetinic acid, 149.30 μg/mL for 10-DAB III, 120.51 μg/mL for baccatin III, 1314.02 μg/mL for taxol, and 122.80 μg/mL for cephalomannine.
3. These stock solutions were mixed to obtain the combined solutions and then were diluted to yield a series of standard working solutions with different concentrations for linear validation.
4. Sample preparation of raw herbs: Three component herbs of this preparation, Hongdoushan, RenShen, and GanCao (10 g each), were dried, ground, and extracted to obtain extracts as outlined for the formula of Hongdoushan capsules. The extracts were dissolved in MeOH to prepare sample solutions.
5. Sample preparation of Hongdoushan capsules: A sample (0.3 g) was placed into a centrifuge tube and extracted ultrasonically (2 × 30 min) with MeOH (10 mL) at room temperature. The pooled extract was centrifuged for five minutes at 9000 g, the supernatant was removed into a 25 mL volumetric flask, and the constant volume was obtained with MeOH. All the solutions were filtered through a 0.45 μm membrane filter for HPLC analyses.
6. Each sample (5 μL) was analyzed by an optimized and validated HPLC-PDA method using a reversed-phase SHIMADZU C_{18} column (4.6 × 250 mm, 5 μm), coupled with a Phenomenex guard column (4.0 × 3.0 mm), setting the temperature at 25 °C and at a flow rate of 0.8 mL/min and utilizing a gradient mobile phase comprising acetonitrile (component A) and water (component B). The elution program was set as follows: 0−35 min kept at 19%A, 35−50 min linear increased from 19% to 55% A, 50−70 min kept at 55% A, 70−85 min linear decreased from 55% to 19% A. An HP1200 Agilent HPLC system equipped with a dual pump, an autosampler, and a PDA detector were used. Detection wave lengths were at 0−30 min (276 nm), 30−50 min (203 nm), 50−85 min (227 nm), and PDA spectra were recorded from 200 to 600 nm.
7. Retention times and associated UV−Vis spectral characteristics of the marker compounds were used to identify the presence of these compounds in the samples (six batches of Hongdoushan capsule).

19.5.1.5 Menoprogen Capsules

The Menoprogen capsule is a well-known traditional Chinese medicine formula generally prescribed for the management of menopause. Wang et al. [64] reported an HPLC-PDA-based quality control method for this formula, utilizing more than one marker compound

and incorporating a fingerprinting approach. A summary of this method is presented below.

1. Ten batches of Menoprogen capsules were obtained from Nanjing Moresoft Manufacturing Company, Nanjing, China.
2. The content of the capsules of each batch (1 g each) was extracted with MeOH (10 mL) with sonication for 60 min at room temperature, filtered through a 0.45 μm membrane filter before HPLC analysis.
3. Chlorogenic acid, rutin, hyperoside, quercetin, and kaempferol were used as marker compounds.
4. Each sample (5 μL) was analyzed by an optimized and validated HPLC-PDA method using a reversed-phase Kromasil ODS C_{18} column (4.6 × 250 mm, 5 μm), setting the temperature at 25 °C and at a flow rate of 1.0 mL/min and utilizing a step-gradient mobile phase comprising 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B) over 135 min: 0–50 min 10–20% B, 50–86 min 20–48% B, 86–135 min 48–100% B. An HP1100 Agilent HPLC system consisting of a vacuum degasser, thermostated column compartment, and a photodiode-array detector was used. Detection wave length was 205 nm.
5. While the marker compounds were identified by the retention times and respective UV–Vis spectra and quantified by peak area, the fingerprinting approach identified 21 "common peaks" representing the characteristic chemicals of this herbal product.

This multiple marker-based HPLC-PDA fingerprinting approach demonstrated a comprehensive quality evaluation method for Menoprogen capsules and for the chemical standardization and batch-to-batch consistency of this product.

19.5.1.6 Arjunarishta

Arjunarishta is an Ayurvedic cardioprotective polyherbal formulation. It is prepared by fermenting the decoction of the bark of *Terminalia arjuna*, fruits of *Vitis vinifera*, and flowers of *Madhuca indica* using flowers of *Woodfordia fruticosa*. Lal et al. [34] reported an HPLC-PDA-based standardization method for this formulation, utilizing more than one marker compound. A protocol of this method is outlined below.

1. The formulation (fermented decoction, 1 mL) was dried under vacuum, suspended in MeOH (5 mL), sonicated for 10 min, centrifuged at 3000 rpm, and the supernatant (1 mL) was filtered (0.45 μm).
2. Gallic acid, ethyl gallate, ellagic acid, quercetin, and kaempferol were used as marker compounds.
3. Sample (20 μL) was analyzed by an optimized and validated HPLC-PDA method using a reversed-phase Phenomenex C_{18} column (4.6 × 250 mm, 5 μm), setting the temperature at 25 °C and at a flow rate of 1.5 mL/min, and utilizing a binary step-gradient mobile phase consisting of 0.5% aqueous acetic acid (solvent A) and acetonitrile with 20% A (solvent B) over 50 min: 0–10 min 10% B, 10–20 min 10–20% B, 20–30 min 20–40% B, 30–40 min 40–60% B, 40–45 min 60–70% B, and 45–50 min 70–10% B. A Shimadzu HPLC system comprising LC-10AT pump, automated gradient controller, Shimadzu SPD-M10 A (Class VP-series, version 6.10) and Rheodyne 7725 I manual injector was used. Detection wave length was 210 nm.
4. While the marker compounds were identified by the retention times and respective UV–Vis spectra, peak area was used for quantification.

19.5.2 Specific Examples of Step-by-step Protocols: Chemical Fingerprinting

There are several examples available in the literature [21,65–76] that describe HPLC-based fingerprinting of various herbal products, both mono- and polyherbals. While probably because of low cost and simplicity, the use of HPLC-PDA seems to be the most common of all, many other sophisticated hyphenated techniques, e.g., LC-PDA-MS [21,67–69,74,76,77], are also used. For example, HPLC and LC-DAD-MS/MS were applied for the qualitative and quantitative analyses of Compound Kushen Injection, a well-known Chinese herbal product [74,78]. In that study, a simple fingerprinting approach was used; eight peaks were selected as the characteristic peaks in the HPLC chromatograms of 27 different batches of this herbal product. Peak identification was carried out by HPLC and LC-MS/MS analyses. A total of 21 chromatographic peaks were identified by comparison of retention time and UV spectra with authentic compounds as well as by summarized MS fragmentation rules. Some variations in the total amounts of marker compounds were observed in different batches of this product.

1.5.2 Some simple examples of step-by-step protocols of chemical fingerprinting of herbal products are presented below.

19.5.2.1 Curculiginis Rhizoma (Curculigo orchioides)

Curculiginis Rhizoma, the dry rhizome of *C. orchioides*, is a well-known Chinese herbal medicinal product and also an important Ayurvedic product used for the treatment of pain, inflammation, immune and hepatic disorders, and as an aphrodisiac [79]. Curculigoside, a phenolic glycoside, is considered to be the major contributor to the medicinal properties of this herbal product. However, as it is a well accepted fact that the overall therapeutic activity (and/or toxicity) of any herbal product is not necessarily owing to just one compound, but most often a result of synergistic effect of a number of

compounds present in the product, an HPLC-based chemical fingerprinting method aiming at the quality evaluation of this herbal product has recently been reported by Bian et al. [79]. A summary of the protocol is presented below.

1. Ten different samples of Curculiginis Rhizoma were collected from China.
2. Each sample (2 g) was refluxed with MeOH (45 mL) for 60 min, and the resulting mass was centrifuged at 3000 rpm for 15 min.
3. The supernatant was transferred to a 50 mL volumetric flask, MeOH was added to bring it to the 50 mL mark, and finally it was filtered through a cellulose acetate membrane (0.45 μm) for HPLC analysis.
4. Each sample (10 μL) was analyzed by an optimized and validated HPLC-PDA method using an Altima C_{18} ODS column (4.6 × 250 mm, 5 μm), setting the detection/monitoring wavelength at 220 nm and temperature at 30 °C, employing solvent A: acetonitrile with 0.02% TFA and solvent B: water with 0.5% TFA in a step-wise gradient (7−30% A in B over 35 min) and a flow rate of 1.0 mL/min. An Agilent 1100 liquid chromatography system, equipped with the G1311A Quatpump solvent delivery system, UV−Vis photodiode array detector G1315B, and degasser G1322A was used.
5. Similarity analysis to establish similarities of different chromatograms was carried out by Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A), as recommended by the State Food and Drug Administration of China (SFDA). Correlation coefficient was used as a measure for similarity analysis.
6. Out of 20 separated peaks, 11 peaks were found in all samples and were termed as "common peaks."

It can be noted that for any HPLC-based fingerprinting analysis, all HPLC conditions have to be optimized prior to any sample run, and all chromatograms must be standardized. The process of standardization generally involves the selection of "common peaks" in chromatograms and the normalization of retention times of all the common peaks. Relative retention time and relative peak area of each characteristic peak related to the reference peak are usually calculated for the quantitative parameters of chemical properties in the chromatographic pattern and for similarity analysis of herbal products.

19.5.2.2 *Qianghuo* (Notopterygium forbesii *or* N. Incium)

Qianghuo is a popular Tibetan and Chinese herbal medicinal product used as a diaphoretic, an antifebrile, and an anodyne. The roots and rhizomes of *Notopterygium forbesii* and *N. incium*, both from the Apiaceae family, are included in the Chinese Pharmacopoeia under the same name (Qianghuo in Chinese). An HPLC-PDA based fingerprinting quality control method was reported by Jiang et al. [80]. Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004 A), as in the above example (see Section 5.2.1), was employed to normalize the chromatographic peaks, to calculate the correlation coefficients between entire chromatographic profiles, and to perform quantitative standard fingerprint/chromatogram for a group of chromatograms. Besides, the relative retention time and relative peak area of each characteristic peak related to the reference peak were calculated for quantification of the chemical properties in the chromatographic pattern of the product. A step-by-step protocol of this method is summarized below.

1. Eighteen different samples of Qianghuo, 15 containing *N. forbesii* and three containing *N. incium*, were collected from China.
2. Each sample (2 g) was extracted with distilled water (25 mL) using an ultrasonic water bath for 15 min. The extraction was repeated twice, and extracts were mixed and filtered.
3. The filtered extract was precipitated by addition of ethanol (final concentration was 50% v/v) at 4 °C for 12 h and filtered again, and diluted by water to the final volume of 100 mL. The final extract solution was filtered again (0.45 μm filter) prior to HPLC analysis.
4. Each sample (10 μL) was analyzed by an optimized and validated HPLC-PDA method using a reversed-phase Thermo C_{18} column (4.6 × 250 mm, 5 μm), setting the detection/monitoring wavelength at 310 nm and column temperature at 30 °C, employing solvent A: water with 0.1% phosphoric acid (H_3PO_4) and solvent B: acetonitrile in a step-linear gradient (10−30% B in A over 12 min; 30−100% B in A in 12−25 min) and at a flow rate of 1.0 mL/min. An Agilent HP 1100 series HPLC-DAD system consisting of a vacuum degasser, thermostated column chamber, and a DAD was used. Ferulic acid, imperatorin, and isoimperatorin were used as standards.
5. Similarities of different chromatograms by calculating the correlative coefficient and/or cosine value of vectorial angle was determined by Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A), as recommended by the State Food and Drug Administration of China (SFDA).
6. A total of 10 peaks were separated, and the highest peak (ferulic acid) in the chromatogram was assigned as the reference peak.

The selection of detection wavelength (310 nm) was one of the key factors that contributed to the reliability and reproducibility of Qianghuo. The relative retention time and relative peak area for all common peaks were determined in comparison with the ferulic acid peak (reference peak).

19.5.2.3 *Ayurved Siriraj Prasachandaeng—An Antipyretic Product*

For the quality control of the Thai traditional medicinal polyherbal product, Ayurved Siriraj Prasachandaeng, HPTLC and HPLC-based fingerprinting approaches were adopted [35]. This product, sold as a powder form, contains 12 medicinal herbs. The examples shown in Sections 5.2.1 and 5.2.2 are monoherbal products and are certainly much easier to deal with, especially in relation to fingerprinting, than a polyherbal product like this one. Relative retention time and applied information content were evaluated in the HPLC fingerprints. Gallic, caffeic, and vanillic acids were used as qualitative and quantitative markers in HPLC. Similarity of the chromatographic pattern among batches was determined by the presence of mathematical parameters in the range of 80−125% of the average. Twelve medicinal herbal components and three batches of the product were analyzed. The chromatograms from three batches were regarded as the original fingerprint of this product. A summary of steps involved in the fingerprinting of this product is presented below.

1. Three batches of Ayurved Siriraj Prasachandaeng and its 12 components (see Table 19.4), a total of 15 samples, were obtained from the Herbal Medicines and Products Manufacturing Unit, Ayurved Siriraj, Faculty of Medicine, Siriraj Hospital, Mahidol, Thailand.
2. All 15 samples were extracted separately in 80% ethanol and lyophilized to dry powder.
3. Each powder was dissolved separately in 50% aqueous MeOH for HPLC and then filtered through a 0.2 μm membrane filter.
4. Reference markers, gallic acid, caffeic acid, and vanillic acid (1 mg each) were dissolved separately in 50% aqueous MeOH. Each solution was vortexed for two minutes and filtered through a 0.2 μm membrane filter.
5. Each sample (10 μL) was analyzed by an optimized and validated HPLC-PDA method using a reversed-phase C_{18} column (4.6 × 250 mm, 5 μm), setting the column temperature at 30 °C, employing solvent A: *o*-phosphoric acid (0.1% v/v) and solvent B: acetonitrile in an isocratic condition of 95% A and 5% B for the first four minutes, a step-gradient elution of 95-85% A at 4−8 min; 85−0% A at 8−13 min and 0−95% A at 13−16 min and at a flow rate of 1.0 mL/min. The UV spectra were recorded over the range of 200−400 nm.
6. Similarity of the chromatographic pattern among batches was determined by the presence of mathematical parameters in the range of 80−125% of the average.

19.5.2.4 *Radix Aconiti (Roots of* **Aconitum carmichaeli***)*

A fingerprinting approach was introduced by means of UPLC-ESI/MSn (ultra performance liquid chromatography-electrospray ionization/mass spectrometry) for the quality control of the Chinese herbal product Radix Aconiti, a widely used toxic traditional herbal medicine [76]. This approach was founded on two processing methods as recorded in the Chinese Pharmacopoeia for the purpose of reducing the toxicity and ensuring the therapeutic efficacy. Various data processing approaches, e.g., similarity evaluation, hierarchical cluster analysis, and principal component analysis (PCA), were carried out to assess the similarity and variation among the samples. A summary of the protocol of this fingerprinting method is presented below.

1. Sample processing and extraction: all samples were processed according to the Chinese Pharmacopoeia 2010. Briefly, Radix Aconiti was cleaned and soaked with water until it was wet thoroughly, water was drained and replaced with fresh water, and the water was exchanged with fresh water and boiled for one to six hours or steamed for one to eight hours. The samples boiled for four, five, and six hours and those steamed for six, seven, and eight hours were designated as the qualified Radix Aconiti. The other Radix Aconiti samples were unqualified ones. Each of the boiled or steamed samples was sliced, dried (50 °C), ground, and sieved (0.45 μm). Ground sample (1.0 g) was placed in a sealed vessel by adding 0.5 mL of 10% aqueous ammonia solution, and diethyl ether (50 mL) was added and extracted using an ultrasonic bath for 30 min at 25 °C. The extraction was repeated three times, pooled together, dried at 50 °C, and the dried extract was stored at −80 °C. For HPLC analyses, the extracts were dissolved in MeOH-water (1:1, 5 mL) and 0.02 mg/mL of reserpine was added as an internal standard. All solutions were filtered through a membrane (0.22 μm).
2. Standard solutions: stock solutions of aconitine-type alkaloids (aconitine, hypaconitine, mesaconitine, benzoylmesaconine, benzoylhypacoitine, and benzoylaconine) were prepared in dichloromethane and kept at −80 °C. An internal standard solution comprising 0.02 mg/mL reserpine in 1:1 MeOH-water was used for further dilution of all standards.

3. HPLC-PDA analysis: each sample (5 μL) was analyzed by an optimized and validated HPLC-PDA as well as a UPLC-ESI/MS method using a reversed-phase Waters ACQUITY UPLC BEH C_{18} column (2.1 × 50 mm, 1.7 μm), setting the column temperature at 30 °C, employing solvent A: water containing 5 mM ammonium bicarbonate and adjusted to pH 10.5 with ammonia, and solvent B: MeOH in a gradient elution mode: 0−5 min from 65% A to 57% A, 5−10 min 57% A to 55% A, 10−30 min from 55% A to 40% A, 30−55 min from 40% A to 10% A, and 55−75 min from 10% A to 5% A, and at a flow rate of 0.3 mL/min. The UV−Vis spectra were recorded over the range of 200−600 nm, but the chromatogram was monitored at 235 nm, which was suitable for aconitine-type alkaloids. An Accela UPLC system equipped with an Accela 1250 pump, autosampler, DAD, and an LTQ ion trap mass spectrometer was used.
4. UPLC-MS analysis: The ESI source of the mass spectrometer was connected to the UPLC system via a capillary to the UV cell outlet. Spray voltage was set at 4.5 kV in the positive ion mode, sheath gas flow rate at 40.5 L/h, and aux gas flow rate at 90 L/h. The capillary temperature was maintained at 250 °C. The scan range was m/z 300−1000 Da. Collision energies for the MS^2 analyses ranged from 25−40 eV, depending on the mass of the precursor ion. Helium (He) was used as the collision gas for the MS^n.
5. For chemometric analysis of the data obtained from the HPLC runs, similarity evaluation (to assess the consistency) [81,82], hierarchical clustering analysis (to classify samples based on the similarities of their chemical properties) [19,24], and principal component analysis (to feature extraction and dimensionality reduction aiming at reduction of data dimensionality and provision of an overview of class separation, clustering, and outliers) were carried out [83].

In this UPLC-UV and UPLC-ESI/MS^n fingerprint study, 39 characteristic MS peaks and 34 UV chromatogram peaks in the common pattern were identified to further characterize the chromatographic fingerprint and contribute to the quality control of this Radix Aconite herbal product through successful application of chemometrics.

19.5.2.5 Folium Turpiniae

Folium Turpiniae, a well-known traditional Chinese medicine derived from *Turpinia arguta* (Staphyleaceae), is used for the treatment of abscesses, fevers, gastric ulcers, and inflammations [84]. A strategy incorporating HPLC-PDA and quadrupole TOF-MS detection, as well as phytochemical and chemometrics analysis (and fingerprinting) for the characterization, isolation, and simultaneous quantification of the chemical constituents of Folium Turpiniae has recently been reported [84]. Nine marker compounds were quantified simultaneously in 10 batches of Folium Turpiniae collected from different regions. Hierarchical clustering analysis (HCA), which is a useful multivariate statistical technique to assign a data set into groups by creating a cluster tree or dendrogram, and principal component analysis (PCA, based on area of common peaks in chromatograms) revealed notable similarities (or differences) among various samples. A summarized protocol is outlined below.

1. Ten batches of samples of Folium Turpiniae were obtained from different parts of China.
2. Nine compounds, ellagic acid-3-*O*-α-L-rhamnopyranoside, apigenin-7-*O*-(2′-rhamnosyl)-gentiobioside, luteolin-7-*O*-β-D-neohesperidoside, ligustroflavone, apigenin-7-*O*-β-D-(6′-rhamnosyl) glucoside, 4′-*O*-methylellagic acid-3-*O*-α-L-rhamnopyranoside, rhoifolin, neobudofficide, and apigenin-7-*O*-β-D-(2′-*O*-α-rhamnosyl)glucuronide, all with >98% purity, were used as marker compounds.
3. Each sample powder (0.5 g) was transferred to a 100 mL round-bottom flask and mixed with 50% MeOH (50 mL), refluxed for 15 min, and cooled to room temperature. MeOH was added to top it up to 50 mL and filtered through a 0.45 μm membrane before injection into the HPLC.
4. Each sample (10 μL) was analyzed by an optimized and validated HPLC method using a reversed-phase Hanbon Phecda C_{18} column (4.6 × 250 mm, 5 μm) with a standard guard column, setting the column temperature at 35 °C, employing solvent A: 1% formic acid (containing 2 mmol/L ammonium acetate) and solvent B: acetonitrile, in a gradient elution mode: 3−9% B at 0−10 min, 9−12% B at 10−26 min, 12−14% B at 26−32 min, 14−17% B at 32−40 min, 17% B at 40−48 min, 17−22% B at 48−58 min, 22−38% B at 58−70 min, and 38−60% B at 70−74 min, and at a flow rate of 1.0 mL/min. The UV−Vis spectra were recorded over the range of 200−600 nm, but the chromatogram was monitored at 262 nm. A Shimadzu LC2010 HPLC system coupled with a quaternary pump, online vacuum degasser, autosampler, thermostat column chamber, PDA detector, and LC solution software was used.
5. The HPLC-PDA system was interfaced to an Agilent 6520 Q-TOF time-of-flight (TOF) mass spectrometer equipped with an electrospray interface operating under the chromatographic conditions mentioned above. The optimized MS operating conditions were negative and positive ion mode, scan spectra from m/z 100−1000, drying gas (N_2) with a flow rate of 8.0 L/min, drying gas temperature of 325 °C, nebulizer

pressure of 40 psi, capillary voltage of 3500 V, skimmer of 65 V, and fragmentor voltage of 120 V. The sample collision energy was set at 35 V. Data were processed using MassHunter Workstation Data Acquisition Software Ver. A.01.00.

6. Samples were analyzed by HCA and PCA using SPSS software (SPSS 11.5, SPSS) and the areas of 31 "common peaks" in chromatograms were selected as the variables.

Using the hyphenated technique HPLC-DAD-Q-TOF-MS (where multiple detectors were used) and phytochemical and chemometrics analyses, it was possible to develop qualitative and quantitative analytical methods for Folium Turpiniae herbal Medicinal products from different regions.

19.5.2.6 Dioscorea zingiberensis

Dioscorea zingiberensis is a Chinese medicinal plant, which is the major component of a traditional Chinese medicinal product. Steroidal saponins have already been shown to be the major bioactive compounds responsible for the therapeutic efficacy of this product [85]. HPLC-based quality control involving saponins as the markers is not an easy task, but Zhang et al. [86] have managed to establish a quality control method for *D. zingiberensis* utilizing a fingerprint approach involving HPLC coupled with evaporative light scattering detector (HPLC-ELSD) and the simultaneous characterization of the steroid saponins by HPLC coupled with electrospray ionization-mass spectrometry and quadrupole tandem time-of-fight mass analyzers detection (HPLC-ESI-Q/TOF). A summarized protocol is outlined below.

1. Twenty batches of samples of *D. zingiberensis* were obtained from different parts of China.
2. Twelve saponins (purity >98%), 10 previously isolated and identified by the authors [86] and two purchased from a commercial supplier, were used as marker compounds.
3. Stock solution (5 mg/mL) of each of these markers was prepared in MeOH, several dilutions were carried out in MeOH, and the resulting solutions were kept at 4 °C for subsequent use.
4. Each sample powder (5 g) was refluxed three times with 50 mL of 70% ethanol at 80 °C. Pooled extracts were evaporated to dryness under a vacuum, and the residue was redissolved in water (30 mL) and centrifuged.
5. The supernatant obtained after centrifugation was passed through aD-101 macroporous resin column (2 × 15 cm) eluting first with water, then with 20% ethanol until the effluent was colorless, and finally with 70% ethanol (80 mL), which was concentrated and transferred to a 25 mL volumetric flask. MeOH was added to top it up to the 25 mL mark and it was filtered through a 0.22 μm membrane before injection into the HPLC.
6. A fully validated HPLC fingerprinting analysis [86] of each sample (10 μL) was performed on a Waters Alliance 2695 HPLC equipped with an online vacuum degasser, a high-pressure quaternary pump, an autosampler, and an Alltech 2000 evaporative light scattering detector (ELSD), utilizing a Welchrom C_{18} column (250 × 4.6 mm, 5 μm) with a binary gradient elution as follows: acetonitrile as solvent A and water as solvent B, initial 25% A, 0–5 min linear gradient 25–30% A, 5–20 min at 30% A, 20–35 min from 30 to 35% A, 35–45 min 35–47% A, 45–47 min 47–70% A, 47–60 min at 70% with a flow rate of 1 mL/min. The effluent was introduced into the Alltech 2000 ELSD, in which the drift tube temperature was 90 °C and the gas flow rate at 2.8 L/min. Data analyses were carried out on an Empower Workstation.
7. A Varian 212-LC equipped with a Q-TOF Premier, a quadrupole, and orthogonal acceleration time-of-flight tandem mass spectrometer, linked to an electrospray ionization interface, was used for the HPLC-ESI-Q/TOF analyses of the samples. MS data were recorded by a Varian MS Workstation software. High purity N_2 gas was used as the nebulizer and auxiliary gas and argon as the collision gas. The same HPLC conditions, including the column, elution program, and flow rate as outlined above were applied. A portion of the column effluent (0.2 mL/min) was delivered into the ion source of the mass spectrometer after a micro split. The conditions of the ESI source were drying gas (N_2) flow rate 9.0 L/min, drying gas temperature 350 °C, nebulizer 35 psig, capillary voltage 5000 V, and spectral scanning range from m/z 100 to 1500.
8. Data processing for the fingerprinting analysis was achieved by using the software named the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A) according to the recommendation made from the State Food and Drug Administration (SFDA) of China, which is mainly applied in the similarity study of chromatographic and spectral patterns. This software helped to synchronize the chromatographic peaks and to calculate the correlation coefficients between entire chromatographic profiles, as well as to compute and generate the mean chromatogram as a representative standard fingerprint chromatogram from a group of chromatograms. The similarities of different chromatographic patterns were evaluated among samples.

The chromatographic patterns obtained from the HPLC-ELSD analyses of 20 different samples were

generally consistent and similar in terms of the presence of similar chemical compounds (saponins), albeit the intensity of the peaks was variable. Out of 12 marker saponins, 10 could be easily determined by the ESI-MS analyses. Among the samples, a total of 68 "common characteristic" peaks, including 22 new steroid saponins with eight aglycone skeletons in the fingerprint, were detected. Zhang et al. [86] demonstrated that trivial HPLC-based fingerprinting, when combined with the online HPLC-MS method, could become a powerful tool in the quality control of herbal products and in the analysis of their chemical constituents. However, a similar approach was previously reported by Man et al. [87].

19.5.2.7 Yuanhu Zhitong Tablet

The Yuanhu Zhitong tablet is a Chinese polyherbal product consisting of *Angelica dahurica* (223 g) and Rhizoma *Corydalis* (445 g) processed with vinegar, and it has been used to treat gastralgia, costalgia, headache, and dysmenorrhea in China [88]. Tang et al. [78] have recently reported a quality control strategy for quantitative and qualitative analysis of "common peaks" in the chemical fingerprint of Yuanhu Zhitong tablets, using photo-diode-array detector and tandem mass spectrometry (HPLC-DAD−MS/MS). A brief outline of this approach is presented below.

1. Twelve batches of Yuanhu Zhitong tablets were obtained from various pharmaceutical companies in China [78].
2. Ten marker compounds (purity >98%) [78] were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).
3. Coatings of the tablet samples were removed, and the content was ground into fine powder. Each ground sample (1.0 g) was extracted with MeOH (35 mL) ultrasonically for 30 min, volume was made to 50 mL with MeOH, filtered, and evaporated to dryness on a water bath (70 °C). The residue was redissolved in MeOH (5 mL) and centrifuged (15,000 rpm) for 10 min. The supernatant was filtered (0.45 μm filter) and transferred to an autosampler vial for HPLC-DAD-ESI-MS/MS analysis.
4. According to the prescription and preparation protocol of Yuanhu Zhitong tablets recorded in China Pharmacopoeia, two negative control samples without Radix, *Angelica dahurica* or Rhizoma *Corydalis*, were prepared to validate the specificity of the method.
5. Stock solution of each of the 10 markers was prepared in MeOH, or MeOH-water (1:1). Several dilutions were carried out in MeOH, and the resulting solutions were kept at 4 °C for subsequent use.
6. A fully validated HPLC quantification and fingerprinting analysis [78] of each sample (5 μL) was performed on an Agilent 1260 series HPLC, utilizing an Agilent Eclipse plus C_{18} column (250 × 4.6 mm, 5 μm) with a binary gradient elution as follows: 0.4% ammonium acetate aqueous solution (pH adjusted to 6.0 with glacial acetic acid) as solvent A and acetonitrile as solvent B, initial 17% B, 0−25 min linear gradient 17−19% B, 25−55 min at 19% B, 55−70 min from 19 to 25% B, 70−80 min 25−28% B, 80−95 min 28−34% B, 95−120 min 34−35% B, 120−140 min 35−42% B and 140−160 min 42−50% B; flow rate 1 mL/min; column temperature at 30 °C; quantitative detection wavelength at 254 nm (xanthotoxin, bergapten, imperatorin and isoimperatorin), 270 nm (berberine), 280 nm (protopine and tetrahydropalmatine) or 345 nm (jatrorrhizine, coptisine, and palmatine), while the wavelength of fingerprinting analysis was set at 280 nm.
7. The above Agilent HPLC system was interfaced with an Agilent 6460 Triple Quadrupole mass spectrometer in a post-column splitting ratio of 4:1. The conditions of ESI source were source voltage 3000 V; drying gas (N_2) flow rate10.0 L/min; drying gas temperature 320 °C; and nebulizer 25 psi. The MS data were acquired from m/z 100 to1000 in positive ion modes. LC-ESI-MS/MS was used to identify "common peaks" in each sample. In the full scan mass spectra, most of the constituents exhibited their pseudomolecular ions $[M + H]^+$ in positive ion mode under the soft electrospray ionization mode. Precursor ions were subjected to collision-induced dissociation to generate the fragment ions, and the fragmentation patterns were proposed for the structural identification of constituents.
8. Data analysis was carried out by the software, Similarity Evaluation System for Chromatographic Fingerprint of TCM (Version 2004A). The relative retention time and relative peak area of each "common peak" related to the marker peaks were calculated for quantitative expression of the chemical properties in the chromatographic pattern of the tablet samples. Based on this, the correlation coefficients of entire chromatographic profiles of samples were calculated, while the simulative mean chromatogram was generated.

A total of 40 peaks were assigned as the "common peaks." For quantification of "common peaks," the detection wavelength was set at 254, 270, 280, or 345 nm [78]. Ten analytes, e.g., protopine, jatrorrhizine, coptisine, palmatine, berberine, xanthotoxin, bergapten, tetrahydropalmatine, imperatorin, and isoimperatorin, were simultaneously determined. For qualification

of "common peaks," 33 compounds including 10 quantitative analytes were identified or tentatively characterized using LC-MS/MS.

19.6 CONCLUSIONS

Hyphenated techniques, originating from the coupling between HPLC and various detection techniques, e.g., UV−Vis, PDA, MS, and NMR, have become indispensable tools for analyzing various complex matrices, including herbal products. Much of the applications of HPLC-based analyses of herbal products are associated with authentication, standardization, and quality assurance/control of various herbal products and often provide better outcome than other conventional techniques. Quality control of herbal products aims to ensure its quality, safety, and efficacy. HPLC analyses involving chemical markers, as well as fingerprinting with chemometrics, are pivotal in the current practice of quality control of such products. Nowadays, to obtain a comprehensive understanding of the characteristic components of an herbal product, chemical fingerprints from hyphenated techniques, especially involving HPLC, are strongly recommended for the purpose of quality control, since they might represent appropriately the "chemical integrities" of the herbal products and therefore be used for authentication and identification. With the continuing progress in computational technology, hyphenation efficiency, and affordability, it is envisaged that HPLC-based analysis of herbal products will become even more attractive to the analysts dealing with efficacy and safety of herbal products in the years to come.

References

[1] Choi CK, Dong MW. Sample preparation for HPLC analysis of drug products. Sep Sci Technol 2005;6:123−44.

[2] Waksmundzha-Hajnos M, Oniszczuk A, Szewczyk K, Wianowska D. Effect of sample preparation methods on the HPLC quantification of some phenolic acids in plant materials. Acta Chromatogr 2007;19:227−37.

[3] Sadek PC. Sample preparation techniques for HPLC and GC analyses. Chichester: John Wiley and Sons; 2008.

[4] Pan J, Zhang C, Zhang Z, Lim G. Review of online coupling of sample preparation techniques with liquid chromatography. Anal Chim Acta 2014;815:1−15.

[5] Wen Y, Chen L, Li J, Liu D, Chen L. Recent advances in solid-phase sorbents for sample preparation prior to chromatographic analysis. Trends Anal Chem 2014;59:26−41. http://dx.doi.org/10.1016/j.trac.2014.03.011.

[6] Li DQ, Zhao J, Xie J, Li SP. A novel sample preparation and on-line HPLC-DAD-MS/MS-BCD analysis for rapid screening and characterization of specific enzyme inhibitors in herbal extracts: case study of α-glucosidase. J Pharm Biomed Anal 2014;88:130−5.

[7] Huie CW. A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. Anal Bioanal Chem 2002;373:23−30.

[8] Kirkland JJ. Development of some stationary phases for reversed-phase high-performance liquid chromatography. J Chromatogr A 2004;1060:9−21.

[9] Latif Z, Sarker S. Isolation of natural products by preparative high performance liquid chromatography (prep-HPLC). In: Natural products isolation. 3rd ed. USA: Humana Press − Springer-Verlag; 2012.

[10] Moldoveanu SC. Mobile phases and their properties. In: Moldoveanu MC, David V, editors. Essentials in modern HPLC separations. Elsevier; 2013. p. 363−447.

[11] Eurby MR, Petersson P. Chromatographic classification and comparison of commercially available reversed-phase liquid chromatographic columns using principle component analysis. J Chromatogr A 2003;994:13−26.

[12] Snyder LR, Kirkland JJ, Glajch JL. Practical HPLC method development. 2nd ed. New York: John Wiley and Sons; 1997.

[13] Dolan JW. Starting out right, part 3-the role of the solvent in controlling selectivity. LC-GC Europe 2000;13:148−56.

[14] Dolan JW. Starting out right, part 6 − the scouting gradient alternative. LC-GC Europe 2000;13:388−94.

[15] Dolan JW. How much is enough? LC-GC Europe 2003;16:740−5.

[16] Pratima NA, Shraddha B, Zibran S. Review of ultra performance liquid chromatography and its applications. Int J Res Pharm Sci 2013;3:19−40.

[17] Qi Y, Li S, Pi Z, Song F, Lin N, Liu S, et al. Chemical profiling of Wu-tou decoction by UPLC-Q-TOF-MS. Talanta 2014;118:21−9.

[18] Novakova L, Matysova L, Solich P. Advantages of application of UPLC in pharmaceutical analysis. Talanta 2006;68:908−18.

[19] Yang G-L, Yang L-W, Li Y-X, Cao H, Zhou W-L, Fang Z-J, et al. Applications of ultra performance liquid chromatography to traditional Chinese Medicines. J Chromatogr Sci 2010;48:19−21.

[20] Sarker S, Nahar L. Hyphenated techniques and their applications in natural products analysis. In: Natural products isolation. 3rd ed. USA: Humana Press − Springer-Verlag; 2012.

[21] Li M, Hou X-F, Zhang J, Wang S-C, Fu Q, He L-C. Applications of HPLC/MS in the analysis of traditional Chinese medicines. J Pharm Anal 2011;1:81−91.

[22] Shaw L-H, Lin L-C, Tsai T-H. HPLC-MS/MS analysis of a traditional Chinese medical formulation of Bu-Yang-Huan-Wu-Tang and its pharmacokinetics after oral administration to rats. Plos One 2012;7:e43848.

[23] McMaster MC. LC-MS: a practical user's guide. New Jersey: John Wiley & Sons; 2005.

[24] Yang DZ, Yin XX, Ong CN, Tang DQ. Multidimensional information-based HPLC technologies to evaluate traditional Chinese medicine. J Chromatogr Sci 2013;51:716−25.

[25] Wold S. Chemometrics: what do we mean with it, and what do we want from it? Chemom Intell Lab Sys 1995;30:109−15.

[26] Brenton RG. Chemometrics: data analysis for the laboratory and chemical plant. Chichester: John Wiley and Sons; 2003.

[27] Gemperline P. Practical guide to chemometrics, CRC. Boca Raton: Florida; 2006.

[28] Jing D, Linfang H. Application of chemometrics in quality evaluation of medicinal plants. J Med Plants Res 2006;5:4001−8.

[29] Lavine B, Workman J. Chemometrics. Anal Chem 2010;82: 4699−711.

[30] Bansal A, Chhabra V, Rawal RK, Sharma S. Chemometrics: a new scenario in herbal drug standardization. J Pharm Anal 2014;4: 223−33. http://dx.doi.org/10.1016/j.jpha.2013.12.001.

[31] Jolliffe IT. Principal component analysis. 2nd ed. Germany: Springer-Verlag; 2002.

[32] Gad HA, El-Ahmady SH, Abou-Shoer MI, Al-Azizi MM. Application of chemometrics in authentication of herbal medicines: a review. Phytochem Anal 2012;24:1−24.

[33] Chowdhury HR, Mondal S. Morphological and ethnomedicinal consideration of Euphorbia fusiformis Buch.-Ham.

Ex D. Don: some new observations from district Birbhum, West Bengal, India. Int J Res Ayurveda Pharm 2012;3:113–6.
[34] Lal UR, Tripathi SM, Jachak SM, Bhutani KK, Singh IP. HPLC analysis and standardisation of Arjunarishta – an Ayurvedic cardioprotective formulation. Sci Pharm 2009;77:605–16.
[35] Akarasereenont P, Thitilertdecha P, Chotewuttakom S, Palo T, Seubnooch P, Wattanarangsan J, et al. Chromatographic fingerprint development for quality assessment of "Ayurved Siriraj Prasachandaeng" antipyretic drug. Siriraj Med J 2010;62:4–8.
[36] Rane R, Gangolli D, Salkar K, Chotalia C, Suthar A. Application of classical and instrumental methods for evaluation of polyherbal capsule formulation. Int J Res Ayurveda Pharm 2014;5:23–5.
[37] Zhu LC, Yang X, Tan J, Wang BC, Zhang X. A validated high performance liquid chromatograph-photodiode array method for simultaneous determination of 10 bioactive components in compounds hongdoushan capsule. Pharmacogn Mag 2014;10: 83–8.
[38] Sulaiman SM, Rajashekhar G, Prakash PJ, Singh DS, Saleem C. Evaluation of safety and immunotoxicity of Immunol, a polyherbal formulation in rats. J Pharm Toxicol 2010;5:262–74.
[39] Tejasvi PR, Satyavati D, Begum SA. Anxiolytic activity of Jessica – *a polyherbal formulation*. Int J Pharm Pharm Sci 2013;5:384–8.
[40] Hewavithana T, Ranaweera KKDS, Tissera MHA, Yapa PAJ. Use of physicochemical properties, chromatographic and spectrophotometric measurements in the standardisation of Srilankan polyherbal formulation "Maha Varthikava Watee". Intl J Res Ayurveda Pharm 2012;3:493–6.22.
[41] Soni H, Patgiri B, Bhatt S. Quantitative determination of three constituents of Rasayana Churna (a classical Ayurvedic formulation) by reversed phase HPLC. Int J Res Ayurveda Pharm 2014;5:17–22.
[42] Kajaria DK, Gangwar M, Kumar D, Sharma AK, Tilak R, Nath G, et al. Evaluation of antimicrobial activity and bronchodialator effect of a polyherbal drug-Shrishadi. Asian Pac J Trop Biomed 2012:905–9.
[43] Neergheen-Bhujun VS, Munogee N, Coolen V. Antioxidant and anti-inflammatory efficacies of polyherbal formulations and elixirs traditionally used in Mauritius for the treatment of rheumatoid arthritis. J Herbal Med 2014;4:1–9.
[44] Chouhan B, Kumawat RC, Kotecha M, Ramamurthy A, Nathani S. Triphala: a comprehensive Ayurvedic review. Int J Res Ayurveda Pharm 2014;4:612–7.
[45] Balammal G, Babu MS, Reddy PJ. Analysis of herbal medicines by modern chromatographic techniques. Int J Preclin Pharm Res 2012;3:50–63.
[46] Liang Y-Z, Xie P, Chan K. Quality control of herbal medicines. J Chromatogr B 2004;812:53–70.
[47] Gao W, Yang H, Qi LW, Liu EH, Ren MT, Yan YT, et al. Unbiased metabolite profiling by liquid chromatography-quadrupole time-of-flight mass spectrometry and multivariate data analysis for herbal authentication: classification of seven Lonicera species flower buds. J Chromatogr A 2012;1245:109–16.
[48] Wang LB, Wang XB, Kong LY. Automatic authentication and distinction of Epimedium joreanum and Epimedium wushanense with HPLC fingerprint analysis assisted by pattern recognition techniques. Biochem Syst Ecol 2012;40:138–45.
[49] Gao X, Yang XW, Marriott PJ. Evaluation of Coptidis Rhizoma-Euodiae Fructus couple and Zuojin products based on HPLC fingerprint chromatogram and simultaneous determination of main bioactive constituents. Pharm Biol 2013;51:1384–92.
[50] Zou H-B, Du A-Q, Zhang X-L, Wei P-H, Lu W-J, Yang G-S, et al. Quality control methodology and their application in analysis on HPLC fingerprint spectra of herbal medicine. Chromatogr Res Int 2012;2012:1–12.
[51] Reyes GCDL, Koda RT. Determining hyperforin and hypericin content in eight brands of St John's wort. Am J Health-Syst Pharm 2002;59:545–7.
[52] Wang B, Shen L, Cong WJ, Lin X, Feng Y, Zhu YY, et al. A simple HPLC method for simultaneous analysis of 7 bioactive constituents for Liuwei Dihuang pill and its application in quality consistency evaluation. Anal Methods 2013;5:2384–90.
[53] Kim JH, Shin HK, Seo CS. Simultaneous determination of 13 chemical marker compounds in Gwakhyangjeonggi-san, an herbal formula, with validated analytical methods. Nat Prod Commun 2014;9:65–9.
[54] Lazarowych NJ, Pekos P. Use of fingerprinting and marker compounds for identification and standardisation of botanical drugs: strategies for applying pharmaceutical HPLC analysis to herbal products. Drug Inf J 1998;32:497–512.
[55] Yuan D, Yamamoto K, Bi K, Zhang P, Liu F, Kano Y. Studies on the marker compounds for standardisation of traditional Chinese medicine "*Polyporus sclerotium*". Yakugaku Zasshi 2003;123: 53–62.
[56] Tang W, Wan M, Zhu Z, Chen G, Huang X. Simultaneous determination of eight major bioactive compounds in Dachengqi Tang (DT) by high-performance liquid chromatography. Chin Med 2008;3:1–6.
[57] Kushwaha SKS, Kushwaha N, Maurya N, Rai A. Role of markers in the standardisation of herbal drugs: a review. Arch Appl Sci Res 2010;2:225–9.
[58] Kwok K-Y, Xu J, Ho H-M, Chen H-B, Li M, Lang Y, et al. Quality evaluation of commercial Huang-Lian-Jie-Du-Tang based on simultaneous determination of fourteen major chemical constituents using high performance liquid chromatography. J Pharm Biomed Anal 2013;85:239–44.
[59] Malasoni R, Srivastava A, Pandey RR, Srivastava PK, Swivedi AK. Development and validation of improved HPLC method for the quantitative determination of curcuminoids in herbal medicament. J Sci Ind Res 2013;72:88–91.
[60] Weon JB, Ma JY, Yang HJ, Lee B, Yun B-R, Ma CJ. Qualitative and quantitative analysis of nine majot compounds in the Bozhougyiqi-Tang using a high-performance liquid chromatography coupled with a photodiode array detector and electrospray ionization mass spectrometer. Pharmacogn Mag 2013;9:271–82.
[61] Sudani RJ, Akbari BV, Vidyasagar G, Sharma P. Quantitative and chromatographic fingerprint analysis of *Embelia ribes* churna formulations by HPLC method. Int J Pharm Biol Arch 2011;2:657–63.
[62] Basar N, Talukdar AD, Nahar L, Stafford A, Kushiev H, Kan A, et al. A simple semi-preparative recersed-phase HPLC/PDA method for separation and quantification of glycyrrgizin in nine samples of Glycyrrhiza glabra root collected from different geographical origins. Phytochem Anal 2014;25:399–404. http://dx.doi.org/10.1002/pca.2507.
[63] Ghafari S, Esmaelli S, Aref H, Naghibi F, Mosaddegh M. Qualitative and quantitative analysis of some brands of Valerian pharmaceutical products. Ethnomedicine 2009;3:61–4.
[64] Wang GL, Wei M, Wang J, Lu Y, Mahady GB, Liu D. High-performanceliquid chromatography with photo diode array (HPLC-PDA) quality control of menoprogen, a Traditional Chinese Medicine (TCM) formula used for the management of menopause. Int J Med Plants Res 2013;2:146–51.
[65] Zhou J, Qi L, Li P. Quality control of Chinese herbal medicines with chromatographic fingerprint. Chin J Chromatogr 2008;26: 153–9.
[66] Feng C, Ruan J-L, Cai Y-L. Study of high-performance liquid chromatography fingerprint for traditional Chinese medicine Yigongningxue oral liquid. J Chem Soc Pak 2010;32:34–9.
[67] Zhou D, Zhu YR, Guan Y, Quan W, Li YW, Guo C, et al. Chemical fingerprint and metabolic fingerprint of Danhong injection by HPLC-UV and HPLC-MS. Asian J Chem 2013;25:6285–92.
[68] Chen F, Li H-L, Tan Y-F, Zhang J-Q. Quantitative analysis of the major constituents in Chinese medicinal preparation Suo-Quan formulae by ultra fast high performance liquid

chromatography/quadrupole tandem mass spectrometry. Chem Cent J 2013;7:131–8.
[69] Chen F-F, Qi H-Y, Shi Y-P. Fingerprint analysis of *Codonopsis Radix* by HPLC coupled with chemometrics analysis. Chin Herbal Med 2013;5:307–12.
[70] Goodarzi M, Russell PJ, Heyden V. Similarity analyses of chromatographic herbal fingerprints: a review. Anal Chim Acta 2013;804:16–28.
[71] Feng X, Kong WJ, Wei JH, Ou-Yang Z, Yang M. HPLC fingerprint analysis combined with chemometrics for pattern recognition of ginger. Pharm Biol 2014;52:362–7.
[72] Funari CS, Carneiro RL, Andrade AM, Hilder EF, Cavalheiro AJ. Green chromatographic fingerprinting: an environmentally friendly approach for the development of separation methods for fingerprinting complex matrices. J Sep Sci 2014;37:37–44.
[73] Kiazolu JB, Zhang LY, Intisar A, Wang Y, Zhang RS, Wu ZP, et al. RP-HPLC separation and statistical data processing of different batches of aerial parts of Jologbo. J Liq Chromatogr Relat Technol 2014;37:48–60.
[74] Ma Y, Gao HM, Liu J, Chen LM, Zhang QW, Wang ZM. Identification and determination of the chemical constituents in a herbal preparation, compound Kushen injection, by HPLC and LC-DAD-MS/MS. J Liq Chromatogr Rela Technol 2014;37:207–20.
[75] Xiong HS, Yu LX, Qu HB. A weighting approach for chromatographic fingerprinting to ensure the quality consistency of botanical drug products. Anal Methods 2014;6:476–81.
[76] Zhu HB, Wang CY, Qi Y, Song FR, Liu ZQ, Liu SY. Fingerprint analysis of Radix Aconiti using ultra-performance liquid chromatography-electrospray isonisation/tandem mass spectrometry (UPLC-ESI/MSn) combined with stoichiometry. Talanta 2013;103:56–65.
[77] Zhang J-I, Cui M, Yu H-I, Guo D-A. Chemical fingerprint and metabolic fingerprint analysis of Danshen injection by HPLC-UV and HPLC-MS methods. J Pharm Biomed Anal 2005;36:1029–35.
[78] Tang D-Q, Zheng X-X, Chen X, Chen X, Yang D-Z, Du Q. Quantitative and qualitative analysis of common peaks in chemical fingerprint of Yuanhu Zhitong tablet by HPLC-DAD-MS/MS. J Pharm Anal 2014;4:96–106.
[79] Bian Q, Yang H, Chan C-O, Mok DK-W, Chen S. Fingerprint analysis and simultaneous determination of phenolic compounds in extracts of Curculiginis Rhizoma by HPLC-Diode Array detector. Chem Pharm Bull 2013;61:802–8.
[80] Jiang F, Tao Y, Shao Y. Fingerprinting quality control of Qianghuo by high performance liquid chromatography-photodiode array detection. J Ethnopharmacol 2007;111:265–70.
[81] Lu HM, Liang YZ, Chen S. Identification and quality assessment of *Houttuynia cordata* injection using GC–MS fingerprint: a standardization approach. J Ethnopharmacol 2006;105:436–40.
[82] Xu CJ, Liang YZ, Chau FTV, Heyden YV. Pretreatments of chromatographic fingerprints for quality control of herbal medicines. J Chromatogr A 2006;1134:253–9.
[83] Theodoridis GA, Gika HG, Want EJ, Wilson ID. Liquid chromatography–mass spectrometry based global metabolite profiling: a review. Anal Chim Acta 2012;711:7–16.
[84] Li L, Zhao Y, Liu W, Feng F, Xie N. HPLC with quadrupole TOF-MS and chemometrics analysis for the characterisation analysis for the characterisation of Folium Turpiniae from different regions. J Sep Sci 2013;36:2552–61.
[85] Tang SR, Yang RT, Pan FS, Zhao AM, Pang ZJ. Steroidal saponin and steroidalsapogenin in Chinese Dioscorea L. J Plant Resour Environ 2007;16:64–72.
[86] Zhang X, Liang J, Liu J, Zhao Y, Gao J, Sun W, et al. Quality control and identification of steroid saponins from Dioscoreazingiberensis C. H. Wright by fingerprint with HPLC-ELSD and HPLC-ESI-Quadrupole/Time-of-fight tandem mass spectrometry. J Pharm Biomed Anal 2014;91:46–59.
[87] Man S, Gao W, Zhang Y, Wang J, Zhao J, Huang L, et al. Qualitative and quantitative determination of major saponins in *Paris* and *Trillium* by HPLC-ELSD and HPLC–MS/MS. J Chromatogr B 2010;878:2943–8.
[88] National Commission of Chinese Pharmacopoeia. Pharmacopoeia of peoples Republic of China. Beijing: Chemical Industry Press; 2010. 525–526.

LIST OF ABBREVIATIONS

ACN Acetonitrile
ELSD Evaporative light scattering detector
EMEA European Medicines Agency
ESI-MS Electrospray ionization mass spectrometry
EtOH Ethanol
EU European Union
HCA Hierarchical clustering analysis
HELP Heuristic evolving latent projections
HPLC High performance liquid chromatography
HPTLC High performance thin layer chromatography
IR Infrared
IT Information theory
LC Liquid chromatography
LDA Linear discriminate analysis
LLS Local least square
MeOH Methanol
MS Mass spectrometry
NMR Nuclear magnetic resonance
NP Normal-phase
OPA Orthogonal projection analysis
PCA Principal component analysis
PDA Photo diode array
RP Reversed-phase
SCC Spectral correlative chromatography
SFDA State Food and Drug Administration
SIMCA Supervised classification procedure using soft independent modeling of class analogy
TCM Traditional Chinese Medicine
TFA Tri-fluoro-acetic acid
TLC Thin layer chromatography
TOF Time-of-flight
UPLC Ultra performance liquid chromatography
UPLC–ESI/MSn Ultra performance liquid chromatography-electrospray ionization/mass spectrometry
UV Ultraviolet
WHO World Health Organization

This page intentionally left blank

CHAPTER

20

Ayurveda — Opportunities for Developing Safe and Effective Treatment Choices for the Future

Pratip K. Debnath[1], *Subhadip Banerjee*[2], *Parikshit Debnath*[3], *Achintya Mitra*[4], *Pulok K. Mukherjee*[5]

[1]Gananath Sen Institute of Ayurvdiya and Research, Kolkata, India; [2]Bengal Institute of Pharmaceutical Sciences, Kalyani, West Bengal, India; [3]S.D.M College of Ayurveda and Hospital, Hassan, India; [4]National Research Institute of Ayurvedic Drug Development, Kolkata, India; [5]School of Natural Product Studies, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India

OUTLINE

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00020-9

20.1 AYURVEDA THE SCIENCE OF LIFE

"Om Sarve Bhavantu Sukhinah
Sarve Santu Nir-Aamayaah
Sarve Bhadraanni Pashyantu
Maa Kashcid-Duhkha-Bhaag-Bhavet
Om Shaantih Shaantih Shaantih"

[May all remain happy, May all remain disease free, May all see what is Auspicious, May no one Suffer, Let Peace prevail. Ayurveda the science of life or living is accepted as one of the ancient documented comprehensive medical system known. The Vedic literature (here Brihadaranyaka Upanishad), the most ancient written document of India presents before us the perception of health of that time. It enumerates that Nirmaya (disease-free healthy living) can only be attained through happiness (Sarve Sukhina Bhavantu) and peace (Shaantih).]

Brihadaaranyaka Upanisad 1.4.14.

Ayurveda developed through communication, the method of transmitting information from one generation to other verbally or nonverbally from one entity to another. Contemporary issues that India confronts are the most daunting challenges to exploring newer alternatives in all socially acceptable norms including health sciences. Ayurveda is one such alternative catering heath care in continuity since Indus Valley Civilization (2300−1750 BC). Despite many reports, there is tremendous upsurge in public interest for Ayurveda. Cutting across the rural to hilly terrain of forests where even today health facility has not reached the distant places that natural medicine has, Ayurveda remains in its purest form and sustainable. Health is an international problem, so cure of diseases is also under the same domain. There are no proprietary rights; we must try to find out this knowledge and bring it within the purview of people globally because they ate the ones who are suffering from handling of adverse market forces. Even to these suffers, Ayurveda must be amenable, the natural, economically, and socially viable and acceptable health care. We must consider human being as a whole with body, mind and soul to be healthy; healthy life is ensured by the harmony of these three entities. In life we must have satisfaction of mind and tranquility of spirit. In our traditional health care system Ayurveda regime all recipes are provided; one has to find out the right things in the right directions. Ayurveda considers individual as a whole, the object of treatment, and not merely a particular expression of that system. Health is considered as the basis of all other development, which is essential for economic, artistic, innovative, and spiritual life, here say "*Arogyam Mulam Uttam*" one must practice for the sake of humanity, treat the individual as a whole and not piece meal like automobiles, obeying the fundamental principles. Health is a major concern which need rationalistic outlook. In order to develop Ayurveda, the science of Life, we need scientific thinking which in turn will answer various problems of healthcare.

20.1.1 Ayurveda *Siddhantas*: Strength of Fundamentals in Ayurveda

The roots of Ayurveda can be traced to the beginning of cosmic creation. In the universe, everything is composed of matter, and according to Ayurveda, all matter consists of five basic elements (*Panchamahabhutas*) [1]: the first element is space (*Akasha*), and the remaining four elements air (*Vayu*), water (*Jala*), fire (*Agni*), and earth (*Prithivi*) exist within the space [2].

Both the systems, human (microcosm) and universe (macrocosm), are linked permanently, since both are built from the same elements. Thus humans are

miniatures of the universe, a replica of nature, and everything that affects human beings also influences the macrocosm. Hence, the evolution of life and the creation of the universe can be concerned with Ayurveda [3]. Along with these *Panchamahabhutas*, functional aspects like movement, transformation, and growth are governed by three biological humors, viz. *Vata* (space and air), *Pitta* (fire and water), and *Kapha* (water and earth), respectively [2]. These three bodily humors usually known as *Tridhatus* regulate every physiological and psychological process in the living organism. Additionally, *Ojas* or *Jeevaniya Shakti* (vital force) developed from tissue metabolism is also essential for healthy functioning of the body [4]. It is believed that after intake of food/diet, it undergoes a process of digestion and ultimately forms two types of products—*Prasadas* (nutritional products) and *Malas* (excretory products). The *Prasada* builds the seven *Dhatus* (tissue) of the body, whereas *Malas* become waste products to be expelled out. Body produces three types of waste products: feces (solid), urine, and sweat (liquid). Both *Prasada* and *Mala* are important, and their presence in the right proportion in the body is indispensable for health and well-being. Health is considered as the balance between body, mind, and consciousness along with three humors *Vata*, *Pitta*, and *Kapha* [5]. They work together to bring the balance (Figure 20.1)

It is an accumulated knowledge base of antiquity; concerned biomedical scientists need to explore it with evidence-based "bedside to bench approach." Ayurveda needs novel methodology for its development in the realm of existing science and technology including updated in silico approach. Recent research on antitubercular adjunct therapy in tuberculosis patients with validation of the molecular mechanism of action of the immunomodulator herb by in silico approach elucidated novel mechanism of action shaping unexplored dimension of Ayurvedic pharmacology [6,7]. The knowledge base of Ayurveda includes Ayurvedic medicine, Ayurvedic principles, therapeutic modalities *Panchakarma*, and preventive aspect through *Rasayana* and veterinary use.

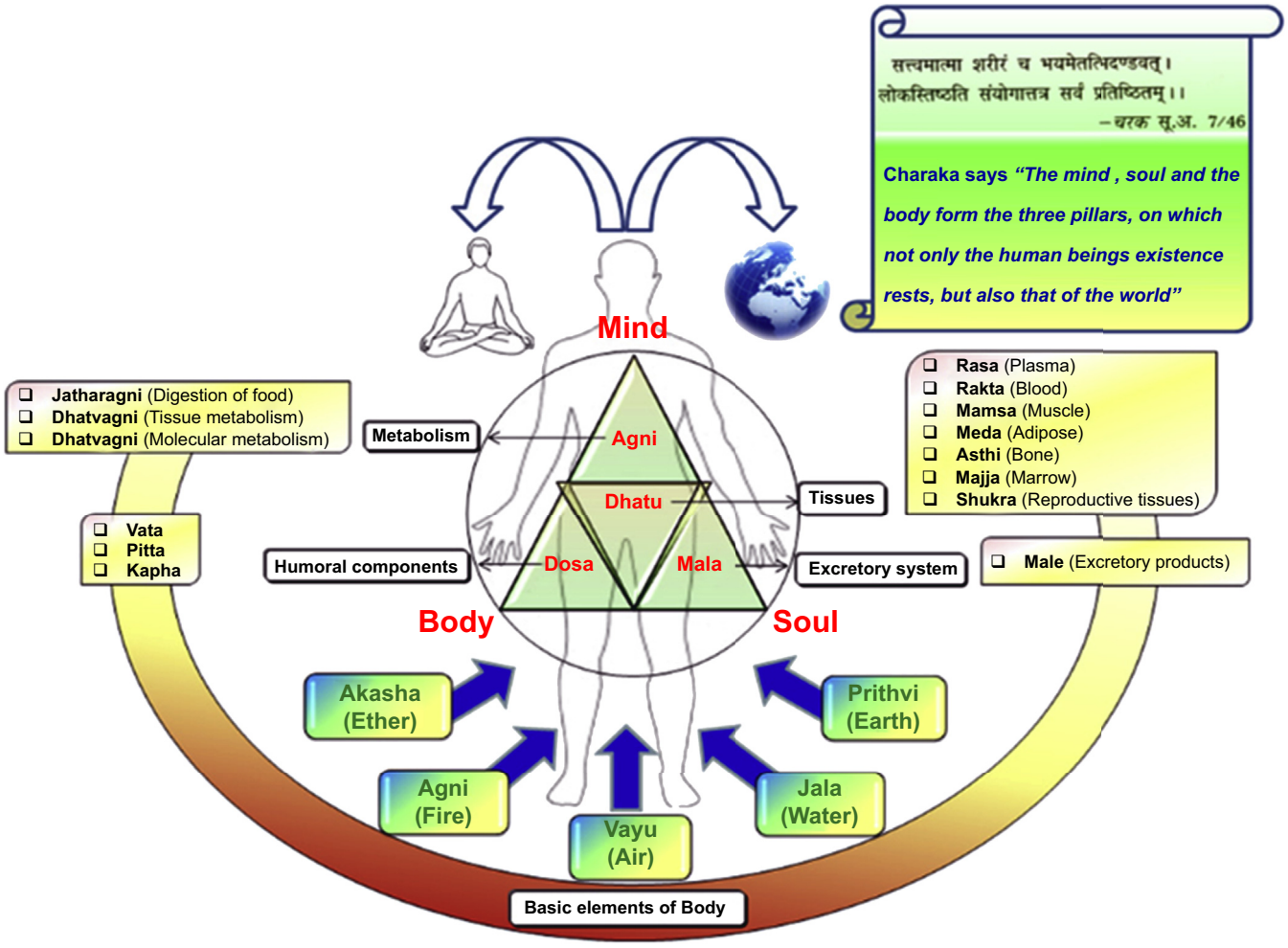


FIGURE 20.1 Ayurvedic principle on the basic elements of human physiology. *Reproduced with permission from P.K. Mukherjee et al., Journal of Ethnopharmacology, 143, (2012) 424–434.*

20.1.1.1 Panchamahabhuta Siddhanta

The "*Panchamahabhutas*" (the five primordial elements) are considered the building blocks of all physical matter that exist on our planet (organic and inorganic, animate and inanimate, the living and the nonliving). These five elements are the *Prithvi* (the earth element), *Ap* (the water element), *Tejas or Agni* (the fire element), *Vayu* (the air element), and *Akasha* (the space or ether element). The *Panchamahabhutas* are the origin of the physicochemical basis of all matter. For a proper grasp of the "five element theory," it is important to desist from interpreting the elements on the basis of a literal translation of the Sanskrit terms, but to take them as symbolizing a unique, subtle analogy to explain the "material" component of the planet, as we perceive it [8,9]. The authors have derived this semantic relationship between *Panchamahabhutas* and *Tridosha* to symbolize *Vata*, *Pitta,* and *Kapha* (See Figure 20.2).

20.1.1.2 Tridosha Siddhanta

The Ayurvedic concept of health and diseases is discussed considering the *Tridosha* theory as conscientious for biological entity in balance state. The structural, functional, and behavioral dimensions of the living organism are being conceptualized with *Doshas* (humoral component), which are of two types, viz., *Sharirika* (somatic)—*Vata, Pitta,* and *Kapha,* and *Manasika* (psychic)—*Satva*, *Rajas,* and *Tamas*. *Sharirika* and *Manasika Doshas* for continuance of physiological homeostasis are interrelated to each other on psychosomatic dimensions. The three "*Doshas*" (*Vata, Pitta, Kapha*), in Ayurveda, give a rationalization for all the psychobiological functions of living beings. They are called "*Doshas*" (i.e., "defective" or "deranged" elements) because they are well-organized elements that are imbalanced and highly susceptible to derangement, although in contradiction, they need to subsist and function in a state of "balance" or "equilibrium" to preserve perfect health (and prevent disease conditions). Diverse qualities and functions have been attributed to each *Dosha*. The kinetic machinery of a system has been attributed to *Vata*, the metabolic mechanism to *Pitta,* and the structural and stability machinery to *Kapha*. For instance, *Vata* contributes to expression of shape, cell division, signaling, movement, excretion of wastes,

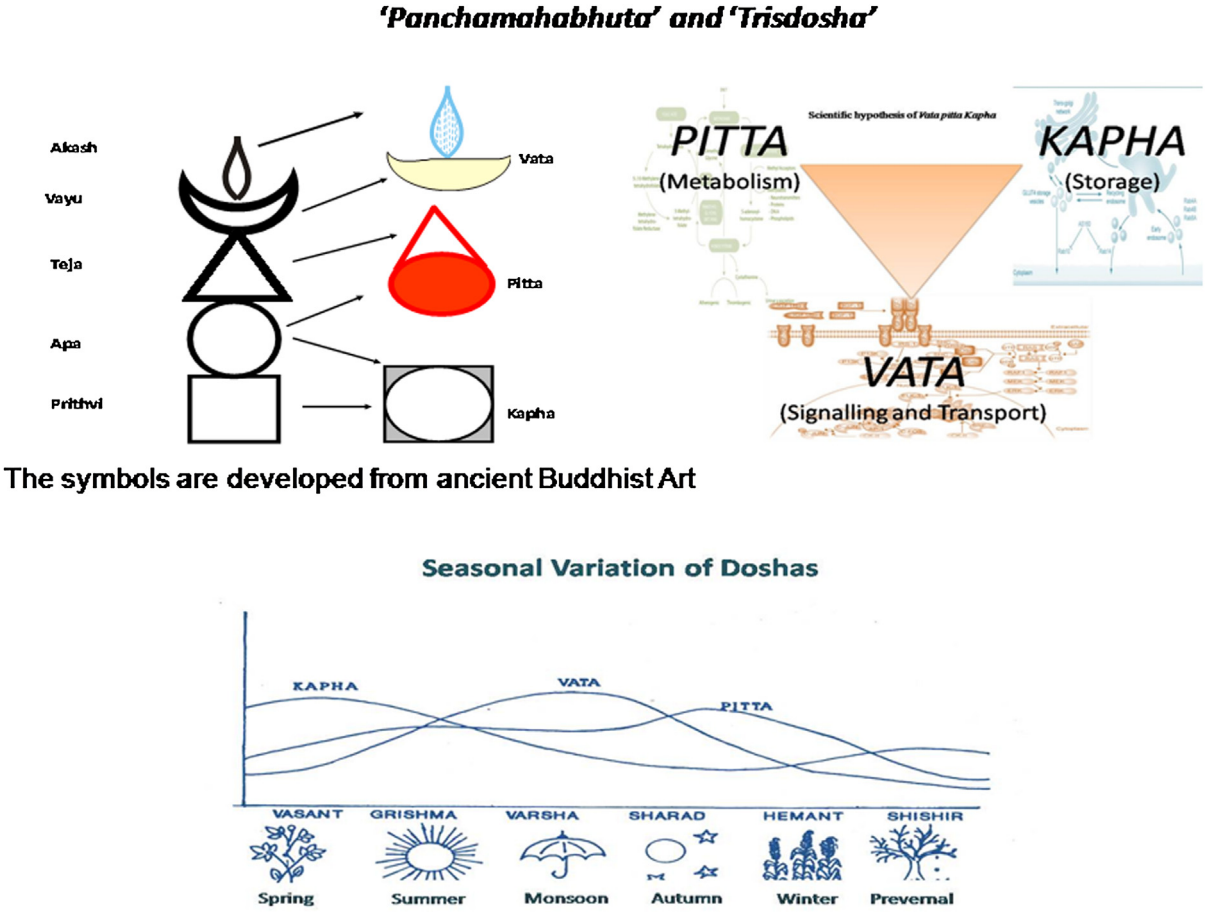


FIGURE 20.2 Relationship between *Panchamahabhutas* (the five primary elements) and *Tridosha* with its seasonal variation.

and cognition and also regulates the activities of *Kapha* and *Pitta*. *Kapha* is accountable for growth and maintenance of structure, storage, and stability. *Pitta* is chiefly responsible for metabolism, thermoregulation, energy homeostasis, pigmentation, vision, and host surveillance. Hence the differences in *Tridoshic* proportions right from the time of fertilization are manifested as diverse phenotypes that can be in accordance with external appearances, body physiology, and response to external environment like seasonal variations (see Figure 20.2). Thus a continuum of relative proportions of *Doshas* results in seven possible constitutional *Prakriti* types, namely, *Vata, Pitta, Kapha, Vata-Pitta, Pitta-Kapha, Vata-Kapha,* and *Vata-Pitta-Kapha* [8,10,11].

20.1.1.3 *Swasthavritta*: Preventive Health Science

The basic objective of Ayurveda as mentioned by Charaka is dual in nature. In the ancient manuscript *Charaka Samhita* (*Sutra Sthana* 30/26) (see Figure 20.3) it is enumerated that the principle objective of Ayurveda is to protect the health of the healthy and to alleviate the disorders in the diseased. This explains the importance of *Swasthavritta* or preventive medicine in Ayurveda. Ayurveda provides a guiding principle for attainment and preservation of perfect health in its *Swasthavritta* (Ayurvedic preventive health), *Dinacharya* (daily regimens), and *Ritucharya* (seasonal regimens). In prevention and management of chronic diseases such as cardiovascular disorders, diabetes, hypertension, arthritic disorders, stress, and cancers, nondrug measures as supplements to medications and surgical principles found in Ayurveda are being advocated routinely in the present era by health care providers in developed nations [1].

20.1.1.4 *Roga Vigyan* and *Chikitsa Siddhanta*: Disease and Treatment Aspect

Prior to written systemic development of Ayurveda, four superspecialities such as *Bhutavidya* (demonology), *Sarpavidya* (dealing with poisons and venoms), *Rasayana* (rejuvenation), and *Vajikarana* (dealing with aphrodisiacs) existed in Vedic literature [10]. After establishment of *Atreya Sampradaya* (school of physician) and *Dhanwantari Sampradaya* (school of surgeons) four more disciplines were included on the development of reason-based Ayurveda like *Kayachikitsa* (internal medicine), *Shalya Tantra* (surgical sciences), *Shalakya Tantra* (eye and ear nose, and throat (ENT)), and *Kaumarabhritya* (maternal and child health). *Sarpavidya* was restructured and renamed as *Agada Tantra* (toxicology and forensic medicine) [12].

This change may be attributed to the influence of a new philosophy in Ayurveda with eight major disciplines known as *Ashtanga Ayurveda*. Presently, there are 17 specialties in Ayurveda, viz. *Ayurveda Siddhanta* (fundamental principles of Ayurveda), *Ayurveda Samhita* (dealing with Ayurvedic classics), *Rachna Sharira* (anatomy), *Kriya Sharira* (physiology), *Dravya Guna Vigyan* (Materia Medica and pharmacology), *Rasa Shastra* (metal and minerals processing), *Bhaishajya Kalpana* (pharmaceuticals), *Kaumarabhritya* (pediatrics), *Prasuti Tantra* (obstetrics and gynecology), *Swasthavritta* (social and preventive medicine), *Kayachikitsa* (internal medicines), *Roga Nidana* (etiopathology), *Shalya Tantra* (surgery), *Shalkya Tantra* (eye and ENT), *Manasa Roga* (psychiatry), *Agada Tantra* (toxicology and forensic medicine), *Sangaharana* (anesthesia), and *Panchakarma* (cleansing for rejuvenation therapy) [13].

Science of *Roga Vigyan and Vikriti Vigyan* (disease process) and *Chikitsa Vigyan* (therapeutic modalities) are guided by different principles. The pathological processes are described as *Panchanidana* (five etiological factors): these are *Nidana* (cause), *Purvarupa* (premodial symptoms), *Rupa* (symptomatology), *Upasaya* (therapeutic measures), and *Samprapti* (pathogenesis) [14—18]. The main aim of *Chikitsa* (therapeutic management/treatment) is to convert unhappiness to happiness basically guided by *Samanya* (general) and *Visesha* (specific) principle [15,16,19].

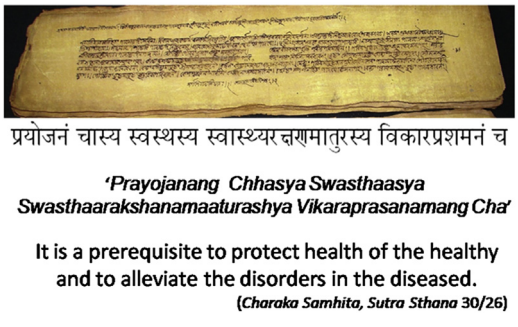


FIGURE 20.3 The object of Ayurveda in *Charaka Samhita*.

20.1.2 Symptoms of Life and Its Language

Ayurveda considered that human body has an inherent intelligence or radar system that reciprocates health protection and maintenance of homeostasis at the cellular level. This is known as *Pragna* (inner instinct), which warns us to do certain things that are congenial to our body and to avoid certain things. With time due to external factors this self-guided signaling system has got feeble or underexpressed, which invites disturbed ease (disease). *Susruta Samhita* [4] described "*Swa Yoni Vardana Anna Prakankshate*," i.e., the *Jivatma* (minute cell) expresses what to take and what to avoid. All the desires are produced at the cellular level. Similar confirmation is available in

Ashtanga Hridaya [12] in support of this theory, "*Kurvatehi Ruchim Dosha, Viparita Samanyoh*"—*Dosha* produced in the organ's minute living cell with consciousness expresses what is congenial to take and what should be to avoid. This *Pragna* or perception is either lost or subdued by the environment over time and are ignored inviting many lifestyle disorders. Recent literature again renewed the importance of this area [16,20]. Desires are generated at the cellular level not in the mind. Habitats and condition reflexes are the guiding force for misunderstanding these desires, and the mind fulfils these desires. For example, certain tastes like sweet, sour, and salty are good for person with *Vata Prakriti*, for example, ice cream is good, but for a person with *Kapha Prakriti* it may lead to ease disturbance. A person with *Kapha* knows well to avoid ice cream, but in certain situations his or her *Pragna* (inner instinct) fails to control this phenomenon, which is known as *Pragnaparadha*. The etiopathogenesis section describes in detail how disease occurs [5].

20.1.3 *Nadi Vigyan*: A Lost Art of Observational Diagnosis

Importance of *Nadi* (pulse) to determine health and disease was a highly esteemed diagnostic procedure exclusive to Ayurveda since *Samhita Kala*, but during the thirteenth century AD *Sarangadhara Samhita* first described the method in detail. *Acharya Kanada* dilated it to a systematic approach. In our body there are more than 70,000 *Nadi* (pulsating vessels); among them the pulse in eight areas could be felt to know the personalized physical status whether in health or in disease. Further, this intuitive science requires experience and practice [21,22]. *Nadi* is determined by the balance of the *Tridosha* in one's body; for beginners' learning purpose different animal movements like snake, frog, and pigeon were used to understand the correlation of *Vata, Pitta*, and *Kapha*. It is described that in the early morning the physician will read the pulse for men in the right radial area and for females on the left hand. By pulse reading experienced physicians could predict the forthcoming disease and place of occurrence, that is, the affected organs like lungs for *Kapha*, brain for *Vayu*, and stomach for *Pitta* [20].

20.2 KNOWLEDGE BASE OF ANCIENT INDIA

20.2.1 History of Knowledge Base

Indian Hindu mythology proclaims Vedic literature to be the most authentic and ancient one, which was documented by the *Aryans*. It comprises *Samhita* (*Rig Veda, Sama Veda, Yajur Veda*, and *Atharva Veda*), *Brahmana, Aranyaka*, and *Upanishad* and in the later period *Puranas* and *Shastras* [23]. References to diseases, herbs, and herbal cures can be distinguished in all the four *Vedas* especially in the *Rig Veda*; this oldest documented repository of human knowledge represents the first mention of medicinal herb in *Ousadhi Sukta* (*RV.10:47,1–23*) [24]. Ayurveda is said to be an *Upaveda* of *Atharva Veda*, which contains 114 hymns related to formulations including plant- and animal-derived products, minerals, and metals for the treatment of different diseases [25,26]. In *Rig Veda* 67 plants and *Sama Veda* 67 plants each are included, while in *Yajur Veda* 81 plants and in *Atharva Veda* 293 plants are included. In *Brahmana* 129 and in *Upanishad* 31 plants are the foundation of informatics on the ancient use of medicinal plants that are still in use (See Tables 20.1–20.3).

The concepts and practice, fully devoted to Ayurveda were first described in text form by Agnivesha in his treatise the "*Agniveshtantra*," which was later amended by Charaka and became known as the *Charaka Samhita*, which primarily focuses on therapeutics of enlisted 526 plants and plant products for medicinal use [27]. Charaka represents the *Atreya Sampradaya* (school of physicians) founded by the great scholar-sage *Punarvasu Atreya* who defined Ayurveda uniquely "Wherein the beneficial and adverse influences leading, respectively,

TABLE 20.1 Identified Plants from Different Vedic Literature

Vedic literature	Number of plants	Scientifically identified plants
Rig Veda	67	
Sama Veda	67	
Yajur Veda	100	120
Atharva Veda	293	
Brahmana	129	
Upanishad	31	

TABLE 20.2 Plants Identified From Puranas

Purana literature	Number of plants	Total identified
Vamana Purana (200 AD)	4	
Vayu Purana (200–1400 AD)	4	
Kurma Purana (700–800 AD)	7	
Matsya Purana (800 AD	11	256
Brahma Vaivarta Purana (700 AD)	10	
Brahma Purana (1200–1600 AD)	200	
Agni Purana (800–900 AD)	19	

TABLE 20.3 Plants in Ayurvedic Literature

Ayurvedic samhita	Number of plants	Scientifically identified
Charaka Samhita	526	526
Sushruta Samhita	573	573
Astanga Hridaya	902	902
Sarangadhara Samhita	689	673
Bhavprakash Nighantu	1203	834
Madanpal Nighantu	480	Not known
Kaiyadeb Nighantu	450	Not known
Sodhala Nighantu	499	Not known

to happiness and misery and to life healthy or ill are described, besides the respective helpful and harmful measures are described and quantified that system is called Ayurveda" [28]. The next landmark in Ayurvedic literature was the *Sushruta Samhita* compiled by Sushruta known as the "Father of Surgery" (600 BC), which places special emphasis on surgery and surgical techniques. It described 573 medicinal plants, 57 drugs of animal origin, and 64 minerals and metals as therapeutic agents. The next important authority in Ayurveda after Charaka and Sushruta is Vagbhata of Sindh, who flourished in about the seventh century AD and described 902 plant species. His treatise called the *Ashtanga Hridaya* is considered unrivaled for the principles and practice of medicine. These three important books are known as the senior triad "'*Brihatrayees*" (big or major three). These books contain basic concepts of health and disease, disease management, anatomy and physiology, hygiene, Materia Medica, pharmacology and therapeutics, herbal formulations, pharmacy, and synthesis of herbomineral formulas. The Ayurvedic texts are much respected in neighboring countries and have been translated into Greek (300 BC), Tibetan and Chinese (300 AD), Persian and Arabic (700 AD), as well as several other Asian languages. Indian Materia Medica provides lots of information on the ethnic folklore practices and traditional aspects of therapeutically important natural products. This includes about 2000 drugs of natural origin; of these 400 are of mineral and animal origin, while the rest are of vegetable origin [29].

20.2.2 *Vrikshayurveda*—Study of Plant Science in Ancient India

Vrikshayurveda is a special subject of Ayurveda that deals with the cultivation, nourishment, management, etc., of medicinal and other edible plants. The study of plant life or science of *Vrikshyayurveda* drew considerable attention of ancient Indians as specialty. Nowadays, transgenic plants are developed with advanced technology. In *Vrikshyayurveda* similar plant character changing procedures are described in detail. By application of some plants products, white-cotton-yarn-producing plants started yielding different color yarn like red, indigo, and blue. These plants seize production of flower and fruits by special treatment, old plants are rejuvenated and start flowering, and creeper plants can be changed to erect plants.

In *Vrikshyaurveda,* we find information's on the advantages and disadvantages of growing trees, selection and location of trees, auspicious and inauspicious plants, and importance of tree planting including land description and suitability of tree planting and green manure application during cultivation. The diagnosis and treatment of diseased plants was done on the basis of *Vata*, *Pitta,* and *Kapha* of Ayurvedic principle. The meteorological forecasts like underground water current (*Siranveshana*) and indications of onset of rains (*Silabhedanam*) were also known at that time.

In the *Vrihat Samhita* written by Varahamihira and in *Agnipurana*, *Arthashastra* described the art of collection and selection of seeds, selection of soil, sowing procedure, successful germination of seeds, and various means of propagation of plants on grafting and cutting. Besides this, treatment of cow's (*Gava* Ayurveda), horses (*Aswa* Ayurveda), elephants (*Gaja* Ayurveda), and poultry and birds (*Paksha* Ayurveda) has also been described.

20.2.3 The Evolution of Written Knowledge and History of Preservation

This knowledge has been passed from one generation to the next through written medium Sanskrit, frequently in palm leaf parchments (*Taalpatra*) in the form of *Shlokas* (Sanskrit verses). Many of them have been enshrined in the form of medical literature, like "*Ayurveda Shastra,*" but a vast number of them are still being passed to the next generation verbally just like the pre-Vedic period when there was no process of documentation and experience and knowledge were percolated to the next generation through *Shruti* (hearing) and *Smriti* (memory). In Vedic medicine, it was noticed that magicoreligious elements and empirical-rational elements were running separately, while *Atharva* Vedic medicine is an amalgam of religion, magic, and empiric-rational elements. It would appear that at the time of *Atharva Veda* there were designated physicians and elaborate pharmacopoeia treating diseases with herbs. In Vedic literature, *Gopatha Brahman*, *Sarpa Veda*, *Pisacha Veda*, and *Asura Veda* were mentioned as specialty. In 800 BC and 600 BC in *Satapatha Brahmana* subjects of study were mentioned as *Atharva Veda, Sarpa Vidya, Pitriya Vidya,* and *Bhuta Vidya* [30].

20.2.4 None Codified Tribal health and AYUSH Initiative

Forests dwellers in three states West Bengal, Bihar, and Orissa were selected for exploratory evaluation study through questionnaires and intensive dialogue with people and traditional tribal practitioners. In the absence of scientific methods for studying this complex and unique system, an appropriate methodology was devised. The inhospitable terrain often makes researchers face problem with communication. To overcome the constrains, it was decided that the tribal doctors would be entrusted with not only the diagnosis of the ailments but also treatment of the patients with the available plants and other products collected from the forests, the ways and means to which they are accustomed. Patients were examined by both the tribal and allopathic doctors. After arriving at independent diagnoses, the patients were treated by the tribal doctors with their medicine. The determining factors were health care study, health status, disease treatment, culture, and rituals related to health. A total of 13 communities of tribal practitioners were studied group from three adjoining states [31].

20.2.5 Documentation of Tribal Health Culture

Attempts are made at present by AYUSH Department of Ayurveda, Yoga, Unani, Siddha, Homeopathy. National policy on Indian System of Medicine and Homeopathy (ISM&H) (2002) was included to enlist folk practices for scientific evidence. As such the North Eastern Institute of Folk Medicine has already started laying importance to documentation and to make it evidence based following the reverse pharmacology approach, i.e., bedside to bench [32]. The age old system of health care still exists in pure form in remote tribal areas only, available to protect from health hazards. The system depends entirely on the environment. Rapid deforestation abuses the natural resources and equilibrium is grossly disturbed.

20.3 *DRAVYAGUNA*: PHARMACODYNAMIC CLASSIFICATION OF HERBS IN AYURVEDA

In order to understand Ayurvedic method of treatment with regard to diet or herbs, a few Ayurvedic terms like *Rasa, Guna, Virya, Vipaka, Karma,* and *Prabhava* need to be understood. These terms/concepts, in fact, encompass the entire gamut of Ayurvedic pharmacology (*Dravyaguna Vigyan*). Ayurveda does not differentiate between food and drugs, in terms of the ultimate "fate" of any substance (*Dravya*) that is ingested by (or orally administered to) a living being. Ayurvedic pharmacology thus deals with "substances" (*Dravyas*) and not necessarily only with "drugs." In fact, Charaka emphatically states that there is no "substance" in this world that cannot be used as a "drug"! *Rasa, Guna, Virya, Vipaka, Karma,* and *Prabhava* of food items (and drugs) are the most important properties: *Rasa* in this context is quite different from the *Rasa Dhatu* (nutrient fluid) and refers to the primary taste of the substance orally ingested. Ayurveda describes six (potency) of the food items and drugs that, in turn, result in specific (often predictable) *Karma* (actions) on the human body–mind unit. Ayurveda also refers to *Anurasas* (secondary tastes).

20.3.1 Pharmacodynamic Classification and Medicinal Plants in Ayurveda

Ayurvedic classification of herbs according to the pharmacological properties of the plants considering their physicochemical and biological characteristics has been the most basic substance–activity relationship in the history of science. A medicinal plant *Dravya* (substance or drug) is characterized by its qualities (*Gunas*) and the effect (*Karma*). Quality and effect cannot exist apart from a substance, in which they coexist in an inseparable concomitance (*Samavaya*). They work either on the principle of sameness (*Samanya)* or on the principle of antagonism (*Visesa*). A substance with the property of *Ushna* accelerates metabolism, it strengthens *Pitta* by the principle of *Samanya* and reduces *Kapha* by the principle of antagonism. The taste perception is known as *Rasa*; there are six types through which drug action was identified in the past, when "taste was the test laboratory." The sense of taste has been designated as the most important tool possessed by Ayurveda for perceiving the healing quality of a substance or botanical (See Table 20.4).

20.3.2 Importance of Plant Identification According to Ayurvedic Nomenclature

Identification of the Ayurvedic medicinal plants according to their traditional description in authorititative texts is quite delicate. The authors share some of their personal experiences. Let us take the example of Jivanti, which is classified under *Jivaniya Varga* (life pounding) acclaimed for its tonic effects in the Ayurvedic texts. It has been generally denoted as *Desmodium fimbriatum*. When preliminary pharmacological screening was done we thought that the plant is *Desmodium* only due to the claim of our supplier whom we trusted. Later, when botanical identification was done we found that the plant was substituted with a Himalayan orchid for

TABLE 20.4 Chemical Biology Relationship of "Taste" and its Therapeutic activity

			Therapeutic phenotype described	
Sl. no	**Taste perception**	**Chemical nature**	**Promotive (Positive)**	**Antagonistic(Negative)**
1	Madhura (sweet)	Carbohydrate, sugar, fats, amino acids	Promotes weight gain, vitalizer	Obesity, respiratory disorder, swelling, goiter, diabetes, lymph nodes
2	Amla (sour)	Organic acids	Stimulate appetite, promote digestion	Blood disorders, swelling, inflammations, flushing, anemia, hemorrhage, visual disorder
3	Lavana (salty)	Salts	Moisten, promote digestion, expectorant, laxative	Impotence, graying and falling hair, bleeding stomach, skin disorder
4	Katu (pungent)	Volatile oils	Promote weight loss, vermifuge, stimulate appetite	Impotence, unconsciousness, hot feeling, thrust
5	Tikta (Bitter)	Bitter principles, alkaloids, glycosides	Antitoxic, vermifuge, febrifuge	Mental weakness, nausea, dry mouth, nerve disorder
6	Kasaya (astringent)	Tannins	Astringents, antiinflammatories that help in absorption	Heart trouble, contraction and blockade of channels

which no systematic botany has been reported or regarding whose pharmacology very little is known. We found that *Leptadenia reticulata* is also used as Jivanti. This experience emphasizes the concept of biological standard in case of plants rather than just its morphological similarity.

Here it may be stressed that concepts of chemotaxonomy and biological quality assurance are very important in case of Ayurvedic medicinal plants. We should also point out that recent transcriptomics research shows that transcriptome associated with the biomarker compounds or secondary metabolite biosynthetic pathway is very important to conserve and effectively harness the medicinally active natural sources. The time has come to study plant genomics and transcriptomics in a serious note to maintain genetic standards like complementary deoxyribonucleic acid (cDNA) library to conserve the genotypes and chemotypes of the plants that might become extinct or mutated due to evolutionary reasons and changing climate. These genetic aberrations may cause change in their chemical or metabolite profiles, leading to loss of their therapeutic relevance.

20.4 *NIDANA SAMPRAPTI*: DISEASE PATHOGENESIS IN AYURVEDA

Ayurveda relies on the inter-relationship of body (*Sharira*), mind (*Manas*), organs (*Avayava*), senses (*Indriya Visaya*), and soul (*Atman*) for the physical, mental, social, and spiritual welfare of human beings. Therefore, Ayurveda describes development of disease as a psychosomatic phenomenon. The general well-being of health (*Swastha*) can only be achieved when a poise of mind, body, and soul is maintained. This coordination is governed by consciousness (*Chetana*) or some kind of intelligence in totality, correlated from the subcellular level right up to the body (*Sharira*) where cell is just a basic functional unit. The etiopathology (*Nidana Samprapti*) of a disease has been projected as phenotypes expressed at mental as well as systems level. The disease components cannot not cause anything until they comes in contact (*Samyoga*) with the body (*Sharira*) through the five sense organs (*Indriya*) and cause upregulation of signaling (*Atiyoga*) downregulation of signaling (*Hinayoga*), or distorted signaling (*Mithyayoga*) in a system to manifest a phenotypic change (i.e., disease). The above process has been termed as "*Asatmya Indriyartha Samyoga Pragnaparadha Parinam.*" The stage that follows is *Pragnaparadha* where due to this aversion in signaling or processing of the body the intelligence or knowledge *(Pragna*) that governs this steady state of functioning is distorted (*Aparadha*) at the systems level or even at the mental level of processing. It is interesting to observe that ancient sciences show such level of understanding that well-being is connected to the symmetry of mental and physiological processing. When the signal or consciousness (*Chetana*) is distorted its facultative control over willpower (*Dhriti*), intellect (*Buddhi*), memory (*Smriti*), and ego (*Ahamkara*) is lost causing negative instincts like greed (*Lobha*), anger (*Krodha*), and confusion (*Moha*) to develop. These are directly connected to dynamic balance of *Vata, Pitta,* and *Kapha,* as summarized in Table 20.5. The concept of *Vata, Pitta,* and *Kapha* has been

TABLE 20.5 Manifestation of Vata, Pitta, and Kapha

Vata	*Utsaha* (initiativeness)
Pitta	*Probha* (lusture), *Prasada* (passion), *Medha* (intelligence)
Kapha	*Kshama* (tolerance), *Dhriti* (will power), *Alobha* (desire)

now perceived to be correlated with genomic expression of *Prakriti* in *Parinama*. According to the Ayurveda system an individual's basic constitution to a large extent determines predisposition and prognosis to disease as well as therapy and lifestyle regimen. Ayurveda describes seven broad constitution types (*Prakriti*) each with a varying degree of predisposition to different diseases. In the realm of predictive medicine, efforts are being directed toward capturing disease phenotypes with greater precision for successful identification of markers for prospective disease conditions. Biochemical estimations of the deoxyribonucleic acid (DNA) isolation, genotyping, and validation of genetic homogeneity, ribonucleic acid (RNA) isolation, and cDNA microarray experiments were conducted with statistical analysis of microarray along with quantitative polymerase chain reaction analysis ultimately to determine functional annotation of differentially expressed genes. On clinical phenotyping, individuals of different *Prakritis* exhibit differences at the biochemical level [33]. Identification of genetic variations that underlie differential expression of genes and biochemical end points are much correlated with *Prakriti* phenotype. The determining factors responsible for health and diseases are the *Prakriti* of individual; identification of risk issue, course, and complication; prognosis of the disease; and drug–body interaction. In the disease pathogenesis how the physiology ends and pathology starts is a unique proposition of Ayurveda. There are diurnal and seasonal variations of *Vata, Pitta,* and *Kapha;* once it goes up and again comes to a balance state [34,35]. The phenomena are subdivided into six stages known as *Shatkriyakala*. At every moment, there are dynamic processes that increase and eventually come to a balance state. This is known as *Sanchaya* (accumulation), *Prokopa* (aggravation), and *Prasama* (alleviation); if *Prasama* does not take place, the pathology sets in (*Prasara*), leading to *Sthanasamsraya* (localization) in any organ. In this state, the prodisease phenomenon develops but no clear-cut disease symptoms could be identified. After a few days or weeks the disease symptoms develop known as *Vyakti*. In this state, diagnosis is possible, but if not treated, it leads to chronicity known as *Bheda*. In Ayurveda, treatment may be initiated from *Prasara* stage, which could be determined by symptoms (*Rupa*) and *Nadi Pariksha*.

20.4.1 *Vyadhikshamatva*: Concept of Immunity in Ayurveda

Disease prevention is an ancient concept prevailing since the Vedic period (*Atharva Veda* 6.13.1-2; 2.17.1-4). The modalities later on percolated to Ayurveda where it was developed as a full-fledged subject of study application and research. The quintessence of all *Dhatus* (tissues) is *Ojas*. The expression of *Ojas* is through *Bala* (resistance power), which is in turn responsible for immunity or resistance against disease. *Ojas* is considered to be an immune material. *Ojas* is of two types *Para Ojas* and *Apara Ojas*. *Para Ojas* has only eight drops (*Ashtabindu*); deficiency or loss results in death. Being an immune material, its optimum quality and quantity maintains homeostasis and regulates immunity (*Charaka Samhita* Su 26/81).

This fundamental concepts of immunity in Ayurveda are expressed as *Vyadhikshamatva,* which means defense mechanism to resist disease (*Vyadhi* = disease; *Kshamatva* = capacity to adapt). The changeover from health to disease was related to the capacity of *Vyadhikshamatva*; this adaptation power is individualized, not general, and thus relates to the general concept of immunity. This concept of *Vyadhikshamatva* is further extended to the terms *Vyadhi Utpadani Bandhakatva* (inherent capacity to oppose the disease) and *Vyadhi Balavirodhita Shakti* (defense toward ongoing disease process). However, this capacity to avert disease is due to some *Bala* (inherent power or strength), which could be assessed clinically. This power or *Bala* is classified into *Sahaja* (genetic), *Kalaja* (environmental or time dependent), and *Yuktikrita Bala* (external factors or artificial). This *Yuktikrita Bala* can be compare to the concept of vaccines.

20.4.2 Concept of Stress in Ayurveda

Hans Seyle (1907–82), celebrated as the pioneer of theory of stress, an Austrian-Canadian endocrinologist of Hungarian origin who conducted much important scientific work on general adaptation syndrome, was amazed by the concept of stress in Ayurveda. In the 1950s, Selye discovered and documented that stress differs from other physical responses in that stress is stressful whether one receives good or bad news, and whether the impulse is positive or negative. He called negative stress "distress" and positive stress "eustress." The system whereby the body copes with stress, the hypothalamic-pituitary-adrenal axis system, was also first described by Selye. He also pointed to an "alarm state," a "resistance state," and an "exhaustion state," largely referring to glandular states. Later he developed the idea of two "reservoirs" of stress resistance, or alternatively stress energy [36]. Hans mentions "*It is certainly remarkable to what extent Ayurveda foresaw the general*

adaptation syndrome and thereby intricate aspects of the stress problem. I always had the greatest respect for the ancient Indian medicinal School but I find it almost impossible to understand how they could have arrived to this degree of prophetic forethought at a time when nothing was known about hormones which is really an appreciable dynamicity of Ayurveda and its concept of stress."

In Ayurveda, stress is described as a stimulus (physical, chemical, or biological) that can be corroborated at the mental, cellular, and systems level. If we think in correlation with Ayurveda, it has been described as a consequence (*Parinama*) associated with *Pragyaparadha* entailed above. This is caused at both the systems (metabolome, proteome, immunome) and the neuropsychiatric level. As it can be seen in Figure 20.4, indiscriminate accumulation (*Kama*) of any substance (may be biological or chemical) leads to loss of the critical buffering capacity (*Kshamahani*) up to which a system can resists an anomaly in it (may be aberration in signaling or accumulation of certain toxic metabolite). This causes the biological system or unit to take evasive action (*Krodha*) which leads to loss of judgment (*Sammohana* or *Medhahani*). This in turn creates an irritated state (*Smritihani*), which finally causes a degenerative change (*Prayatnahani*) due to loss of functionality or redundancy. The pathophysiological model can be explained in terms of stress-mediated loss of immunity, preferably in a clinical model that connects the body to mind. The pathophysiological model can only be rationalized if perceived at the systems or metabolomic level. In Ayurveda it has been stated that accumulation of unassimilated food or toxic metabolites (*Ama*) causes blocking or derangement of the major channels (*Srota Dusti*), which is important for immune signaling or immunity (*Ojas*). This causes the body to lose its normal functioning manifested as loss in body luster (*Prabhahani*) at the physical level and the loss of happiness (*Prasadhani*) at the mental level. This ultimately causes loss of initiative (*Utsahahani*) of the body and mind. This may lead to derangement of *Tridosha* manifesting a disease in *Parinama* at the physiological, mental, or systems level [5,37].

20.4.3 Prodisease Concept (*Rogi Chikitsa*): Prevention and Treatment

Diagnosis was envisaged a great importance in the treatment module of Ayurveda. *Madhava Nidana* among the classics was entirely devoted to it. But diagnosis was focused on identifying the specific disturbances of *Doshas* in a given patient and not so much on affixing the label of a disease. Treatment had to start early before the disease struck roots and progressed through the six stages (*Shatkriyakala*) to its full-blown manifestation. Before this full-blown manifestation some features develop; these are known as prodisease (*Purva Rupa*),

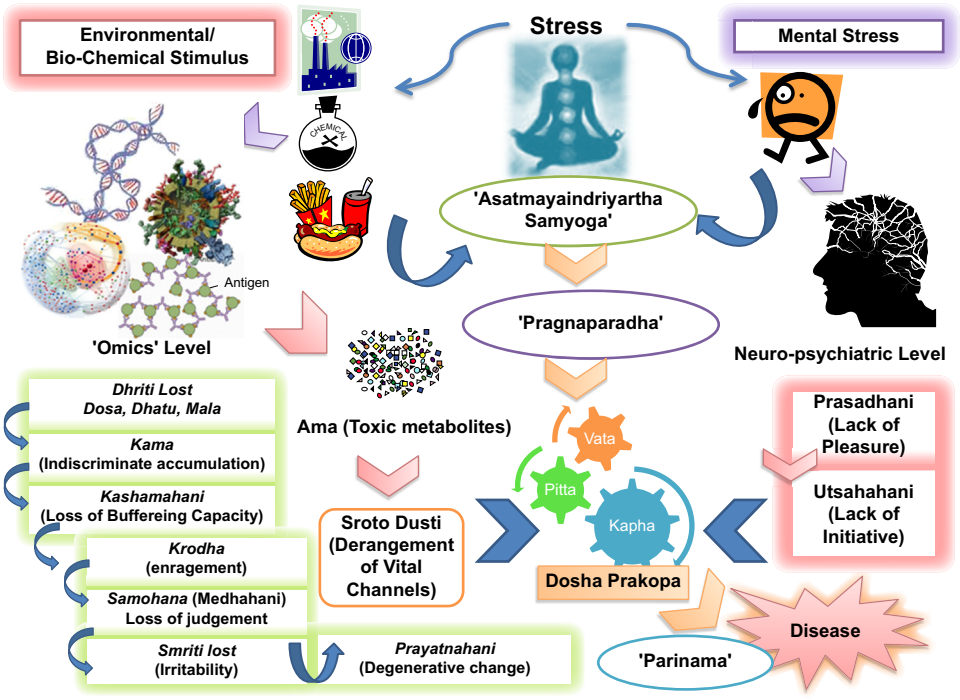


FIGURE 20.4 Conceptual model of stress in Ayurveda.

which have been developed as a unique concept in Ayurveda [15,16].

The concept of preventive medicine is a common practice where a person has been advised to follow a dietary regimen (*Ahara*) and healthy lifestyle and dietary habit has been fine-tuned according to one's constitution (*Vataja, Pittaja, or Kaphaja* evenly mixed, i.e., *Tridoshaja*) and seasonal changes (*Kala*) encompassing the concept of *Ritucharya* (seasonal regimen).Due to various physiological perturbations caused by seasonal changes and imbalances in the *Vata, Pitta,* and *Kapha* level, which spread to get localized to a particular system or organ, disease develops (see Table 20.6). Clinically these symptoms and disease development can be differentiated according to basic genetic or physiological conditions of a person, that is, one's *Prakriti*. Some common examples are given in Table 20.7.

TABLE 20.6 Factors Causing Development of Disease

1. Stress and strain	2. Contaminated food—raw material (chemical pesticides), cooking materials frozen for long period of time
3. Overactivity or less activity	4. Movement on high-speed vehicle
5. Diet antagonistic to personal health	6. Over use or faulty use of sense organs
7. Habitual toxicity—consumption of alcohol, coffee, tea, cold drinks, chewing, and smoking tobacco	8. Injudicious use of medicine or self medication
9. Faculty sex health management	

TABLE 20.7 Prodisease Alarming Signals

Sanskrit term	Manifestation
1. *Gouraba*	Feeling of heaviness
2. *Arochak*	Loss of appetite and anorexia
3. *Daha*	Burning sensation, internal or external
4. *Twaka Chhaya Vikriti*	Skin changes
5. *Krodha*	Getting irritated with small reasons
6. *Vedana*	Malaise or pain all over the body
7. *Dourbalya*	Feeling of weakness
8. *Nidranasa*	Disturbed sleep

20.5 *PANCHAKARMA* THERAPY: PERSONALIZED DETOXIFICATION AND REJUVENATION THERAPY

Panchakarma is a specialty of Ayurveda having diversified defensive, healing, and support actions indicated in wide range of diseases and health conditions. It is a personalized medicine concept where a therapeutic regimen is designed according to *Prakriti* (individual characteristics governed by Ayurgenomics) and other factors. These therapeutic procedures are employed to get rid of impurities of various systems of the human body and force out the toxic metabolites from the body by maintaining normal functions of systems. This is fundamentally a biocleansing regimen that facilitates the body system for better bioavailability of the pharmacological therapies and for achieving homeostasis of inherent factors. It also enhances the acceptability of the body to various dietary regimens and use of rejuvenation therapy for promoting health as well as for specific therapeutics.

There are three major steps: *Purvakarma* (preoperative), *Pradhanakarma* (main operation of the five variants), and *Paschatkarma* (postoperative) (see Figure 20.5). The group of five major detoxification measures composed of *Vamana* (therapeutic emesis), *Virechana* (therapeutic purgation), *Anuvasana Basti* (induced enema with medicated oil), *Niruha Basti* (induced enema with decoction of plants), and *Nasya* (nasal administration of medicaments) are technically termed *Panchakarma,* which stands on the basic principles and practices of Ayurveda. These major procedures require preoperative and postoperative measures to achieve the goal. The preoperative measures are *Deepana Pachana* (enhancement of enzyme activities), *Snehana* (oleation), *Swedana* (fomentation), etc., which are mandatory to perform in sequence before the major procedure targeting the subject as a whole followed by the disease specifically. *Snehana* or oleation is lubricating the system by using unctuous substances like oil or ghee externally and internally. *Swedana* is performed by applying heat over the body to stimulate the action of sweat glands, to secrete sweat, as well as to increase body temperature and circulation. There are so many procedures to perform according to the conditions.

Vamana Karma (see Figure 20.5) is one of the *Panchakarma* procedure indicated for *Kapha* disorders as mentioned in the Ayurvedic pathology. It is done by oral administration of emetic drugs in a particular dosage form with large amounts of liquids like milk or decoction of Madhuka (*Glycyrhizza glabra*) where the body toxins are expelled through the mouth. Strict diet regimen is followed for 7 days to enhance the *Agni* (enzymes activity) after *Vamana Karma,* which is known

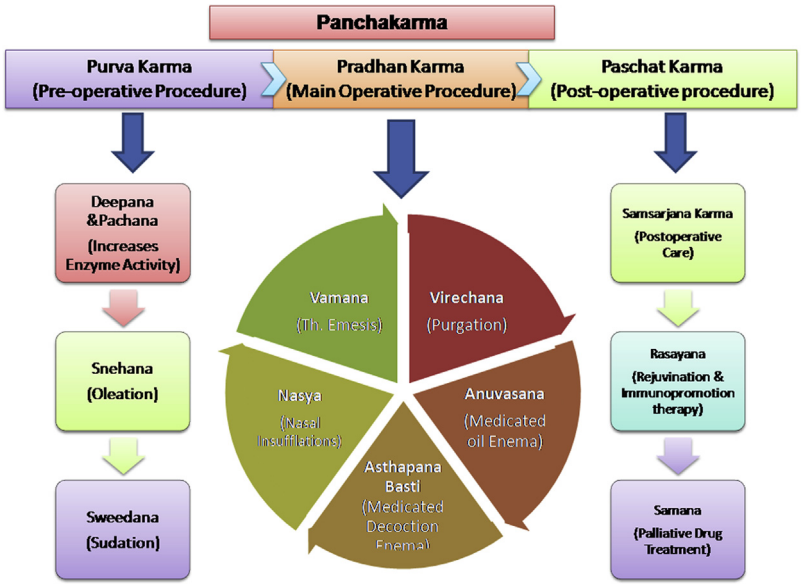


FIGURE 20.5 The conceptualization of *Panchakarma* (the five processes).

as *Samsarjan Krama*. This procedure is effective in bronchial asthma, psoriasis, and other chronic skin diseases. *Virechana Karma* (therapeutic purgation) is done by the oral administration of specific purgative drugs in a specific dosage form. It is highly effective in *Pitta*- and *Vata*-predominant diseases like skin diseases, urinary disorders, diabetes, anorectal diseases, arthritis, and hemiplegia. *Anuvasana* (induced enema with medicated oil) and *Niruha Basti* (induced enema with decoction of plants) are indicated in *Vata*-predominant diseases, such as arthritis, constipation, sciatica, hemiplegia, and amenorrhea. *Nasya Karma* (nasal application of drugs) is especially employed in specific disease conditions targeting the head and neck. Delivering of medicaments in the form of oils, powders, juices, etc., into the nostrils is performed. It is indicated in chronic nonspecific headache, brachial neuralgia, ear ache, chronic sinusitis, facial palsy, etc.

Postoperative regimen is the major step in *Panchakarma* after completion of the principle therapy. This is done to revitalize the *Agni* (metabolic enzyme system), which gets imbalanced due to the operative processes. This regimen should be strictly followed. Commonly, *Peyadi Samsarjana Krama* (postoperative medicated drink therapy) is widely practiced in all cases of *Pradhan Karma* causing the enhancement of enzyme activities of the patient. In this procedure, no oral medicine targeting the disease is administered. However, the diet schedule from liquid, semisolids, to solid is followed in that order. The schedule is to be designed by the physician according to the subject and disease. *Peya* (medicated drink), *Vilepi, Manda, Krita Yusha, Mamsa Rasa*, etc., are the different types of diet incorporated in the schedule. The period of treatment varies from 7 days to 3 months in different conditions.

20.5.1 *Panchakarma* Therapy in Psoriasis Treatment

Psoriasis is an autoimmune noninfectious, chronic inflammatory skin disorder in which altered keratinization of epidermal cell takes place with well-defined erythematous lesions and silvery plaque with a prediction for the extensor surface and scalp. In psoriasis, *Panchakarma* therapy is highly beneficial (see Figure 20.6) for its immunoprotection mechanism. In plaque psoriasis, *Vamana Karma* (therapeutic emesis) is generally practiced using some herbal emetic drugs. *Deepana, Pachana, Snehana,* and *Swedana Karma* are carried out before *Vamana Karma*. Recent study has shown that *Panchakarma* treatment can control psoriasis cases without any adverse drug reactions [38].

FIGURE 20.6 *Panchakarma* therapy in psoriasis with *Vamana Karma*.

20.5.2 *Jalaukavacharana* (Leech Therapy): Management of *Vicharchika* (Eczema)

In Ayurveda, the various skin disorders are detailed under the nomenclature of *Kushta*. *Vicharchika* is specially mentioned under the heading *Kshudra Kushta* (minor skin diseases) in Ayurvedic classics, and it is similar to the disease eczema of modern medical science [39,40]. The Ayurveda classics advocate several lines of conservative treatment for *Kushta* disease specially *Vicharchika* [41]. As per Ayurvedic understanding, diseases that do not respond to various medical treatments are definitely disorders in which blood is contaminated [42]. Among the *Shodhana Karmas* (purificatory treatments), bloodletting is considered as the best treatment for skin diseases (see Figure 20.7). According to Sushruta, if a person regularly undergoes bloodletting, he can develop resistance against all types of skin diseases [43]. Bloodletting is one of the ancient and important parasurgical procedures described in Ayurveda for treatment of various diseases. Various methods are employed for bloodletting such as the use of *Shringa* (horn), *Jalauka* (leech), *Alabu* (gourd), *Prachhana* (scarification), and *Siravyadha* (vein puncture). Among them, leech therapy (bloodletting using leech) has gained greater attention globally, because of the medicinal values it possesses. Being a disease caused due to vitiated blood, the bloodletting therapy using leeches can be advocated in case of eczema also. Keeping this view in mind, we have started and evaluated the efficacy of leech therapy in the patients with eczema.

20.5.3 *Sarpavisha Chikitsa*: Bioprospecting Snake Venom as Drugs

Poisonous substances (*Visha*) have been traditionally used in therapy since the time of *Charaka Samhita* (1000 BC) [44]. According to Sushruta it is profoundly used in Ayurvedic toxicology (*Agada Tantra*) for the treatment of other toxins [45]. In Ayurveda toxins are classified into two major sources: plant source (*Sthavara*—the immobile) and animal source (*Jangama*—the mobiles); another artificial source has been termed as *Gara visha* [46]. According to Indian mythology, it is believed that *Visha* originated when the *Devatas* (deities or demigods) and *Asuras* or *Rakshasas* (evil demons) were deeply engaged to *Samudra Manthana* or *Ksheera Sagara Mathanam* (event referring to "Churning of the Ocean of Milk") for the quest of *Amrita* (nectar of immortality) [47]. Both Charaka (Ch. Chi. 23/24) and Sushruta (Su. Kal 2/19-20) and later Vagbhata (Ash. Utt 35/7) explained various aspects of toxins and their pharmacological prospects. Toxins of various sources and their phenotypical symptomatic treatment have been extensively detailed by Charaka (Ch. Chi. 23). This commentary indicates that the use of a poison cures poisoning due to its *Prabhava* or specific action (Ch. Su. 26/69). Sir Jagadish Chandra Bose's Research on *Suchikavaran Rasa* reflected on the use of cobra venom as drug [48].

FIGURE 20.7 Nonpoisonous leech sucking the toxic blood in eczema patient.

In Indian Science Congress 13th session (1927) at Lahore, Sir J.C. Bose in his Presidential Address said *"Cobra venom acts on the animal as a deadly poison even in minute quantities. I found the effect on the plants was identical. I was greatly interested to find that a preparation of cobra venom known as Suchikavaran Rasa,the principal constituent of which is minute quantities of cobra venom, has been employed as a cardiac stimulant in the Hindu System of Medicine for nearly thousand and years. I found that minute doses of prepared cobra venom a great stimulation of the pulsating activity of the plants. Similarly injections of Suchikavaran Rasa in the blood stream of the animals in a state of depression, was found to produce a marked improvement in the frequency and amplitude of the pulsation of its heart beat."*

In Ayurveda snake venom is being used in *Dusyadaro* (a type of *Udararoga* i.e. diseases of abdomen). Different formulations that were prepared using snake venom are mentioned, among them *Suchikavaran Rasa* is advised to be used in collapse stage. Dr. J.C. Bose carried out an experiment with crude cobra venom (CV) and observed that crude-venom-administered plant died, while processed drug formulation did not showed any change. Further, in animal experiments, he observed that crude venom caused a decrease in tissue response, while after treatment with processed snake venom drug, it regained response and further stimulation was observed. Usually, CV is used after processing it with different plants products, which is known as *Shodhana*. The purification was done following the Debnath method and the *Shodhita* venom (SV) was used for pharmacological and toxicological studies [49,50]. Acute toxicity study reveals that the LD_{100} dose of crude CV was 0.7 mcg/kg, while all

the animals survived after treatment of with the same dose of SV. The calculated LD_{50} dose of CV and SV were found to be 0.62 and 5.4 mcg/kg i.p. intraperitoneal, respectively. SV showed antiinflammatory, analgesic, anticonversant, and positive ionotropic effect on perfused heart preparation, while CV produced stoppage of heart in systole (100 mcg/ml) but SV up to 250 mcg/ml did not produce any toxic effect on the heart. In frog rectus muscle, CV and SV antagonized acetylcholine response. The calculated ED_{50} was 45 mcg/ml for CV and 138 mcg/ml for SV. It is apparent that after *Shodhana* toxicity is reduced keeping intact the therapeutic effect. A traditional herbomineral preparation "*Mahammadsha*" was used for the treatment of osteoarthritis or *Sandhivata* [49,50].

20.5.4 *Rasayana Chikitsa*: Rejuvenation and Immunomodulation Therapy

Rasayana therapy is a comprehensive regimen that encompasses the concept of "rejuvenation" of mental and physical health. In Ayurveda *Rasayana* refers to acquisition, movement, or circulation of nutrition needed to the body tissue (known as *Dhatus* in Ayurveda). The *Rasayana* therapy is one among eight branches that are primarily of promotive value and are essentially meant to rejuvenate the body and mind to impart longevity aging and immunity against disease. The herbs and foods mentioned under this are perceived as immunomodulators, adaptogenic, antiaging, antistress, and memory enhancers.

It has been mentioned in *Charaka Samhita* in the very first chapter Chikitsa Sthana. However, other texts like *Sushruta Samhita* and *Ashtanga Hridaya* by Vagbhata mentioned it briefly with few additions or changes. Acharya Dalhana in his commentary on *Sushruta Samhita* (Su. Chi. 27/1-2) mentions a very rational classification, namely, *Ajasrika* (nutrition), *Kamya* (vigor and vitality), *Naimittika* (disease specific).

Rasayana is attained through direct enrichment of the nutritional flow (referred as *Poshaka Rasa*). Foods like Shatavari (*Asparagus racemosa*), milk, ghee etc., 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 (*Semicarpus anacardium*) is an example that acts at the metabolomic 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. Herbs and foods like Guggulu (*Commiphora mukul*) work in a similar way exhibiting hypolipidemic and antiatherosclerotic activity.

There is general idea that *Rasayana* is geriatrics, but it is actually science of nutrition that encompasses application for all ages to augment our vitality. *Charaka Samhita* clearly mentioned that nutrition is the primary aim of *Rasayana* and other effects such as antiaging, adaptogenic, and memory enhancer are secondary functions or attributes associated with the main aim. It is important to emphasize that *Rasayana* is not just a drug therapy that encompasses nutrition (*Ajasrika*) and good conduct (*Achara Rasayana*). Medicated ghee, milk, and foods have been mentioned under this *Ajasrika Rasayana*. Different *Rasayana* herbs and food supplements are used nowadays. Classical food supplements such as *Chyawanprasha* and *Brahmi Rasayana* are quite effective for all age groups. However, *Sharangadhara Samhita* has augmented further personalization or specificity of the *Rasayana* herbs according to the age group of a person. At present, we observe that many herbal preparations alter immune function and display an array of immunomodulatory effects [51]. In various *in vitro* and *in vivo* studies, herbal medicines have been reported to modulate cytokine secretion, histamine release, immunoglobulin secretion, class switching, cellular coreceptor expression, lymphocyte expression, phagocytosis, and so on [52,53]. Ayurveda lists a separate class of immunomodulatory botanicals named *Rasayanas*. Several botanicals from these texts have been studied for their immunomodulatory properties and have the potential to provide new scaffolds for safer, synergistic, cocktail immunodrugs [54]. 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 (i.e., asthma, respiratory problems) [55,56].

20.5.5 *Parpati Chikitsa*: The Last Option for Complex Disorders

"Vidya Vitarko VijnangSmriti Stattparata Kriya
Yasyate Sadagunmstattsaya Nang Sadyomutivartate"

(Learning, reasoning, proficiency, memory, devotion and experience, one credited with the six merits never fails in healing a curable disease)

(*Charaka Samhita*, Chikitsa Sthana 13)

In Ayurveda, therapeutic management is categorized into curable (*Sadhya*), palliative (*Yapya*), and incurable (*Asadhya*). *Udara Roga* (abdominal diseases), *Rajyakshma* (tuberculosis), and some others are considered to be incurable. Among the *Udara Roga*, *Jalodara* (ascites) was first described in *Atharva Veda*. Later, eight types of *Udara Roga* have been described in *Charaka Samhita* (Ch. Chi. 13) and *Sushruta Samhita* (Su. Chi. 14). *Jalodara* is still considered as an incurable disease devoid of any medicine to

cure it. Some specialized treatments are provided to improve life expectancy and quality of life, where accumulation of peritoneal fluid and scanty urine affecting liver and spleen are common. Ascites may develop due to many causes; among them cirrhosis of liver, tuberculosis, and cancer are very common. To these patients, *Parpati Chikitsa* is provided by experienced physicians. *Parpati* prepared with mercury and sulfur is known as *Rasa Parpati*, while on addition of gold to it *Swarna Parpati* is prepared. The process of preparation is very crucial as directed in *Rasashastra* (see section 20.7). During this treatment regime water and salt are restricted; only herbal extract with milk is provided as food and drink. *Parpati* is given at a daily dose of 1 Rati (120 mg) once daily for 21 days; if patients can sustain this therapy, then *Vardhaman Parpati Chikitsa* (ascending dose therapy from 1 to 10 Rati then back to the smallest therapy in a stepwise manner) is prescribed. Similarly *Vardhaman Pippali* (*Piper longum*) can be prescribed with increasing dose from 1 to 10 pieces. Clinical trial on the treatment of *Jalodara* (ascites) following the herbomineral preparation *Arogyavardhani Rasa* was done in 1962 at the IMS-BHU (Institute of Medical Sciences, Benaras Hindu University) (then college of medicine) for 5 years time; 390 patients were registered and 191 could complete the study. It was observed that 67.1% recovered from ascites and the remaining 12.5% were declared to be cured. A total 24 patients could be followed up for 4 years. The condition relapsed in some patients after 3 years and in some after 2 years. Patients discharged with no ascites may have chances of relapse if they do not follow proper diet and dietetics or discontinue the drugs [57].

20.6 AYURVEDA: REDISCOVERING NOVEL THERAPIES

20.6.1 Ayurveda Systems Biology

Ayurveda presents before us a bounty of clinically proven alternatives toward drug discovery as a function of evidence-based medicine [58]. Ayurvedic principles are developed on the basis of innumerable clinical observations (time tested). The scientific rationality of these principles is based not merely on ancient texts but because they can demonstrate results as can be found in *Ashtanga Hridaya Uttaratantra* in verse 40:81 [59]. The concepts of Ayurvedic biology deal with how the biological world has evolved to develop different stratifications of variability from the basic chemical level to the complex organism and beyond (see Figure 20.8). The Ayurvedic fundamentals have substantiated the view at different levels of associations and variable interactions giving rise to complexity. Ayurveda deals with this complexity taking a didactic modeling approach with overall view of system following the unifying interpretation of the *Advaitic* philosophy rather than a dualistic Cartesian approach.

Understanding the systems biology of a disease or a disease complex gives us the strategic advantage to identify target and also related challenge to find out ways that may help us to mitigate, treat, or manage the disease. The Ayurvedic internal medicine known as *Kayachikitsa* mainly consists of this etiopathogenesis, diagnosis, and treatment of the diseases. The novel effort like "Genome-wide association studies" are focused to determine where variations in its entirety for many diseased and healthy subjects could be compared to identify regions in the genome that had sufficiently different frequency and could be associated with a disease [60]. We find that Ayurvedic drugs and diseases have been classified according to phenotypic classifications (symptomatic complex) correlated with the genomic concept of *Prakriti* (*Vata, Pitta, Kapha*) [61]. In rational drug discovery process, we find that understanding the molecular pathogenesis is important for target validation and drug discovery process. It should be incorporated in Ayurveda as well. The diseases or phenotypes should be properly classified, and research should be initiated to know the molecular pathways or network associated with the diseases. This will help us to know the pharmacology of the herbs that interfere with the disease complex, which positively modulates the system (body and mind) against the disease. This translation of disease and treatment philosophy in terms of recent findings of "omics" level of research may discover novel targets or strategy to develop drugs or combinations of drugs against diseases [62,63]. Mechanism of action of the herbs may be demystified by combining Ayurvedic principles and functional genomics with systems biology approach [64,65].

20.6.2 Ayurgenomics and Personalized Medicine

Ayurveda has a personalized approach in predictive, preventive, and curative aspects of medicine, which intersects the mind and body [66]. Ayurgenomics deals with interindividual variability in assessing susceptibility and establishing diagnosis and prognosis mainly on the basis of constitution type of the individual (*Prakriti*) [33,61]. Selection of a suitable dietary, therapeutic, and lifestyle regime is made on the basis of clinical assessment of the individual keeping one's *Prakriti* in mind [33,67,68]. *Prakriti* is a consequence of the relative proportion of *Tridoshas* (three entities), *Vata*, *Pitta*, and *Kapha*, which are not only *Shukra Shonita* (genetically determined) but also influenced by *Mahabhuta Vikara* (environment), especially maternal diet and lifestyle (*Matura Ahara Vihara*) and age of the transmitting parents (*Kala Garbhashaya*). The ethnicity, familial characteristics, as

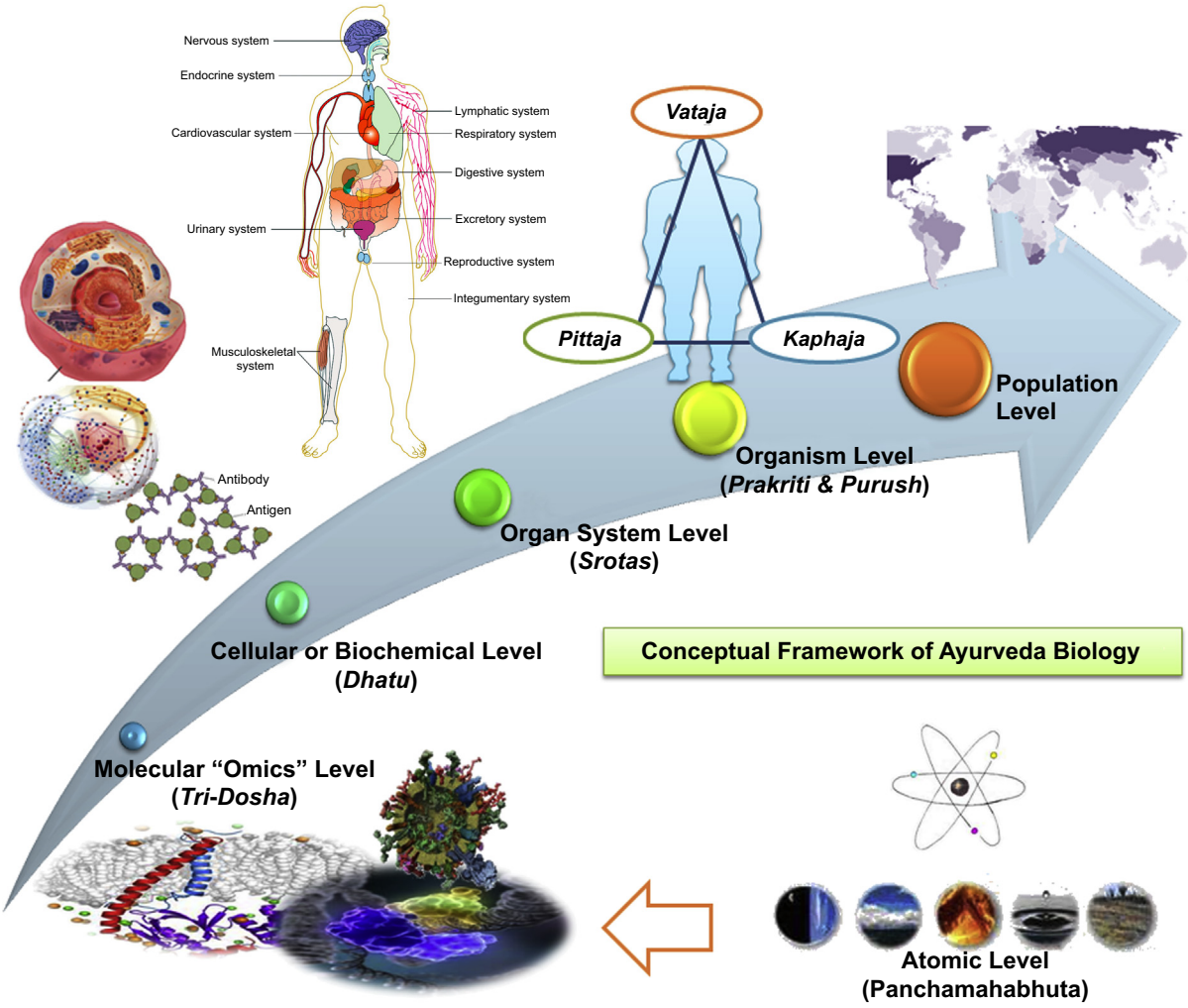


FIGURE 20.8 Conceptual framework (dogma) of Ayurveda biology.

well as place of origin of an individual are also described to influence development of *Prakriti* besides the aforementioned individual-specific factors. Several multiscale dimensions and stratification have been stressed by workers to increase the resolution of understanding about the disease mechanism to that of the genetic variome and related factors [61]. Metabolic variability correlated with CYP2C19 genetic variability and HLA gene polymorphism to elucidate the concept of pharmacogenomics with the *Prakriti* types is interesting [67,69]. However, understanding of population-wide variability across Indian populations (IGV Indian Genome Variation Consortium) provided a major thrust to further our quest for understanding the variability in healthy individuals. Transcriptional profiles of pooled RNA from *Vata*, *Pitta*, and *Kapha* revealed differences in core biological processes between the *Prakriti* groups, which overlapped with the biochemical pathways and biochemical profiles to signify the existence of genetic variation and their cellular manifestation as mentioned in Ayurvedic text. We find a glimpse where such genetic association may be connected with cardiovascular function [70], systemic inflammation [71,72], gut microbiota axis [73,74], anthropometry [75] and related diseases, which opens up a new age of Ayurgenomics-guided therapy based on Ayurvedic herbal products. Very simply it can solve the variation of the disease pattern and treatment efficacy variations among the populations seen from personal clinical experiences. As stressed by the authors in the above section integration of the knowledge of *Nidan Samprapti* (prognosis) with phenotypic ensembles and the upcoming genetic association can elucidate disease pathways, hence drug discovery will be accentuated in the lines of Ayurveda systems biology.

20.6.3 Reverse Pharmacology: Toward Integration from Clinics to Laboratory

Novel drug discovery from natural sources is contributing a huge share to the modern trends [76,77]. The terminology reverse pharmacology in natural product

drug discovery has been common to us for more than a decade. Its successful example can be traced back to the 1930s in India. Sir Ram Nath Chopra and Gananath Sen started the underpinning of reverse pharmacology of medicinal plants by following the clinically documented effects of Ayurvedic drugs [78]. Sarpagandha (*Rauwolfia serpentina*) was a major breakthrough where Sen and Bose were able to persuasively demonstrate the antihypertensive effects of the plant. They also noticed its side effects such as depression, extrapyramidal syndromes, and gynecomastia among others [79].This effort helped in the evolution of a new antidepressant, anti-Parkinson drug and prolactin-reducing drugs [80]. Reserpine is an example of an antihypertensive alkaloid from *Rauwolfia* that was marketed successfully. Most convincingly we find Gananath Sen and Kartick Bose as one of the oldest Indians to get cited in the celebrated pharmacology textbook Goodman and Gillman.

Later on Dr. Vaidya defined it as a reverse discipline that goes from "clinic to laboratories" [32] apart from the concept where it has been mentioned in relation to orphan G-protein coupled receptor (GPCR's) (Angelique and Ralph, 2008). Most lucidly it can be defined as an interdisciplinary scientific approach that integrates clinical and experimental knowledge toward safe and effective drug development [81]. Here it can be mentioned that the recent trend of translational research in Ayurveda from "bedside to bench" is also talking of the same direction [82].

This bedside-to-bench effort can be extended to the field of Ayurveda also where clinical observations are the main model to test the safety and efficacy because in ancient literature humans were treated as the only subjects. It can be strategic to develop clinical candidates, modify bioactivity of existing herbal drugs, and also develop vaccine adjuvants [83].

20.6.3.1 Reverse Pharmacognosy: From Molecule to Target

Recently, a concept called reverse pharmacognosy [84] just like reverse pharmacology has emerged, which utilizes natural molecules from a natural source, preferably plant, to find their biological activities, that is, their binding to specific target protein using cheminformatics or traditional knowledge (like Ayurveda) about the plant. In order to find the target the process uses in silico (like docking, molecular dynamics, pharmacophore matching) or in vitro methods. Together with chemogenomics and Ayurgenomics it can find application in drug repositioning, i.e., exploiting existing knowledge for innovation [64,85]. These are exclusively important when US Food and Drug Administration is advocating a Critical Path Initiative to modernize drug development by incorporating recent scientific advances to enhance innovative opportunities in public/private partnership model. The reason behind such policy may be postmarketing withdrawal in spite of stringent regulatory mechanism, thus safety is a major problem nowadays [86]. Pharmacogenomics is now a significant factor influencing drug discovery, which has been connected to the *Prakriti* concept, thus advocating personalized medicine [87].

20.6.3.2 Ayurvedic Pharmacology: Toward Safety and Personalized Efficacy

Ayurvedic drugs presume to work on multiple targets in a disease network. Ayurvedic pharmacology has the primary goal of ensuring safety and maintain efficacy by rationalizing the mode of action of drugs. A recent review shows various strategies of phenotypic screening like biochemical methods; genetic interactions together with computational inference have important roles in target identification and deciphering mode of action [88]. A successful example may be observed in the case of inflammation and cancer where Ayurvedic knowledge-based approach toward target identification strategy has been appreciated [89,90]. Mechanism of action is being elucidated in the most critical cases of herbomineral drugs, where studies encompassing immunomodulation and *Rasayana* (rejuvenation) using phenotypic disease screening method in *Drosophila* model has opened a novel dimension of Ayurvedic pharmacology [91].

Ayurveda has a huge knowledge base in pharmacology that needs rediscovery and rational interpretation. We eagerly need to consider research tools that provide extensive data including the "omics" tools, such as genomics, proteomics, and specially metabolomics, which have lots of role to play in natural product drug discovery [92–96]. Chronic diseases such as diabetes and obesity are caused by defects in multiple genes and pathways [97]. Thus, it is not surprising that the current one-target-one-compound approach in drug discovery and development has failed to deliver, and rise of polypharmacology and network pharmacology is observed as expected in the postgenomic era [98,99]. Network pharmacology and systems approach are gaining pace in deciphering traditional herbal therapies in complex diseases [100–102]. Furthermore, network-based approaches have been applied to understanding disease mechanisms [103].

It has been shown experimentally and accepted traditionally that the combination of certain drugs could be more potent than the simple sum expected from the action of two individual drugs [104]. Moreover, there should be unique treatment that fits best the molecular profile (*Prakriti*) of a person's disease and lifestyle habits (*Satmya*). Recently, an Ayurgenomics study on rheumatoid arthritis revealed that persons with different *Prakriti*

showed different pathways involved in causing the same disease. Thus it is logical that drugs should be personalized. Targeting individual mechanism of action is observed to be more efficacious ensuring safety [105].

Considering such scenario we should also find suitable translational technologies that can help us to interpret these traditional concepts of medicine with advanced level of scientific rationality. Translational systems biology approach may accelerate Ayurvedic medicine research as it can be found in Traditional Chinese Medicine in recent literature [106–108]. Understanding the mechanism of action and interactions at the level of molecular networks of complex diseases or systems level is very useful to bridge gaps in natural product drug discovery and has huge potential of research in drug discovery and development [109–111]. Diseases of pathogenic origin with complex pathogenesis can also be treated with Ayurvedic adjunct therapy. Recently, "bedside-to-bench" initiatives pertaining to use of Ashwagandha in multidrug-resistant tuberculosis patients have shown success [6]. Clinical trials followed by in silico target fishing effort have established a possible mode of action about the herbal adjunct therapy. Adjunct therapy of herbal drugs with conventional Anti-tubercular Drug (ATD) therapy also opened up a novel strategy of immunoprotection that may lead to new direction in chemotherapy.

Considering the various knowledge-based resources available and the presence of technological platforms in the field of pharmacology, systems biology, and genomics, we find the need to integrate various disciplines and ideas following the essence of reverse pharmacology. We propose a conceptual workflow (see Figure 20.9) considering a continuous relationship between the scientist, physician, and patient. The in silico pathway pertaining to Ayur-chemogenomics, a concept embracing Ayurvedic Pharmacology and chemogenomics, can be readily used to predict mode of action of Ayurvedic drugs as seen in the recent literature [112,113]. We find that Ayurveda reverse pharmacology research should start from traditional knowledge followed by clinical study (bedside) following Ayurgenomics and Ayur-pharmacoepidemiology (see section). Then on the basis of clinical data laboratory studies may be undertaken. The laboratory study may be in silico or it may follow an experimental pathway where phenotypic assays may reveal molecular-level interactions. This information can then be processed using computational inference technologies for developing association model to understand the network-level interaction of the drugs and reveal the network or systems pharmacology. As herbs and herbominerals are multicomponent drugs, their polypharmacological network will provide a clear picture.

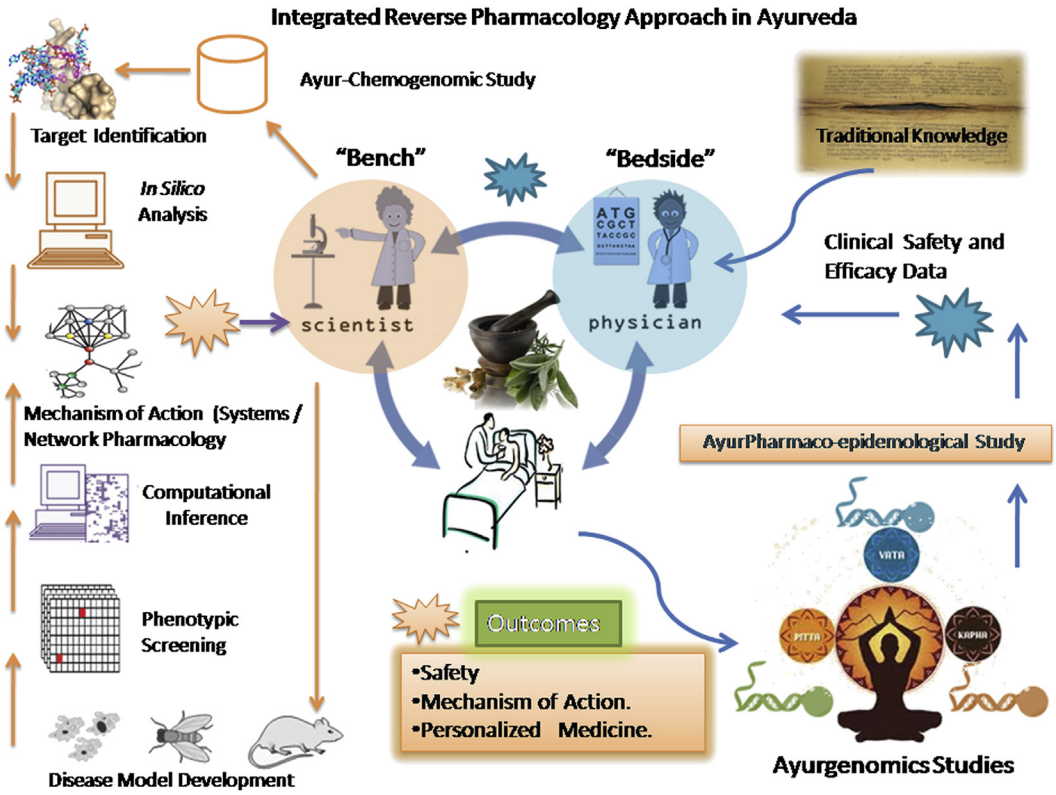


FIGURE 20.9 Conceptual Workflow of Integrated Reverse Pharmacology approach in Ayurveda.

20.7 *RASASHASTRA*: THE MYSTERY OF ANCIENT CHEMISTRY

In the evolution of nature, inorganic matter is observed prior to organic matter, after which creation of life has occurred. Therefore it can be easily verified that inorganic materials are the primary constituents of life processes [114]. The main source of foods and medicine are therefore the principal support of life on earth; Plants derive their micronutrients from minerals and metals [115,116]. Plants convert these inorganic substances into bioassimilable forms. However, little is known about inorganic materials and about how they provide such an important resource to life forms and thus a growing interest in science. In this section, we will try to decipher and appreciate how ancient medical science like Ayurveda was able to successfully use these metals and minerals in safe and effective therapies by developing specialized technologies to tap their therapeutic power.

Rasashastra developed during eighth century AD, *Rasashastra* (*Rasa*—mercury, *Shastra*—knowledge) is one of the specialized, almost secretive branch of Ayurveda that deals with herbomineral/metal/nonmetal preparations [117]. *Rasashastra* is centrally based on the medical use of *Shodhita Parada* (pharmaceutically processed mercury). Mercury is termed as "*Rasa*" or "*Rasendra*" and "*Parada.*" It is called "*Rasa*" because it has the power to transform or associate with other metals to from amalgams. *Rasayana* (immunomodulation and antiaging quality) and *Yogavahi* (ability to target drugs to the site) are characteristics of a properly prepared herbomineral/metal/nonmetal preparation (see Figure 20.10), which is also nontoxic, biologically acceptable, and assimilable by the patient's body [118,119]. It is regarded as the most powerful form of Ayurvedic drugs, which are known for their rapid and prompt action at low dose due to their nanoparticle level [120,121]. However, it needs the highest form of expertise to prepare and use these drugs, where adherence to strict code of practices and adequate expertise has been mandated. It has been mentioned about drug preparation that "*Mardanam Guna Vardhanam.*" It means that the more a drug is processed to reduce its particle size, the more enhanced id its efficacy; nowadays concepts of nanomedicine highly focus on this. It is very much true, as from practical experience while manufacturing drugs it has been found that after prescribed *Marana* regimen, the "*Rasa*" drugs achieve nanoparticular size range, which helps its easy absorption and elimination, thus showing better effect. The drugs are administered in small dose as they have high efficacy compared to herbs, but if not prepared properly, they may be detrimental as the minerals and metals are toxic themselves. It is regarded as the complex branch of Ayurvedic pharmaceutics that has described the preparation of most powerful drugs in Ayurveda encompassing medicinal uses of around 70 different metals and minerals along with herbs [122–124].

FIGURE 20.10 Facets of *Rasasashtra*: *Bhasmas, Kuppipakwa, Makaradhwaja, Marana, Rasausadhis.*

20.7.1 Traditional Bioinorganic Medicinal Chemistry

Metals have been used in treatments since ancient times. The *Ebers Papyrus* from 1500 BC is the first written account of the use of metals for treatment and describes the use of copper to reduce inflammation and the use of iron to treat anemia. Recently, metals have been used to treat cancer, by specifically attacking cancer cells and interacting directly with DNA. Metals, minerals, and their use in Ayurveda as therapeutic modalities are being much celebrated and practiced due to their small dose requirement and rapid onset of action contrary to the herbal drugs [125,126]. Bioinorganic chemistry focuses on the function of inorganic substances in living systems, including the transport, speciation eventually leading to mineralization of inorganic materials, and the use of "inorganics" in medicinal therapy and diagnosis [127,128]. Therefore traditional use of "inorganics" as drugs may be referred to as traditional bioinorganic chemistry while dealing with the ancient science like *Rasashastra*.

The traditional knowledge about chemistry flourished in ancient India since Indus Valley Civilization (2500–1500 BC). During the Vedic period (1500–600 BC) we find mentions of the use of metal and medicinal plants for curing diseases in *Atharva Veda* (eleventh century BC). *Atharva Veda* mentions use of gold, *Harita* (yellow); silver, *Rajata* (white); and copper, *Lohita* (red). The hymns of *Atharva Veda* for cure of

diseases are referred to as *Bhaishajyani*, while those that have as their object the prolongation of life and preservation of youth and health are known as *Ayushyani*, a term that later gave place to *Rasayani*, the Sanskrit equivalent of alchemy. Gold is mentioned to provide longevity, strength, or as *Rasayana*. Thus it can be observed that the origin of alchemical notions gathered around gold, lead, and other medicinal plants. However, medicinal use of chemistry evolved to a great height during the Ayurvedic period (600 BC–800 AD). Although, mercury was not used until the Transition period (800–1100 AD). Then we find the rise of Tantric cults during the Tantric period (700–1300 AD) and use of mercury got dominance. Several books on *Rasashastra* developed the bioinorganic chemistry knowledge during this time (see Table 20.8). This was followed by Iatrochemical period (1300–1550 AD) during which there was further development of different aspects of use of minerals and metals as medicine [129]. These significant developments of Traditional Bioinorganic Medicinal Chemistry can be observed in several ancient literature over a period of time (see Table 20.9) Drug preparations from metals and minerals are included in the Ayurvedic pharmacopoeia. The quality control, standardization starting from procurement of raw material and their processing to finished product, is well enumerated. The standard operational procedure is maintained on the aegis of Ayurvedic literature. The following metallic drugs are safe and nontoxic as they are backed by scientific evidences for standards based on observation and hands-on experiences [130].

TABLE 20.9 Significant Mention of Metals and Minerals for Medical Purpose in Different Ancient Literatures

Ancient literature	Significance in traditional bioinorganic medicinal chemistry
Atharva Veda (eleventh BC)	Mentions use of gold, silver, copper in medicine with some other medicinal plant juice
Charaka Samhita (1000 BC–fourth century)	Mentions six metals. Chemical methods of preparation of copper sulfate, iron sulfate orpiment in combination with herbs Rust of iron and pyrite has been mentioned as ingredient of pills. Processing of metals involving roasting with sulfur before being used for medical purpose referred to as *Marana* (killing of metals)
Sushruta Samhita (1000 BC–fifth century)	Mentions six metals, viz. tin, lead, copper, silver, *krishna loha* (iron), and gold. Sulfates of iron and copper, alum, red ocher, realgar, etc., were meant for external use. Roasting of metals was mandated before internal use Compound of arsenic such as white arsenic (*Phenasma Bhasma*) and orpiments were recognized as poisons Practical methods like preparation of metallic salts have been referred to as *Ayaskriti*
Astanga Hridaya (800–850 AD)	Minerals and natural salts along with herbs have been prescribed In one instance, roasting of metals in closed crucible (*Ardhamusha*) i.e., a mixture of 64 parts of stilbium (*Srotanjana*) and one part each of copper, iron, silver, and gold, is mentioned Equal parts of mercury and lead are mixed to make them into a cillyrium with equal proportions of stilbium and camphor

20.7.1.1 Pharmacology of Metals and Minerals in Ayurveda

The most commonly used metals in Ayurveda preparations are gold, silver, iron, copper, tin, lead, zinc, mercury, and arsenicals. A combination of metals such as *Sarva Louha* (containing tin, lead, iron, copper, and silver) finely pulverized in myrobalan juice (*Phyllanthus emblica*, *Terminalia chebula*, and *Terminalia bellirica* separately or in combination) was known to be a very good tonic [131]. Usually, sulfur preparation is used externally for the treatment of skin diseases. Antimony sulfide, iron sulfate, and copper sulfate preparations were used extensively for external application. Alchemy was transformed in Ayurveda as *Rasashastra* (Hindu chemistry). It may be noted that in *Rasashastra*, mineral drugs especially those of mercury and sulfur, were used after processing (*Samasakara*), as it was recognized that those drugs are toxic. In order to reduce or remove their toxicity, a number of *Shodhana* or purification process were performed. These include grinding with plant extract and with other acidic and alkaline liquids, heating and dipping in various liquids, or by boiling, fusion, sublimation, etc. Through this purification process, all washable and volatile impurities of minerals were removed. Sometimes organic or inorganic materials are added either in traces or in large quantities for detoxification or potentiating the efficacy. The drugs for mineral origin are subjected to *Bhavana* (grinding with plant extracts and acidic liquids) and then the material is ready for *Putapaka* [132,133].

Bhasma is the ultimate therapeutic state of minerals or metals that are administered to the patients. It may be defined as the alkaline ash, which is prepared by

TABLE 20.8 Eminent Books and Contributors of *Rasasashtra*

Contributors	Book name
Nagarjuna	*Rasaratnakara*
Shankaracharya	*Rasahridayatantra*
Govinda Bhagabat	*Rasahridaya*
Rasaratnasamuchaya	*Yasodhara*
Rasachintamani	*Madantadeva*

calcination or incineration of minerals drugs along with herbs and/or their extracts [118,134]. The process by which a *Bhasma* is prepared is called *Marana* and *Jarana* and the method used is known as "*Putas.*" Different types and numbers of *Putas* and were described for different metals and minerals. The outcome of *Putas* on the minerals and metals are anaerobic oxidation or calcination. Calcined minerals and metals are compressed into very minute form, so that they may be absorbed easily. After intake of the minerals in the *Bhasma* state it is converted into colloidal form and can pass through the minutest capillaries due to nanoparticular size [119–121].

20.7.1.2 *Makaradhwaja:* A Panacea?

Mercury is known to mankind for a long time; mercury amalgamation for gold recovery has been employed for the past 2000 years. Mercury was one of the first metals known to mankind. Aristotle and Theophrastus in 300 BC mentioned that liquid silver also known as quick silver is obtained by rubbing cinnabar with vinegar in a copper mortar and pestle. Diseriodes in first century AD stated that mercury could be obtained by heating cinnabar with charcoal in an iron pot. It is one of the most toxic metals; it is toxic in every form, but depending on the compound the degree of toxicity depends on the actions on the critical target organ [135]. The natural mercury compound is cinnabar. It is an amalgam of mercury and sulfur. In Ayurveda, Unani and Siddha sublimates of mercury ($HgSO_4$) are used.

Makaradhawaja is a well-known Ayurvedic mineral preparation containing mercury, sulfur and gold, which have been used in continuity through the ages. The inclusion in the therapeutic modalities relates with the evolution of life. On the basis of philosophical background and scientific reasoning, its uses were intricate with Ayurveda. The term *Makaradhwaja* bears a close resemblance and symbolizes the power of vitality and rejuvenation.

The *Makaradhwaja* a product, crystalline in texture; it looks like cinnabar and signifies the emblem of *Makara*. *Makara* is a fabulous creature, its anterior half resembles a crocodile, while its posterior half looks like a fish. Water is the universal life essence, and the primordial element is the greatest creative power. Crocodile lives mostly in large rivers of the world and became the symbol of water and thus symbolizes the life-protecting power with both fertility and fecundity. The drug that carries the flag of *Makara* symbolizing the activity is known as *Makaradhawaja*. Naturally occurring cinnabar or mercuric sulfide symbolize virtues of fish and crocodile, representing reproductive cum creative power. In Indian mythology Lord Shiva is the symbol of creation and mercury is the agent that he employed. As such for creation, God Shiva and Goddess Parvati's fusion is essential. Mercury symbolizes semen of Shiva and sulfur is the *Rajah* (generative material) of Parvati. With Shiva –Parvati's fusion starts are created. Surprisingly, this correlation could be interlinked with the preparation of *Makaradhwaja*. The drug is actually prepared by Ayurvedic physicians using 1 part gold, 8 parts mercury, and 16 parts sulfur added and triturated in stone mortar and pestle; the product is a black mixture ready for sublimation on sand bath.

While on drug preparation, mercury is extracted from cinnabar. Naturally occurring cinnabar is an amalgam of sulfur with other trace elements. Moreover, mercury has got natural affinity for gold. And since ancient times, in gold extraction mercury is being used. The ratio of mercury and gold is always 8:1 by weight. The amalgamation depends on the coordination number with ratio. It is surprising how the ancient scientists without knowing the coordination number fixed this ratio of mercury and gold for the preparation of *Makaradhawaja* [136].

When these crystals were pulverized they became like red vermilion (See Figure 20.10). When the fine powder is made fine enough nanoparticles are formed known as *Anu Makaradhawaja* [129]. It is thought that the atomized form turns to a living form like soul and is the best drug to save life from death and decay. In the early 1930s it was marketed by Merck (Germany) and The Bengal Chemical, India. Recent pharmacological studies show that these traditionally processed mercuric substances are not toxic and are almost 1000 times safer than methyl mercury [137].

Traditional medicines (TM) are widely used by one-third of the world's population [138] due to affordability and because they tally with patient's beliefs. In fact, clinical and animal studies also render them to be nontoxic and safe [139,140].

20.8 AYUR-PHARMACOEPIDEMIOLOGY

20.8.1 Background

Almost 50% of poorest Asian and African population does not have regular access to essential drug [141], and evidence of increased cost of allopathic medication, polypharmacy, and chronic diseases also supports this trend [142–144]. Plant materials are used throughout developed and developing countries as home remedies, over-the-counter drug products, and raw materials for the pharmaceutical industry, and represent a substantial proportion of the global drug market [145]. Thus, clients, policy makers, pharmaceutical establishments, government organizations, clinicians, and patients are all putting interest in drugs with cost-effectiveness and

comparative effectiveness research, cheering evidence-based medicine to make sure patients get quality care, and focusing on helping patients use medications properly [142–144].

In the Indian context, TM is used by approximately 70% of the population for primary health care [146]. The World Health Organization emphasizes that the quality, safety, and efficacy of medicinal plants should be evaluated, ensuring rational use of plant-based products in an integrated approach [147]. As a matter of fact Ayurveda with its unique fundamental principles and systematic approach documents a diversity of health care practices [148]. Agreed upon the needs to study the use of drugs in the factual domain, continuous surveillance of drug use and ways to evaluate how patient characteristics influence drug utilization and clinical outcomes in large populations can be met through the use of pharmacoepidemiologic research designs [149].

20.8.2 Concept of Ayur-Pharmacoepidemiology

There is a widespread misconception that all drugs of "natural" origin are "safe" and long-term use of a medicine based on tradition assures both safety and efficacy. Currently, the majority of adverse events related to the use of herbal/traditional products that are reported are attributed either to poor product quality or to improper use [150]. Ayurvedic products and therapies are recognized and endorsed globally. So, it is important to ensure that the health care provided by Ayurveda is safe and reliable maintaining standards for the safety, efficacy, and quality control. Ayurvedic pharmacotherapy may offer many challenges to clinicians as they are not aware of the potential benefits, efficacy, and risks of Ayurvedic drugs to individual and population-based patient care. It is not realistic to believe that large, prospective clinical trials can be conducted to understand all issues of Ayurvedic medication due to financial and time constraint. Ayur-pharmacoepidemiology research is one such technique that can be implemented to acquire facts about Ayurvedic medication practice and safety.

Ayur-pharmacoepidemiology studies can usually be less expensive and provide some evidence as to the use and safety of Ayurvedic medications in populations. By combining the interest of Ayurveda, pharmacology, and epidemiology, an Ayur-pharmacoepidemiologist will apply epidemiological principles to study the effects of Ayurvedic medications/medicinal herbs in human populations. Ayur-pharmacoepidemiology studies will quantify Ayurvedic drug use patterns and adverse Ayurvedic drug effects including common predictable adverse drug reactions as well as the uncommon and unpredictable ones. In this context the term Ayur-pharmacoepidemiology is defined as *"The study of the use of and the effects of Ayurvedic herbs/drugs in large numbers of people with the purpose of supporting a rational and thereby cost-effective use of safe and effective Ayurvedic herbs/drugs in the population."* The field of Ayur-pharmacoepidemiology will use the effects of Ayurvedic medicinal products on large populations in order to describe and analyze the practices and conditions of use, evaluate the safety and efficacy as an alternative to a clinical trial (including pharmacovigilance surveillance), evaluate the effectiveness in a routine situation (comparative effectiveness research), and carry out economic and medicoeconomic evaluations.

20.8.3 Good Pharmacoepidemiology Practices in Ayurveda

Pharmacoepidemiology is being used progressively to appraise health care systems, interventions, and health-related behaviors. It is the scientific mainstay of assessing a drug's benefits and risks, and developing, executing, and assessing strategies to enhance the overall balance of such benefits and risks. The International Society of Pharmacoepidemiology (ISPE) identified that pharmacoepidemiologic research designates the study area in which uses and effects of health care products expanding to clinical, economic, and other health outcomes are measured. The ISPE Guidelines for Good Pharmacoepidemiology Practices (GPP) are intended to assist investigators with issues pertaining to the planning, conduct, and evaluation of pharmacoepidemiologic research. The adopted GPP guideline addresses areas such as protocol development; responsibilities, personnel, facilities, resource commitment, and contractors; study conduct; communication; adverse event reporting; and archiving [151]. In perspective of this, Ayur-pharmacoepidemiologic studies may be able to provide valuable evidence about the health effects of Ayurvedic herbs/drugs (Anonymous, 2008).

GPP in Ayurveda will assist researchers in following good Ayur-pharmacoepidemiologic research principles, including the use of Ayur-pharmacoepidemiologic studies for risk management events of Ayurvedic herb/drugs; promote comprehensive Ayur-pharmacoepidemiologic research by encouraging arduous data collection, analysis, and reporting; provide a framework for conducting and evaluating Ayur-pharmacoepidemiologic studies; and facilitate suitable use of technical resources by inspiring vigilant study design and planning of study conduct. This in turn will help to understand the usage of Ayurvedic medicines (generic \proprietary), their safety and novel beneficial effects, as well as adverse events/effects. Also, new

indications for a given plant/drug are captured and they serve as the major resource for reverse pharmacology.

20.8.4 Pharmacovigilance in Ayurveda

Inclusion of Ayurveda in pharmacovigilance systems has become increasingly important given the growing use of Ayurvedic products and medicines globally. Pharmacovigilance of Ayurvedic herbs/drugs can be stated as "*the detection, assessment and prevention of adverse drug reactions from Ayurvedic origin in humans.*" The process monitors Ayurvedic medicines as used in everyday practice to identify previously unrecognized adverse effects or changes in the patterns of their adverse effects; assessing the risks and benefits of Ayurvedic medicines in order to determine the obligatory actions to expand their safe use; providing information to users to optimize safe and effective use of Ayurvedic medicines; and monitoring the impact of actions taken [150].

Ayurveda advocates the personalized methodology in treating patients. Furthermore, instead of using single therapeutics, Ayurveda uses combinatorial therapy, which is more complex in nature. Systematically determined seasonal and daily regimes including lifestyle modification and Ayurvedic dietetics based on Ayurvedic *Prakriti* (phenotype) are the unique features of Ayurvedic management. It is reasonable to arbiter the success of Ayurvedic treatments in terms of Ayurvedic end points and not be restricted to endpoints designated by the biomedicine approach. The Ayurvedic system of medicine has endured and flourished over an extensive period of time, which is a signal that it works. Better record keeping tracking the effectiveness and safety of the therapeutic regimen sustained by a vigorous pharmacovigilance program is already taking place. The Ayurvedic pharmacovigilance program should focus more on effectiveness rather than on safety, as is the case with conventional medicine databases. The conceptual framework for new models of Ayurvedic clinical studies and guidelines for AYUSH Good Clinical Practices (GCP) have stemmed from the principles and practices of Ayurveda [152]. If practiced properly, the global scientific community should also benefit.

20.8.5 Future of Ayur-Pharmacoepidemiology: A New Beginning

Progress in pharmacoepidemiology arises from increased use of computerized data. The major drawback of Ayurveda is the paucity of well-documented clinical records. Poor quality of data management needs to be rectified with more attention to the special needs of the Ayurvedic sector. Reverse pharmacology or observational therapeutics, metaanalyses, case studies and case series, and an effective pharmacovigilance program are much appreciated rather than performing randomized controlled trials of Ayurveda managements. Hence, Ayur-pharmacoepidemiology should be initiated from the Primary Health Care (PHC) level. Major emphasis should be on Ayurvedic drug utilization, prevalence of disease, and safety of drugs with provision of special training to Ayurvedic doctors. Thus, future research efforts must focus on Ayur-pharmacoepidemiological research, which will take into account full-bodied documentation and accepting their contrivances thus promising a epidemiologically safe and effective health care system.

20.9 CONCLUSION

Present is formed admitting the past, without the knowledge of ancient past, present and future remains incomprehensible

[It will be appropriate to divulge the issue on health, healthcare and its management in the realm of past. India has a rich heritage of traditional medicine and traditional health care system. In realm of science it is realized that the ancient medicine Ayurveda emphasized that the science as the father of knowledge and experiences as the mother of science.] [153]

Sir Jagadish Chandra Bose.

Ayurveda is a contemporary science. It is a misnomer to state that Ayurveda is an ancient science. On the contrary, Ayurveda has survived throughout the country and is still very much alive in India today, like some other ancient systems of medicine. Existing concepts of Ayurveda are to be revaluated in the modern concept strategies. This is required to make Ayurveda more scientifically validated and acceptable to the world community. As we start the millennium, it is imperative that the rich heritage of Ayurveda be rationalized and revamped for its use for the suffering million all over the world. With the perpetuated traditional knowledge many events of life remained unanswered. Amidst the enormous growth of science and the available plethora of knowledge base, we are still unable to solve questions [154] like how we are alive, how disease sets in, and what govern life and death. In Ayurveda cure, and healing are distinctly different. Cure basically reflects the physical reality, which is the body is free from specific symptoms and one is returned to a familiar state of function. Healing occurs at different levels of human existence. One can be healed without physically being cured of specific symptoms or disease [155]. The holistic therapeutic model of Ayurveda needs moderation with cutting-edge technology being ecofriendly for connecting people. The Government of India Department of AYUSH had made a positive

stride, and the initiatives had attained hike in this millennium.

The following needs to be done: identify gaps in disease and drugs, brainstorming session on each disease condition to identify formulations. research and development in identified formulations and drugs, evaluation of safety and efficacy data, preparation of dossiers of effective formulations, interaction with industry for manufacturing of selected formulations, operational research, publicity and awareness. In education sector, the All India Institute of Ayurvedic Medical Sciences is already under the process of construction. Through the Central Council for Indian Medicine (CCIM) (1970), minimum standard of education in undergraduate and postgraduate (PG) teaching, specialties in PG course, awareness on Ayurveda in foreign countries, cultivation of medicinal plant, and revival of traditional health care are being focused. In the area of research, reverse pharmacology approach, Ayur-pharmacoepidemiology, and Golden Triangle Project (GTP) initiatives must be prioritized with special interests on research related to metals and minerals.

References

[1] Sharma RK, Dash B. Agnivesa's Charaka Samhita, text with English translation & critical exposition based on Cakrapani Datta's Ayurvedadipika. 2nd ed. Varanasi: Chowkhamba Sanskrit Series Office; 2001.

[2] Atreya. Perfect balance: Ayurvedic nutrition for mind, body, and soul. New York: Penguin Penguin Putnam Inc; 2002.

[3] Heyn B. Ayurveda: the ancient Indian art of natural medicine & life extension. Vermont: Inner Traditions/Bear & Co; 1990.

[4] Shastri A. Sushruta: Sushruta Samhita with Ayurveda Tattva Sandipika Hindi commentary. 14th ed. Varanasi: Chaukhambha Sanskrit Sansthan; 2003.

[5] Datta GK, Debnath PK. Stress adaptation in Ayurveda by immunomodulatory Rasayana. pp. 60–75. In: Proceedings of the National Seminar on Rasayana. New Delhi: Central Council for Research in Ayurvedic Sciences; 2001.

[6] Banerjee S, Debnath PK, Saikia AK. Herbal adjunct therapy from "Bedside to bench" Chromatography and in-silico studies of vedic plant Aswagandha (*Withania somnifera*) as anti-tubercular adjunct therapy. Germany: Lambert Academic Publishing; 2014.

[7] Debnath PK, Chattopadhayay J, Mitra A, et al. Adjunct therapy of Ayurvedic medicine with antitubercular drugs on the therapeutic management of pulmonary tuberculosis. J. Ayurveda Integr Med 2012;3:141–9.

[8] Ray P, Gupta HN, Roy M. Susruta Samhita (A scientific synopsis). 1st ed. New Delhi: Indian National Science Academy; 1993.

[9] Ray P, Gupta HN. Charaka Samhita (A scientific synopsis). New Delhi: National Institute of Sciences of India; 1965.

[10] Kutumbiah P. Ancient Indian medicine. Reissued 1999. Chennai: Orient Longman Limited; 1962.

[11] Athavale VB. Ayurveda the Science of life (Health and Vigour forever). New Delhi: Chaukhamba Sanskrit Pratisthan; 2003.

[12] Srikanthamurthy KR. Vagbhat's Ashtanga Hridayam. Varanasi: Chowkhamba Krishnadas Academy; 2010.

[13] Misra RP. Geography of health: a treatise on Geography of life and death in India. New Delhi: Concept Publishing Company; 2007.

[14] Srikantamurthy KR. Madhava Nidanam (Roga Vinischaya) of Madhavakara. Varanasi: Chaukhambha Orientalia; 2003.

[15] Dhyani SC. Kayachikitsa (Hindi). Lucknow: Ayurvedic & Tibb Academy; 1982.

[16] Dhyani SC. Nidan Panchaka (Hindi). Varanasi: Chowkhambha Sura Bharati Prakasana; 1983.

[17] Roy A, Debnath PK. Rogavinischaya (Bengali version). Kolkata: J.B. Roy State Ayurvedic Medical College & Hospital; 2000.

[18] Sen G. Siddhanta Nidanam. Calcutta: Kalpataru Press; 1926.

[19] Singh RH. Ayurvediya Nidan Chikitsa ke Siddhanta (Hindi version). Varanasi: Chowkhamba Amar Bharati Prakashan; 2005.

[20] Sharma P. Sharangadhara Samhita, Subodhini Hindi commentary. 7th ed. Varanasi: Chaukhambha Amarbharati Prakashana; 1988.

[21] Sensharma K, Bhattacharya SS. Nadi Prakash O Nadi Vigyan by Mahamuni Kanad. Kolkata: Deepayan Publication; 1998.

[22] Bandopadhyay AK. Nadi Prakasham including Nadivigyan of Kanada. Kolkata: Balaram Prakashani; 1996.

[23] Singh J, Bagchi GD, Khanuja SPS. Manufacturing and quality control of Ayurvedic and herbal preparations. In: Verpoorte R, Mukherjee PK, editors. GMP for botanicals. New Delhi: Business Horizons Ltd.; 2003. p. 201–30.

[24] AYUSH, Govt. of India. Medicinal plants used in Ayurveda. New Delhi: R.A.V. Publication; 1998.

[25] Dikshith TSS. Safe use of chemicals: a practical guide. Boca Raton: CRC Press; 2008.

[26] Clements M. Ayurveda: mother of traditional medicine. In: Mackenzie ER, Rakel B, editors. Complementary and alternative medicine for older adults: a guide to holistic approaches to healthy aging. New York: Springer; 2006. p. 215–32.

[27] Mukherjee PK, Maity N, Nema NK, et al. Bioactive compounds from natural resources against skin aging. Phytomedicine 2011; 19:64–73.

[28] National Commission on Macroeconomics and Health. Financing and delivery of health care services in India. New Delhi: Ministry of Health & Family Welfare, Government of India; 2005.

[29] Mukherjee PK, Venkatesh M, Gantait A. Ayurveda in modern medicine: development and modification of bioactivity. In: Mander L, Lui HW, editors. Comprehensive natural products-II—Chemistry and biology. United Kingdom: Elsevier Science; 2010. p. 479–507.

[30] Mukherjee PK, Nema NK, Venkatesh P, et al. Changing scenario for promotion and development of Ayurveda - way forward. J Ethnopharmacol 2012;143:424–34.

[31] Chatterjee K. In search of an alternative. In: Mukhopadhaya A, editor. State of India's health. New Delhi: Voluntary Health Association of India; 1992.

[32] Vaidya ADB. Reverse pharmacological correlates of Ayurvedic drug actions. Ind J Pharmacol 2006;38:311–5.

[33] Prasher B, Negi S, Aggarwal S, et al. Whole genome expression and biochemical correlates of extreme constitutional types defined in Ayurveda. J Transl Med 2008;6:48.

[34] Dhyani SC. Rasa-panchaka (Ayurvedic principles of drug action). Varanasi: Chowkhamba Krishnadas Academy; 2007.

[35] Athavale VB. Pathogenesis in Ayurveda (Samprapti). New Delhi: Chukhamba Sanskrit Pratisthan; 2001.

[36] Selye H. Stress and disease. Science 1955;122:625–31.

[37] Debnath PK. Stress adaptation. Paper presented at the National Seminar on Rasayan. New Delhi: Central Council for Research in Ayurvedic Science; 1999.

[38] Mitra A, Banerjee M, Das B, et al. Acquiscence of Ayurvedic principles and practices in *Kitibha* (Psoriasis) and excellent clinical responses- a case study. Indian J Tradit Know 2011;10:689–92.

[39] Yadavji TA. Susruta Samhita. Varanasi: Chaukamba Krishnadas Academy; 2008.

[40] Yadavji TA. Sushruta Samhita with Nibandhasangraha commentary of Dalhanacharya and Nyayachandrika Panchika of Gayadasa. Varanasi: Chaukhamba Krishnadas Academy; 2004.
[41] Datar VK. The Charaka Samhita by Agnivesha with Ayurveda Dipika commentary of Chakrapani Datta. Mumbai: Nirnaya-Sagar Press; 1922.
[42] Prakash A, Debnath P, Arun Raj GR, et al. A review on the role of Jalaukavacharana (Hirudotherapy) in the management of venous ulcer. UJP2 2013:38–43.
[43] Prakash A, Arun Raj GR, Debnath P, et al. Exploratory study to assess the efficacy of Jalaukavacharana (Hirudotherapy) in the management of vicharchika (Eczema). Int J Ayur Pharma Research 2013;1:60–5.
[44] Macht DI. Experimental and clinical study of cobra venom as an *analgesic*. Proc Natl Acad Sci USA 1936;22:61–71.
[45] Van Esveld LW. Preparation of "Cobrataxin" for clinical purposes, especially for the treatment of cancer pains. Biochem Zeitschrift 1936;283:343–57.
[46] Hills RG, Firor WM. The use of more potent cobra venom for intractable pain. Am Surg 1952;18:875–9.
[47] Taren JA. A clinical evaluation of cobra venom extracts for control of pain. Med Bull (Ann Arbor) 1953;19:206–11.
[48] Bose JC. The Unity of life, presidential address of Indian Science Congress Lahore, 1927. Everyman's Science 2004;29:208–23.
[49] Debnath PK, Chaturvedi GN, Bhattacharya SK, et al. Comparative pharmacological study of crude and shodhita cobra venom. J Res Ind Med 1971;7:54–62.
[50] Debnath PK, Chaturvedi GN, Bhattacharya SK, et al. Comparative study of the anti-inflamatory and antiarthritic effect of the crude and shodhita venom in albino rat. Rheumaism 1971;6:55–9.
[51] Patwardhan B, Gautam M. Botanical immunodrugs: scope and opportunities. Drug Discovery Today 2005;10:495–502.
[52] Nurul I, Mizuguchi H, Shahriar M, et al. *Albizia lebbeck* suppresses histamine signaling by the inhibition of histamine H1 receptor and histidine decarboxylase gene transcriptions. Int Immunopharmacol 2011;11:1766–72.
[53] Plaeger SF. Clinical immunology and traditional herbal medicines. Clin Diagn Lab Immunol 2003;10:337–8.
[54] Rege NN, Thatte UM, Dahanukar SA, et al. Adaptogenic properties of six Rasayana herbs in Ayurvedic medicine. Phytother Res 1999;13:275–91.
[55] Patil D, Gautam M, Gairola S, et al. Effect of botanical immunomodulators on human CYP3A4 inhibition: implications for concurrent use as adjuvants in cancer therapy. Integr Cancer Ther 2014;13:167–75.
[56] Harwansh R, Mukherjee K, Bhadra S, et al. Cytochrome P450 inhibitory potential and RP-HPLC standardization of trikatu-a Rasayana from Indian Ayurveda. J Ethnopharmacol 2014;153: 674–81.
[57] Anonymous. Jalodara Evam Uski chikitsa. Clinical trial report. Varanasi: Institute of Medical Sciences, Banaras Hindu University; 1963.
[58] Patwardhan B, Mashelkar R. Traditional medicine-inspired approaches to drug discovery: can Ayurveda show the way forward? Drug Discovery Today 2009;14:804–11.
[59] Valiathan MS. Towards Ayurvedic biology: a decadal vision document. Bangalore: Indian Academy of Sciences; 2006.
[60] Indian Genome Variation Consortium. Genetic landscape of the people of India: a canvas for disease gene exploration. J Genetics 2008;87:3–20.
[61] Sethi T, Prasher B, Mukerji M. Ayurgenomics: a new way of threading molecular variability for stratified medicine. ACS Chem Biol 2011;6:875–80.
[62] Chen R, Mias G, Li-Pook-Than J, et al. Personal omics profiling reveals dynamic molecular and medical phenotypes. Cell 2012; 148:1293–307.
[63] Joyce A, Palsson B. The model organism as a system: integrating "omics" data sets. Nat Rev Mol Cell Biol 2006;7:198–210.
[64] Deocaris CC, Widodo N, Wadhwa R, et al. Merger of Ayurveda and tissue culture-based function genomics: inspiration from system biology. J Transl Med 2008;6:1–8.
[65] Patwardhan B, Vaidya A, Chorghade M, et al. Reverse pharmacology and systems approaches for drug discovery and development. Curr Bioact Compd 2008;4:201–12.
[66] Patwardhan B, Bodeker G. Ayurvedic genomics: establishing a genetic basis for mind-body typologies. J Altern Complement Med 2008;14:571–6.
[67] Patwardhan B, Joshi K, Chopra A. Classification of human population based on HLA gene polymorphism and the concept of prakriti in ayurveda. J Altern Complement Med 2005;11:349–53.
[68] Aggarwal S, Negi S, Jha P. EGLN1 involvement in high-altitude adaptation revealed through genetic analysis of extreme constitution types defined in Ayurveda. Proc Natl Acad Sci USA 2010;107:18961–6.
[69] Ghodke Y, Joshi K, Patwardhan B. Traditional medicine to modern pharmacogenomics: Ayurveda prakriti type and CYP2C19 gene polymorphism associated with the metabolic variability. Evid Based Complement Alternat Med 2011;2011:1–5.
[70] Shiomi T, Guilleminault C, Sasanabe R, et al. Augmented very low frequency component of heart rate variability during obstructive sleep apnea. Sleep 1996;19:370–7.
[71] Das UN. Metabolic syndrome X: an inflammatory condition? Curr Hypertens Rep 2004;6:66–73.
[72] Haensel A, Mills PJ, Nelesen RA, et al. The relationship between heart rate variability and inflammatory markers in cardiovascular diseases. Psychoneuroendocrinology 2008;33:1305–12.
[73] Kau AL, Ahern PP, Griffin NW, et al. Human nutrition, the gut microbiome and the immune system. Nature 2011;474:327–36.
[74] Arumugam M, Raes J, Pelletier E, et al. Enterotypes of the human gut microbiome. Nature 2011;473:174–80.
[75] Manning JT, Bundred PE. The ratio of second to fourth digit length and age at first myocardial infarction in men: a link with testosterone? Br J Cardiol 2001;8:720–3.
[76] Zhu F, Ma X, Qin C, et al. Drug discovery prospect from untapped species: indications from approved natural product drugs. PloS One 2012;7:e39782.
[77] Bauer A, Brönstrup M. Industrial natural product chemistry for drug discovery and development. Nat Prod Rep 2014;31:35–60.
[78] Ramalingaswami V, Satyavati GV. Sir Ram Nath Chopra. Indian J Med Res 1982;76:5–6.
[79] Sen G, Bose K. *Rauwolfia serpentina*, a new Indian drug for insanity and hypertension. Indian Med World 1931;21:194–201.
[80] Svensson TH. Effects of chronic treatment with tricyclic antidepressant drugs on identified brain noradrenergic and serotonergic neurons. Acta Psychiatr Scand Suppl 1980;280:121–3.
[81] Angelique L, Ralf J. Alternative drug discovery approaches for orphan GPCRs. Drug Discovery Today 2008;13:52–8.
[82] Aggarwal B, Prasad S, Reuter S, et al. Identification of novel anti-inflammatory agents from Ayurvedic medicine for prevention of chronic diseases: "reverse pharmacology" and "bedside to bench" approach. Current Drug Targets 2011;12:1595–653.
[83] Patwardhan B, Vaidya A. Natural products drug discovery: accelerating the clinical candidate development using reverse pharmacology approaches. Indian J Exp Biol 2010;48:220–7.
[84] Blondeau S, Do QT, Scior T, et al. Reverse pharmacognosy: another way to harness the generosity of nature. Curr Pharm Des 2010;16:1682–96.
[85] Keiser M, Setola V, Irwin J, et al. Predicting new molecular targets for known drugs. Nature 2009;462:175–81.
[86] Lasser KE, Allen PD, Woolhandler SJ, et al. Timing of new black box warnings and withdrawals for prescription medications. JAMA 2002;287:2215–20.

[87] Weinshilboum R, Wang L. Pharmacogenomics: bench to bedside. Nat Rev Drug Discov 2004;3:739–48.
[88] Schenone M, Dančík V, Wagner B, et al. Target identification and mechanism of action in chemical biology and drug discovery. Nat Chem Biol 2013;9:232–40.
[89] Aggarwal B, Ichikawa H, Garodia P, et al. From traditional Ayurvedic medicine to modern medicine: identification of therapeutic targets for suppression of inflammation and cancer. Expert Opin Ther Targets 2006;10:87–118.
[90] Garodia P, Ichikawa H, Malani N, et al. From ancient medicine to modern medicine: Ayurvedic concepts of health and their role in inflammation and cancer. J Soc Integr Oncol 2007;5:25–37.
[91] Dwivedi V, Anandan E, Mony R, et al. In vivo effects of traditional Ayurvedic formulations in *Drosophila melanogaster* model relate with therapeutic applications. PloS One 2012;7:e37113.
[92] Choi S. Systems biology approaches: solving new puzzles in a symphonic manner published in systems biology for signaling networks. pp. 3–11. USA: Springer; 2010.
[93] Chen R, Snyder M. Promise of personalized omics to precision medicine. Wiley Interdiscip Rev Syst Biol Med 2013;5:73–82.
[94] Patti G, Yanes O, Siuzdak G. Innovation: metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol 2012;13:263–9.
[95] Hollywood K, Brison D, Goodacre R. Metabolomics: current technologies and future trends. Proteomics 2006;6:4716–23.
[96] Commisso M, Strazzer P, Toffali K, et al. Untargeted metabolomics: an emerging approach to determine the composition of herbal products. Comput Struct Biotechnol J 2013;4:e201301007.
[97] Keller MP, Choi Y, Wang P, et al. A gene expression network model of type 2 diabetes links cell cycle regulation in islets with diabetes susceptibility. Genome Res 2008;18:706–16.
[98] Hopkins A. Network pharmacology: the next paradigm in drug discovery. Nat Chem Biol 2008;4:682–90.
[99] Rask-Andersen M, Almén M, Schiöth H. Trends in the exploitation of novel drug targets. Nat Rev Drug Discov 2011;10:579–90.
[100] Zhou W, Wang Y. A network-based analysis of the types of coronary artery disease from traditional Chinese medicine perspective: potential for therapeutics and drug discovery. J Ethnopharmacol 2014;151:66–77.
[101] Zhao J, Jiang P, Zhang W. Molecular networks for the study of TCM pharmacology. Brief Bioinform 2011;11:417–30.
[102] Shi S, Cai Y, Cai X, et al. A network pharmacology approach to understanding the mechanisms of action of traditional medicine: Bushenhuoxue formula for treatment of chronic kidney disease. PLoS ONE 2014;9:e89123.
[103] Ideker T, Sharan R. Protein networks in disease. Genome Res 2008;18:644–52.
[104] Lehar J, Zimmermann GR, Krueger AS, et al. Chemical combination effects predict connectivity in biological systems. Mol Syst Biol 2007;3:80.
[105] Juyal R, Negi S, Wakhode P, et al. Potential of ayurgenomics approach in complex trait research: leads from a pilot study on rheumatoid arthritis. PloS One 2012;7:e45752.
[106] Li B, Xu X, Wang X, et al. A systems biology approach to understanding the mechanisms of action of Chinese herbs for treatment of cardiovascular disease. Int J Mol Sci 2012;13:13501–20.
[107] An G, Hunt CA, Clermont G, et al. Challenges and rewards on the road to translational systems biology in acute illness: four case reports from interdisciplinary teams. J Crit Care 2007;22:169–75.
[108] Borisy AA, Elliott PJ, Hurst NW, et al. Systematic discovery of multicomponent therapeutics. Proc Natl Acad Sci USA 2003;100:7977–82.
[109] Gu J, Gui Y, Chen L, et al. Use of natural products as chemical library for drug discovery and network pharmacology. PloS One 2013;8:e62839.
[110] Gu J, Zhang H, Chen L, et al. Drug-target network and polypharmacology studies of a traditional Chinese medicine for type II diabetes mellitus. Comput Biol Chem 2011;35:293–7.
[111] Ru J, Li P, Wang J, et al. TCMSP: a database of systems pharmacology for drug discovery from herbal medicines. J Cheminform 2014;6:13.
[112] Bredel M, Jacoby E. Chemogenomics: an emerging strategy for rapid target and drug discovery. Nat Rev Genet 2004;5:262–75.
[113] Mohd Fauzi F, Koutsoukas A, Lowe R, et al. Chemogenomics approaches to rationalizing the mode-of-action of traditional Chinese and Ayurvedic medicines. J Chem Inf Model 2013;53:661–73.
[114] Ba L, Doering M, Burkholz T, et al. Metal trafficking: from maintaining the metal homeostasis to future drug design. Metallomics 2009;1:292–311.
[115] Dabrowiak JC. Metals in medicine. Chichester: Wiley; 2009.
[116] Kaim W, Schwederski B, Klein A. Bioinorganic chemistry–inorganic elements in the chemistry of life: an Introduction and Guide. New Jersey: Wiley; 2013.
[117] Kumar A, Nair A, Reddy A, et al. Bhasmas: unique Ayurvedic metallic-herbal preparations, chemical characterization. Biol Trace Elem Res 2006;109:231–54.
[118] Sarkar P, Das S, Prajapati P. Ancient concept of metal pharmacology based on Ayurvedic literature. Anc Sci Life 2010;29:1–6.
[119] Savrikar S, Ravishankar B. Introduction to "Rasashaastra" the Iatrochemistry of Ayurveda. AJTCAM 2011;8:66–82.
[120] Pal D, Sahu C, Haldar A. Bhasma: the ancient Indian nanomedicine. J Adv Pharm Technol Res 2014;5:4–12.
[121] Paul S, Chugh A. Assessing the role of ayurvedic "Bhasmas" as Ethno-nanomedicine in the metal based nanomedicine Patent regime. J Intellec Prop Rights 2011;16:509–15.
[122] Mamtani R, Stern P, Dawood I, Cheema S. Metals and disease: a global primary health care perspective. J Toxicol 2011;2011:1–11.
[123] Subhose V, Srinivas P, Narayana A. Basic principles of pharmaceutical science in Ayurvĕda. Bull Indian Inst Hist Med (Hyderabad) 2005;35:83–92.
[124] Chaudhary A. Ayurvedic bhasma: nanomedicine of ancient India–its global contemporary perspective. J Biomed Nanotechnol 2011;7:68–9.
[125] Heinz-Bernhard K, Metzler-Nolte N. Concepts and models in bioinorganic chemistry. John Wiley and Sons; 2006.
[126] Lippard SJ. Bioinorganic chemistry: metals in medicine. pp. 505–583. Mill City: University Science Books; 1994.
[127] Raven E, Le-Brun N, McMaster J, Reedijk J, Robinson N. Bioinorganic chemistry. Dalton Transactions 2013;42:3027–8.
[128] Kaluđerović G, Gómez-Ruiz S, Maksimović-Ivanić D, Paschke R, Mijatović S. Metals in medicine. Bioinorg Chem App 2012;2012:705907.
[129] Ray PC. History of chemistry in ancient and Medieval India incorporating the history of Hindu chemistry. Calcutta: Indian Chemical Society; 1956.
[130] Prajapati P, Sarkar P, Nayak S, Joshi R, Ravishankar B. Safety and toxicity profile of some metallic preparations of Ayurveda. Anc Sci Life 2006;25:57–63.
[131] Gupta K, Pallavi G, Patgiri B, Galib, Prajapati P. Critical review on the pharmaceutical vistas of Lauha Kalpas (Iron formulations). J Ayurveda Integr Med 2012;3:21–8.
[132] Pandit S, Biswas T, Debnath P, Saha A, Chowdhury U, Shaw B, et al. Chemical and pharmacological evaluation of different Ayurvedic preparations of iron. J Ethnopharmacol 1999;65:149–56.
[133] Krishnamachary B, Rajendran N, Pemiah B, Krishnaswamy S, Krishnan U, Sethuraman S, et al. Scientific validation of the different purification steps involved in the preparation of an Indian Ayurvedic medicine, Lauhabhasma. J Ethnopharmacol 2012;142:98–104.

[134] Kumar G, Stivastave A, Sharma SK, Gupta YK. Safety evaluation of mercury based Ayurvedic formulation (SidhMakaradhwaj) on brain cerebrum, liver, kidney in rats. Indian J Med Res 2014;139: 610–8.
[135] Sigel A, Sigel M. Metal ions in biological systems: mercury and its effects on environment and biology, vol. 34. New York: Marcel Dekker; 1997.
[136] Khedekar S, Patgiri B, Ravishankar B, Prajapati P. Standard manufacturing process of Makaradhwaja prepared by SwarnaPatra - Varkha and Bhasma. AYU 2011;32:109–15.
[137] Liu J, Shi JZ, Yu LM, Goyer R, Waalkes M. Mercury in traditional medicines: is cinnabar toxicologically similar to common mercurials? Exp Biol Med 2008;233:810–7.
[138] World Health Organization. Legal status of traditional medicine and complementary/alternative medicine: a worldwide review. Geneva: World Health Organization; 2001.
[139] Kamath S, Pemiah B, Sekar R, Krishnaswamy S, Sethuraman S, Krishnan U. Mercury-based traditional herbo-metallic preparations: a toxicological perspective. Arch Toxicol 2012;86:831–8.
[140] Sinyorita S, Ghosh C, Chakrabarti A, Auddy B, Ghosh R, Debnath P. Effect of Ayurvedic mercury preparation Makaradhwaja on geriatric canine–a preliminary study. Indian J Exp Biol 2011;49:534–9.
[141] World Health Organization. The world medicines situation. Geneva: World Health Organization; 2004.
[142] Rodriquez GLA, Gutthann SP. Use of UK general practice research database for pharmacoepidemiology. Br J ClinPharmacol 1998;l45:419–25.
[143] Wertheimer AI, Andrews KB. An overview of pharmacoepidemiology. Pharm World Sci 1995;17:61–6.
[144] Etminan M, Samii A. Pharmacoepidemiology I. A review of pharmacoepidemiology study designs. Pharmacotherapy 2004; 24:964–9.
[145] Bosnić T, Softic DZ, Jerg-Simanovic D, Pilipovic S. The Microbiological quality of herbal teas and herbal medicinal products. Planta Med 2007;73:236.
[146] World Health Organization. WHO traditional medicine strategy 2002-2005. Geneva: World Health Organization; 2002.
[147] Report of the Regional Meeting, World Health Organization. The use of herbal medicines in primary health care. India: World Health Organization, Regional Office for South-East Asia; 2009.
[148] Patwardhan B. Traditional medicine: modern approach for affordable global health. WHO-CIPIH Study Nine on TM; 2005. Available from: http://www.who.int/intellectualproperty/studies/B.Patwardhan2.pdf.
[149] Luo X, Doherty J, Cappelleri JC, Frush K, et al. Role of pharmacoepidemiology in evaluating prescription drug safety in pediatrics. Curr Med Res Opin 2007;23:2607–15.
[150] AYUSH, Govt. of India. National pharmacovigilance protocol "accAUfor Ayurveda, Siddha and Unani (ASU) drugs. New Delhi: Dept of AYUSH, Ministry of health and family welfare; 2008.
[151] ISPE Commentary. Guidelines for good pharmacoepidemiology practices (GPP). Pharmacoepidemiol Drug Saf 2008;17:200–8.
[152] Patwardhan B. Ayurveda GCP Guidelines: need for freedom from RCT ascendancy in favor of whole system approach. J Ayurveda Integr Med 2011;2:1–4.
[153] Bose J,C. Avyakta (Bengali version). Kolkata: Bose Institute; 1916.
[154] Debnath PK. Medical and veterinary sciences. Presidential address 89th session. Kolkata: Indian Science Congress Association; 2002.
[155] Kasture HS. Concept of Ayurveda for perfect health and longevity. Nagpur: Shree Baidyanath Ayurved Bhawan Private Ltd.; 1991.

CHAPTER

21

Discovery and Development of Lead Compounds from Natural Sources Using Computational Approaches

José L. Medina-Franco

Facultad de Química, Departamento de Farmacia, Universidad Nacional Autónoma de México, Mexico City, Mexico

OUTLINE

21.1 INTRODUCTION

Computer-aided drug design has gained enormous momentum in drug discovery and its contributions to drug discovery are increasing. Computational (also referred to as in silico) techniques play important roles in the various stages of lead discovery and development. In silico methods applied to drug discovery can be roughly classified into two major approaches: structure-based and ligand-based. This classification depends on the level of target structural information available to support the computational calculations. Structure-based methods use the available three-dimensional (3D) information on the molecular target of interest, which is typically obtained, for example, from X-ray crystallography, nuclear magnetic resonance, or homology modeling. Ligand-based approaches use the available information on a series of active ligands (and inactive compounds, when available) in a given assay or set of assays. Despite the fact that substantial progress has been made in a number of computational methodologies, there is still significant

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00021-0

room for improvement, not only in the development of the techniques themselves but also in the rational and adequate application of such approaches by experts and nonexperts [1]. Cases are exemplified by reviews of the progress in molecular docking [2,3], quantitative structure–activity relationships (QSARs) [4,5], virtual screening [6,7], molecular dynamics [8], pharmacophore modeling [9], and chemoinformatics [10], to name a few.

Natural products (NPs), from either terrestrial or aquatic organisms, have a long tradition as sources of active compounds for health-related benefits. It is well acknowledged that over millions of years, Nature has optimized and selected chemical structures to generate chemical scaffolds and compounds enriched with biological function. Drawbacks of NPs that frequently diminish the enthusiasm to pursue active compounds from natural origin include challenges in the isolation and purification procedures, very small available amounts of lead compounds, the difficulty in synthesizing NPs with high structural complexity, and the associated synthesis scale-up issues. Also, in a drug discovery context, caution should be taken with natural compounds that have been designed by Nature for defense and are toxic. As such, one can expect that not all NPs have a beneficial effect on health. However, the large success of using NPs to produce bioactive compounds or bioactive mixtures has inspired the preparation of synthetic molecules that have become drugs. In addition, as reviewed later in this chapter, the unique structural features of NPs represent a promising opportunity to identify active compounds for emerging targets and for "tough targets" difficult to address with classical organic small molecules.

NP research is increasingly being combined with computer-aided drug design techniques to accelerate the identification of novel and improved drug candidates from natural origin and to further understand and quantify the coverage of NPs in chemical space. In this chapter, we discuss the progress on the synergy between NP-based drug discovery and chemoinformatic and molecular modeling methods. Representative applications are schematically outlined in Figure 21.1. This chapter represents an update of previous reviews of such synergistic combination [11–13]. The chapter is divided into eight major sections. After this general introduction, we briefly discuss an overview of the role of NPs in drug discovery. This section is not intended to be a comprehensive analysis of the status of NP research because this point is extensively discussed in other chapters of this book. Instead, the purpose of this section is to put the reader into the context of the challenges faced by the NP drug discovery that are being addressed by the use of computational methods. The third section discusses major structural

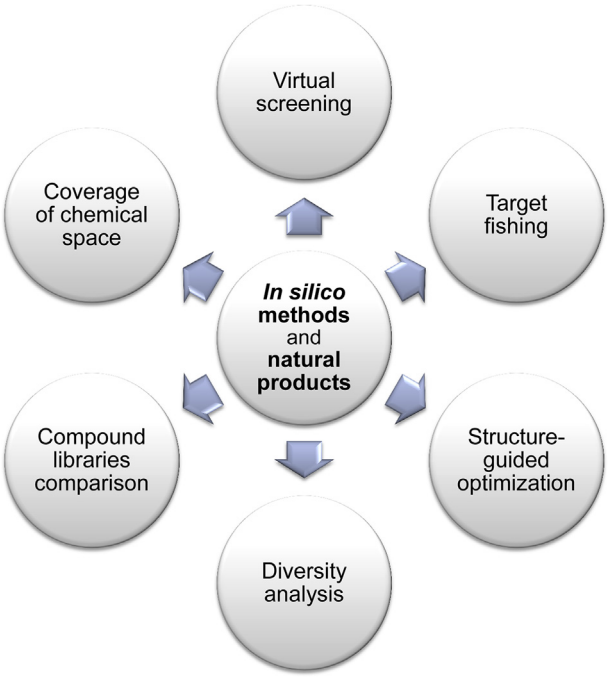


FIGURE 21.1 Examples of the many roles of computational approaches applied to NP-based drug discovery. NP, natural product.

classes of NPs and emphasizes the computational approaches used to characterize their structural diversity and coverage in chemical space. We also discuss measures of structural complexity, which is a distinct feature of NPs. Section 4 focuses on NP databases including collections available in the public domain. In the same section we present examples of chemoinformatic analysis of various NP collections and discuss the pharmacological profiling of such collections, which, in light of the increasing importance of chemogenomics, is a current trend in modern drug discovery. In the next section, entitled "Virtual Screening and Target Fishing," we discuss the application of computational techniques to help identify bioactive compounds of natural origin for a given molecular target (virtual screening). We also present the complementary approach, that is, identifying possible molecular targets for NPs (target fishing). Thus, this section shows how virtual screening and target fishing are used to filter out compounds or molecular targets, respectively, to focus the experimental screening efforts (which usually are time consuming and expensive) on smaller sets of ligands or targets with increased probability of having the desired biological effect. Section 6 discusses the critical role of NPs in addressing molecular targets not easily tackled with typical small molecules, such as protein–protein interactions (PPIs). In this section we also discuss the use of NPs against emerging targets, such as epigenetic targets. The next section is dedicated to presenting the application of computational methods to uncover bioactivities

of NPs of dietary origin. It also shows the intersection of NPs and food chemicals to systematically identify compounds with health-related benefits. Finally, in the last section we present a summary and conclusions.

21.2 NPs IN DRUG DISCOVERY

From ancient times to the modern era, NPs have been extensively used as medicines, dietary products, and nutritional supplements [14]. For example, for a number of years, 80% of drugs were either NPs or their derivatives. Even after the widespread use of techniques such as high-throughput screening (HTS) of synthetic libraries, 50% of the new drugs approved from 1981 to 2010 were NPs, derivatives, or structural analogues [15,16]. It has been estimated that more than 100 NP compounds are currently in clinical trials. NPs are valuable not only as potential therapeutic agents but also as molecular probes to identify targets of pharmaceutical interest and facilitate the characterization of biological processes underlying a disease [17]. As reviewed below, NPs have the advantage of uncovering distinct structural classes [18,19] because of their better coverage of chemical space relative to large synthetic compounds [20]. Therefore, the chemical diversity of NPs can be used to access bioactive compounds with novel scaffolds [20–22]. Some combinatorial libraries are inspired by NP frameworks [23,24].

The reader is referred to extensive and excellent reviews of the role of NPs in modern drug discovery [14,16,20]. The rationale that botanicals may exert their activity owing to the interaction of the bioactive mixtures with multiple biological endpoints in a synergistic manner is contributing to the shift of the current paradigm of drug discovery from single-target to multitarget drug discovery [25]. Authors of that work also envision that a next paradigm in NP-based drug discovery is the synergistic combination of traditional NP research with other drug discovery strategies. The next sections of this chapter are focused on the productive combination of NP-based drug discovery with computational approaches.

21.3 CHEMOINFORMATIC ANALYSIS OF NATURAL PRODUCTS

NPs can be classified in several different manners, for example, by the source, e.g., terrestrial or marine; by kingdom, e.g., plants, bacteria, fungi; or by chemical class, e.g., small molecules, macrocycles, peptides. Of note, although peptides and macrocycles (among which NPs make an important contribution) are less attractive than small molecules in typical drug discovery campaigns, the development of chemical libraries focused on these chemical classes has gained interest [26]. Computational approaches, in particular using chemoinformatics methods (vide infra), have enabled the methodical structural analysis and classification of NPs.

Structural diversity and complexity are distinct features of the chemical structures of NPs that have particular significance in lead discovery and development. Despite the fact that noncomputational experts can easily recognize the distinct structural features of NPs by eye, systematic and quantitative studies are required to derive metrics of such features. A number of well-validated chemoinformatic methodologies are available to characterize the structures of NPs and compound collections of various origins commonly used in screening campaigns [27,28].

Briefly, chemoinformatics, also referred as "cheminformatics" or "chemical information science," can be understood as "the application of informatics methods to solve chemical problems" [29]. Willet further adds that chemoinformatics is focused on "the manipulation of information about chemical structures, either in the form of planar two-dimensional (2D) structure diagrams or (increasingly) in the form of 3D atomic coordinates, with the manipulations encompassing a range of searching, modeling, and statistical approaches" [30].

Chemical libraries are frequently compared using one or more or the following major criteria:

1. Whole molecule properties such as physicochemical properties,
2. Molecular scaffolds and substructural features,
3. Molecular fingerprints.

As discussed below, because each of this criteria has its own advantages and disadvantages, it has been proposed that several of these criteria should be considered for a comprehensive analysis of compound collections [31].

Using one or more of the general criteria above, compound databases are usually compared in terms of the concept of chemical space [32]. Chemical space has several definitions [32], e.g., one formulated by Virshup et al.: "an M-dimensional Cartesian space in which compounds are located by a set of M physicochemical and/or chemoinformatic descriptors" [33]. In simpler terms, the concept of chemical space is intuitive if one makes an analogy with the cosmic universe in which chemical compounds would be represented by the stars [34,35]. However, in contrast to the cosmic universe, the chemical space is relative and depends strongly on the representations used to define the space [36]. A number of chemoinformatic techniques have been developed to characterize and generate visual representations of the chemical space [32,37–39].

Here we review a representative chemoinformatic analysis of NPs that has been published [40]. For the sake of discussion, we divide this section of the chapter based on the major type of criteria, although more than one molecular representation has been used across several studies.

21.3.1 Physicochemical Properties

Physicochemical properties are intuitive and straightforward to interpret. These types of properties are frequently used to define metric-based or empirical rules that attempt to predict "drug-likeness" or to classify compounds as drug-like/non-drug-like [41,42]. A prominent example is Lipinski's Rule of Five [43] to predict passive oral absorption based on molecular weight (MW), octanol/water partition coefficient (*S*log *P*), hydrogen bond acceptors (HBAs), and hydrogen bond donors (HBDs). Over the years other rules have been proposed that also highlight the importance of the number of rotatable bonds (RBs) and the polar surface area for drug development [44,45].

Shultz has presented a critical discussion of metric-based rules frequently used in drug discovery [41]. Of note, these rules are commonly formulated based on existing data and may exclude novel compounds that are outside of the traditional relevant chemical space, e.g., chemical space relevant to emerging molecular targets. One needs to have in mind that existing data are highly influenced by the "relevant" molecular targets heavily investigated by the scientific community. In many instances, the relevance of the molecular targets is guided (and biased) by market/profit interests or the likelihood of receiving funding [26]. Therefore, it has been pointed out that metric-based rules should be used only as a guide to design libraries or select compounds from existing libraries for further development [26].

Numerous studies have been reported comparing the physicochemical properties of NPs with other types of compounds relevant in drug discovery [40,46–48]. Indeed, the unique properties of NPs represent an excellent exception to Lipinski's Rule of Five. It is not uncommon to find NPs that have an MW well over 1000 Da and break many of the other "rules" that define the structures of so-called "drug-like" molecules, and yet are orally bioavailable and have acceptable pharmaceutical properties [49].

Ganesan analyzed the drug-like properties of 24 distinct NPs that were discovered and led to an approved drug in the period 1970–2006. He identified two major types of NPs, those that comply with the Rule of Five and those that are outside of the so-called "Lipinski universe." However, despite the fact that several NPs that have reached the market are very large and flexible, they do comply with the number of HBDs and, more importantly with log *P* values. Indeed, Ganesan concludes that "the single most important lesson from NP lies in their ability to maintain low log P values regardless of other characteristics" [50].

Singh et al. compared NPs contained in the ZINC database [51] with drugs, combinatorial libraries, and the Molecular Libraries Small Molecule Repository (MLSMR) [31]. In that study, three important molecular properties of size, flexibility, and molecular polarity were described by MW, RB, and *S*log *P*; topological polar surface area (TPSA); and HBA and HBD, respectively. In the same work other criteria such as molecular scaffolds and fingerprint-based diversity (vide infra) were included. Concerning the molecular properties it was concluded that NPs from the ZINC database have similar distributions of HBAs, HBDs, and RBs compared to drugs. The distribution of *S*log *P* values showed that NPs are slightly more hydrophobic than drugs and overall have a slightly larger MW, as previously observed for other NP data sets [46,52].

A total number of 28,000 compounds from the Traditional Chinese Medicine (TCM) database [53] (vide infra) were compared to a database of a commercial vendor library and a collection of small molecules obtained with combinatorial chemistry and containing 30 different core scaffolds. The same set of six drug-like physicochemical properties was used: MW, RB, HBA, HBD, TPSA, and *S*log *P*. Results of that study showed that, overall, TCM has the largest values of HBD, HBA, *S*log *P*, and TPSA compared to the other collections. Concerning the size, in general, TCM had the largest molecules as measured by MW [54].

In a separate study, the chemical space of 2477 NPs from a commercial vendor was compared to that of 5963 synthetic compounds from academic sources using diversity-oriented synthesis (DOS) [55] and 6152 synthetic compounds from a commercial vendor typically employed in screening campaigns. Six drug-like properties (MW, RB, HBA, HBD, TPSA, and *S*log *P*) were used in the comparison. It was concluded that DOS compounds considered in that study are heavier and more lipophilic than the NPs or the synthetic commercial compounds [56].

Manallack et al. conducted an analysis of the distribution of ionization constants of 89,425 NPs from ZINC [57]. The profile was compared to drugs, a chemogenomics data set, and other compound databases from ZINC. Results indicated that NPs have a distinct distribution of ionization constants, e.g., higher proportions of complex ionizable compounds and a greater number of zwitterionic molecules. However, NPs from ZINC have some overlap with approved drugs. The distribution of pK_a values of single acids and single bases in

NPs was more similar to that of drugs than that of screening compounds [57]. In a subsequent study, Manallack et al. performed a similar characterization of the acid/base profile and physicochemical properties of 25,566 NPs obtained from ChEMBL [58]. In this second work, the profile was compared with that of human small-molecule metabolites and drugs.

A visual representation of 24 ADME (absorption, distribution, metabolism, and elimination)-related properties for the TCM database and NPs from ZINC was obtained with principal component analysis. The so-called "ADME space" of the NP libraries was compared to a collection of approved drugs, commercial vendor compounds, a general diverse collection obtained from the National Cancer Institute database, and combinatorial libraries. It was concluded that TCM covers a vast region of this property space, including areas uncharted by drugs. NPs from ZINC occupy the same area as drugs [27]. Of note, physicochemical properties along with substructural features, e.g., functional groups, are also used as criteria to filter out compounds with potential toxicity issues early in the drug discovery process [59].

Figure 21.2 shows a visual representation of the property-based chemical space of 1200 approved drugs, 2000 natural products for screening from the commercial vendor AnalytiCon, and 13,387 (synthetic) molecules for screening. The visual representation was obtained using the Web-based public tool ChemGPS-NP$_{Web}$ [60,61]. ChemGPS-NP [60,62] is a principal components analysis-based global chemical positioning system [63] that utilizes principal components analysis to assist with navigation through biologically relevant chemical space [61]. The first four dimensions of the ChemGPS-NP map capture 77% of data variance. The first dimension (principal component 1, PS1) represents size, shape, and polarizability (main contribution is size); PS2 is associated with aromatic and conjugation-related properties (main influence is aromaticity); PS3 describes lipophilicity, polarity, and hydrogen bond capacity (major contribution is lipophilicity); and PS4 expresses flexibility and rigidity. Small molecules can be positioned onto this map using interpolation in terms of principal component analysis score prediction. Details of ChemGPS are provided elsewhere [61]. Figure 21.2 clearly shows that NPs (green spheres) occupy regions of the chemical space that are different from the regions explored by the synthetic compounds (blue spheres) and also cover regions sparsely populated by drugs (orange spheres). The synthetic compounds show a large overlap with the property space of drugs. ChemGPS-NP$_{Web}$ has been used to compare the chemical space of approved drugs with TCM and compounds derived from combinatorial libraries available in PubChem [64].

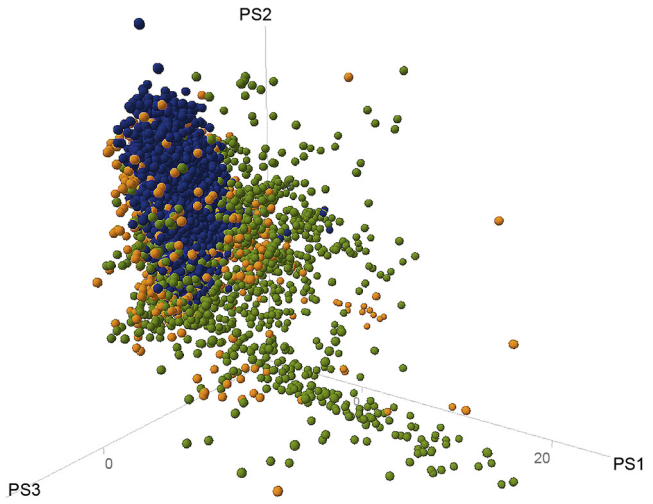


FIGURE 21.2 Visual representation of the chemical space of a collection of 2000 NPs (green), 1200 drugs approved for clinical use (orange), and a general screening collection with 13,387 synthetic compounds (blue). The plot was generated using the ChemGPS-NP prediction scores calculated using the online tool ChemGPS-NP$_{Web}$. NP, natural product.

21.3.2 Molecular Scaffolds and Substructural Features

Although physicochemical properties are broadly used by the scientific community to compare compound data sets, a disadvantage is that they do not provide direct information on the structural features such as chemical connectivity, structural novelty, or complexity. Indeed, different chemical structures can have the same or similar physicochemical properties. A complementary approach is to use molecular scaffolds or frameworks [65]. These terms are used to describe the core structure of a molecule [66]. Similar to physicochemical descriptors, molecular scaffolds (also called chemotypes) are straightforward to interpret and enable easy communication with medicinal chemists and biologists. For example, molecular scaffolds are strongly associated with the concepts of "scaffold hopping" [67] and "privileged structures" [68,69].

There are a number of ways to represent the molecular scaffold in a consistent and systematic manner [65]. One definition is the "cyclic systems" that result from iteratively removing the side chains of the molecule. Cyclic systems are part of the chemotype methodology relying on the molecular equivalence indices developed by Johnson and Xu [70,71] and are similar to the "atomic frameworks" of Bemis and Murcko [72]. A remarkable feature of the scaffolds of Johnson and Xu used to compare compound collections is that molecules classified in a scaffold do not lie in any other chemotypic

class [73]. This approach has been extensively used to classify compound collections [31,73–75].

One of the disadvantages of the scaffold analysis is the lack of information regarding the side chains around the molecular framework and the structural relationship between the scaffolds themselves. A straightforward solution is the analysis not only of the molecular scaffolds but also of the side chains and functional groups and other substructural analysis strategies [76]. Scaffold analysis is used to compare compound data sets, to assess the performance of virtual screening methodologies, to retrieve novel scaffolds, and to analyze the SAR of a set of molecules with measured activity. For instance, we reported a chemotype-based hierarchical classification of the NCI AIDS database to systematically identify the scaffolds associated with anti-AIDS activity [73].

Measuring and comparing the scaffold diversity of compound collections depend on several factors, including the specific approach to describe the scaffolds, the size of the database, and the distribution of the molecules in those scaffold classes [77]. Often, scaffold diversity is measured based on frequency counts. Although these measures are correct in the way they are defined, they do not provide sufficient information concerning the specific distribution of the molecules across the different scaffolds, particularly the most populated ones. Medina-Franco et al. [77] proposed the use of an entropy-based metric to measure the distribution of the molecules across different scaffolds, particularly the most populated ones, as a complementary metric for the comprehensive scaffold diversity analysis of compound data sets. Using this metric, Yongye et al. measured the scaffold diversity of five NP databases available in the public domain (see Section 21.4 of this chapter).The NP libraries were compared with a general screening collection and libraries frequently used in in vivo screening. They found that the general screening library had the largest scaffold diversity. In addition to benzene and acyclic molecules, flavones, coumarins, and flavanones were identified as the most frequent scaffolds across the NP collections analyzed in that work [78].

Koch et al. reported a structural classification of NPs (SCONP) [79]. The SCONP was based on scaffold analysis of the comprehensive (although not publicly available) *CRC Dictionary of Natural Products* (Table 21.1). The scaffold classification can be visualized in a tree-like fashion that resembles the approach previously published by Medina-Franco et al. [73]. Koch et al. employed that structural classification to develop a novel class of selective and potent inhibitors of 11β-hydroxysteroid dehydrogenase type 1 [79].

Feher and Schmidt compared NPs, molecules from combinatorial synthesis, and drug molecules. They used a number of physicochemical properties, atom counts, and other substructural features. They concluded that NPs are different from compounds derived from combinatorial synthesis based on the number of chiral centers, the prevalence of aromatic rings, the introduction of complex ring systems, and the degree of the saturation of the molecule, as well as the number and ratios of different heteroatoms [46].

As part of an effort to compute a natural product-likeness score, P. Ertl et al. showed that natural products consisted of fewer aromatic rings and were less flexible relative to drugs and an in-house set of synthetic compounds [80].

Chen et al. compared the molecular topologies of natural products with those of drugs, human metabolites, clinical candidates, and general bioactive compounds [81]. Chen et al. showed that biologically relevant NPs and human metabolites had the highest ratios of single ring system compounds, among other measures.

21.3.3 Structural Fingerprints

Typical molecular and structural fingerprints encode the information of the entire compound structure, i.e., not only molecular scaffolds, and they have been applied to a number of computer-aided and chemoinformatic applications. The reader is referred to detailed descriptions of the various types of molecular fingerprints commonly used [82].

A disadvantage of some fingerprints is that they are more difficult to interpret. Also, it is well known that chemical space will depend on the type of fingerprints used [83]. To reduce the dependence of chemical space on the structure representation, it has been proposed that multiple methods be used, for example, multiple fingerprint representations, and common or consensus conclusions be obtained [84]. Indeed, the aggregation or combination of methods is a common practice in similarity searching (called “data fusion”) [85], in molecular docking (“consensus scoring”) [86], in activity landscape modeling (e.g., “consensus activity cliffs”) [87,88], and in clustering [89].

Molecular fingerprints are frequently used as a molecular representation for diversity analysis. Singh et al. analyzed the structural diversity of NPs from ZINC using three types of molecular fingerprints from different design, namely Molecular Access System (MACCS) keys, graph-based three-point pharmacophores (GpiDAPH3), and typed graph distance (TGD) [31]. As discussed above, fingerprints with different designs were used to reduce the dependence of chemical space on structure representation. The diversity was compared with approved drugs, MLSMR, and four combinatorial libraries. Results showed that the

TABLE 21.1 Examples of Databases of Natural Products Commercially or Publicly Available

Database	Description and size	URL
Dictionary of natural products	Comprehensive and fully edited database of NPs. It contains more than 259,859 compounds in over 68,000 entries. The database is frequently updated.	www.crcpress.com/; dnp.chemnetbase.com/intro/
SEARCHABLE ONLINE		
HIM—Herbal Ingredients In vivo Metabolism database	Collects in vivo metabolism information for active herbal ingredients, as well as their corresponding bioactivity, organ and/or tissue distribution, toxicity, ADME, and clinical research profile. Information for 361 ingredients and 1104 metabolites from 673 herbs.	58.40.126.120/him/
NuBBE database	Approximately 640 compounds collected from publications of the NuBBE group in Brazil.	nubbe.iq.unesp.br/nubbeDB.html
Super Natural II	352,811 purchasable compounds. It includes information on the 2D structures, physicochemical properties, and vendors.	Bioinf-applied.charite.de/supernatural_new/index.php
Traditional Chinese Medicine (TCM) database @ Taiwan	Database with 37,170 (32,364 nonduplicate) TCM compounds from 352 TCM ingredients.	tcm.cmu.edu.tw
Universal Natural Products Database (UNPD)	Repository of 197,201 compounds obtained from other NP databases.	pkuxxj.pku.edu.cn/UNPD
UNIIQUIM database	More than 3000 NPs collected from publications of the Chemistry Institute, UNAM in Mexico.	uniiquim.iquimica.unam.mx
COMPOUNDS FOR IN SILICO AND/OR EXPERIMENTAL SCREENING		
AfroDb	Representative subset of 954 compounds from African medicinal plants.	zinc.docking.org/catalogs/afronp
Analyticon—MEGx	Pure natural compounds from plants and 1300 pure natural compounds from microorganisms.	www.ac-discovery.com
MicroSource pure natural products collection	800 compounds fully characterized with 95% purity or more.	www.msdiscovery.com/natprod.html

NPs, natural products; TCM, Traditional Chinese Medicine; 2D, two-dimensional.

magnitude of the similarity values computed with different fingerprints strongly depends on the design and resolution of the fingerprints, which has been observed in other studies [84,87,90]. Overall, it was concluded that drugs are the most structurally diverse (showing the lowest mean and median similarities for the three fingerprints). The NP collection was the second most diverse database. In that study, fingerprint-based characterization of the NPs complemented the analysis conducted with physicochemical properties and molecular scaffolds [31].

The interlibrary similarity of TCM with drugs and a commercial vendor library was assessed using MACCS keys, GpiDAPH3, and TGD fingerprints by means of nearest-neighbor curves (distribution of the maximum similarity values to the reference collections). It was observed that TCM has compounds with chemical structures different from those of drugs and the diverse collection of commercial compounds. In the same study, the intralibrary similarity of TCM was compared to the other reference compound databases using MACCS keys by means of pairwise similarity values with the

Tanimoto coefficient [91,92]. It was concluded that, in general, NPs in TCM have lower structural diversity compared to drugs and commercial compounds as captured by MACCS keys/Tanimoto coefficient [54].

The intralibrary similarity of five NP collections whose chemical structures are in the public domain has also been measured using pairwise similarity values computed with MACCS keys and the Tanimoto coefficient. Results showed that the intralibrary similarity strongly depends on the type of NP database analyzed [78]. The fingerprint-based characterization of the five collections was part of a chemoinformatic characterization of the databases that also included a comprehensive scaffold analysis (vide supra) [78].

To illustrate the comparison of compound databases using molecular fingerprints, Figure 21.3 shows the distribution of the maximum MACCS keys/Tanimoto similarities of 13,388 compounds from a general screening collection (blue curve) and 2000 compounds in an NP database from a commercial vendor, AnalytiCon (green curve) with a set of 1200 approved drugs for clinical use (not represented in the plot). Compounds in the general screening collection have a median similarity of 0.71, whereas the NPs have a median similarity of 0.77. These statistics and the curves of the distribution indicate that, in general, the chemical structures of NPs are slightly more similar to those of drugs than the general screening collection (considering the MACCS keys structural representation). Similar conclusions were obtained comparing different general screening and NP databases with a set of drugs [54].

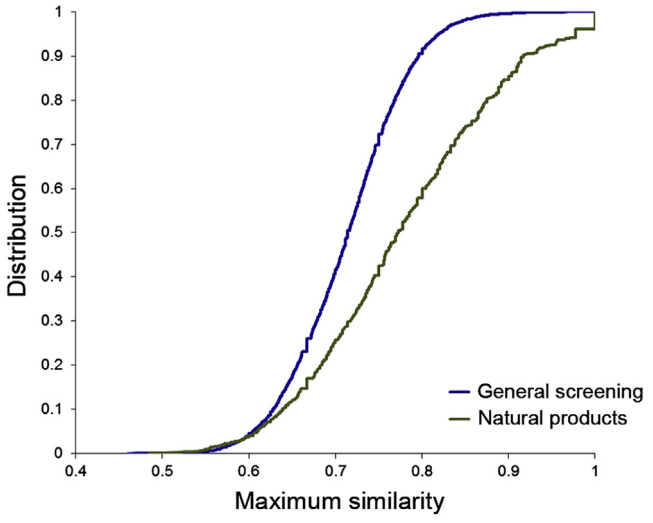


FIGURE 21.3 Nearest-neighbor curves comparing a natural products collection from a commercial vendor and a general screening collection with a collection of approved drugs for clinical use. The curves represent the cumulative distribution function of the maximum similarity of each compound in the screening and natural products collection to all the drugs as measured with MACCS keys and the Tanimoto coefficient. MACCS, Molecular Access System.

21.3.4 Molecular Complexity

Molecular complexity is a concept of growing interest to select compounds from existing collections for screening or designing chemical databases [26]. Lovering et al. showed that structurally complex molecules, as measured by the fraction of saturated carbons, have higher success rates in the drug discovery pipeline [93]. The authors of that work also hypothesized that compounds with increased complexity may increase selectivity. This hypothesis is supported by the study of Clemons et al. that screened three types of compound collections across 100 diverse proteins [94]. This study showed that increasing the content of sp^3-hybridized and stereogenic atoms relative to compounds from commercial sources improves selectivity and frequency of binding. Evidently, increasing molecular complexity is not the only criterion to consider when screening existing collections or designing new libraries. As the authors discussed, other properties have to be balanced [26].

Quantifying molecular complexity is not a trivial task, and several metrics have been suggested, including MW [95–98]. One metric commonly used is the carbon bond saturation defined by fraction sp^3 (Fsp^3) where Fsp^3 = (number of sp^3 hybridized carbons/total carbon count) [93]. Using this metric, López-Vallejo et al. compared the molecular complexity of NPs in the TCM database with the structural complexity of 30 small-molecule synthetic combinatorial libraries [54]. Results of this study demonstrated the high structural complexity of NPs, suggesting the possibility of using these collections to interrogate novel regions in the currently neglected chemical space [54].

21.4 MOLECULAR DATABASES FOCUSED ON NPs AND NP DERIVATIVES

The advancement of synthetic chemistry and HTS has largely contributed to the growth of the number of molecules available. Large numbers of compounds can be conveniently stored in chemical databases, which play a key role in modern drug discovery [99].

Compound databases may contain existing or virtual compounds. The second type usually comprises hypothetical molecules that could be synthesized later. A comprehensive collection of virtual compounds in the public domain is the Generated Database of Chemical Space (GDB) [37,100]. GDB has been used in virtual screening followed by chemical synthesis and biological testing [101,102]. Libraries of existing compounds may be proprietary, also called in-house libraries; commercial; or public. Sources of screening libraries have been reviewed [103–105].

Depending on the goals of the project or the screening campaign, distinct types of compound libraries can be developed and screened [50]. Examples include DOS [55], focused libraries, diverse libraries, combinatorial libraries, Libraries from Libraries [106], and NPs or synthetic analogues of NPs [107].

Recently Medina-Franco et al. [26] reviewed different approaches to designing focused libraries with confined chemical spaces. In that work, the authors discussed two broad types of confinement: (1) library design focused on a relevant therapeutic target or disease and (2) library design focused on chemistry or a desired molecular function [26]. NP databases are important sources of molecules from which to select compounds or inspire the synthesis of molecules for a target of therapeutic interest or with a desired molecular function [108,109]. In this regard, Over et al. analyzed and validated the role of NP-derived fragments for fragment-based ligand discovery [110].

Table 21.1 summarizes examples of NP databases either commercially available or in the public domain [111–115]. Some databases are designed to conduct structure and properties searches online, whereas others are collections of compounds for purchase intended for virtual and experimental screening. A more complete set of NP catalogs for screening is collected in the ZINC database [51]. Of note, by July 2014, ZINC contained over 35 million molecules and 13 NP catalogs (available at http://zinc.docking.org/browse/catalogs/natural-products).

In 2012, Yongye and Medina-Franco compiled one of the first lists of NP databases whose structures are readily accessible on the Web [78]. At the time of that study such databases contained between 560 and 89,000 compounds. Subsequently, the number of NP databases with structure in the public domain doubled.

The TCM database is one of the major sources of natural products freely available online [53]. TCM has been extensively characterized using physicochemical properties and structural fingerprints (vide supra) [54]. Based on this database, the cloud computing system iScreen (available at http://iScreen.cmu.edu.tw/) was developed, which is a Web server for docking TCM followed by customized de novo drug design [116]. TCM has been used successfully to identify pancreatic triacylglycerol lipase inhibitors using in silico approaches [117].

Another important source of NPs freely available online is the Universal Natural Products Database (UNPD) [118]. UNPD is a collection of 197,201 chemical structures obtained from plants, animals, and microorganisms. The physicochemical profile of this database has been analyzed. The physicochemical properties were employed as a basis to generate a visual comparison of the chemical space covered by UNPD and approved drugs for clinical use, concluding that there is a large overlap [118].

Ntie-Kang et al. published the ConMedNP library [119], an extension of the previously published database CamMedNP, which contains 1859 NPs and derivatives obtained in Cameroon [120]. The augmented library ConMedNP represents a recollection of 3177 compounds, not only from Cameroon but also from the Central African flora. Ntie-Kang et al. published a physicochemical characterization of ConMedNP using typical drug-like properties and ADME-related descriptors. In addition, that group made publicly available the 3D structures for other computational applications such as virtual screening (vide infra) [119]. Ntie-Kang et al. also published AfroDb [111], which is a relatively small but representative subset of African medicinal plants containing around 1000 3D structures. AfroDb is available in ZINC.

Esquivel and colleagues at the Informatics Unit of the Chemistry Institute of the National Autonomous University of Mexico is assembling a database of NPs that have been published by the Chemistry Institute of the same university. It is estimated that the database will have information for more than 3000 chemical substances isolated and characterized. Other representative databases of NPs are summarized in Table 21.1 and others are reviewed elsewhere [20,107].

21.4.1 Pharmacological Profiling of NP Databases

Chemogenomics is evolving as a multidisciplinary research field that uses in vitro and in silico methods to better understand a ligand–target SAR matrix or chemogenomics knowledge space [121,122]. The relationship between chemogenomics and related topics of major interest in current drug discovery, such as polypharmacology, drug repurposing, phenotypic screening, and high-throughput in vivo testing, has been discussed in an integrated manner [25]. In two independent publications, Bajorath and Rognan, respectively, reflected on the perspectives of computational chemogenomics [123,124].

Along this line of thinking, there is increased awareness that drugs exert their clinical effects through interactions with multiple targets. This is illustrated by the drugs dabrafenib and trametinib, approved in 2013 by the Federal Drug Administration (FDA) of the United States for the treatment of "unresectable or metastatic melanoma with $BRAF^{V600E}$ mutation as detected by an FDA-approved test" [125]. Both drugs target multiple kinases [125]. This awareness has increased the relevance of and interest in systematically screening chemical compounds, including NPs, across different

biological endpoints, for example, against multiple molecular targets. This is a common practice after isolating and characterizing novel NPs.

An example of a large-scale NP profiling was reported by Clemons et al., who tested the binding specificity of 15,000 compounds, including 2477 NPs, with 100 sequence-unrelated proteins. The results of the screening were made freely accessible to the scientific community and the reader has access to the chemical structures and the corresponding biological profiles [94]. This data set has been subjected to a series of computational studies aimed at elucidating the SAR and identifying structural patterns associated with the selectivity or promiscuity of the molecules using fingerprint or substructure representations [126–128]. For example, this data set was the basis for developing approaches for identifying structural changes that have a significant impact on the number of proteins to which a compound binds. For instance, Yongye and Medina-Franco [126] proposed the structure–promiscuity index difference (SPID) metric. SPID encodes the relationship between structure similarity and the number of different proteins to which each pair of compounds binds [126]. In a separate study, Dimova et al. analyzed the same large microarray data set, using the concept of matched molecular pairs [129]. Dimova et al. identified single-site substitutions that lead to large differences in compound promiscuity [127].

21.5 VIRTUAL SCREENING AND TARGET FISHING

21.5.1 Virtual Screening

Because experimental testing of molecules is expensive and time consuming, virtual screening, also called in silico or computational screening, represents a valuable tool to guide and focus experimental efforts on smaller, filtered sets of compounds with increased probability of showing the desired biological activity [6,130,131]. This is particularly attractive for filtering NPs for experimental testing because, in many instances, NPs are available in small quantities. Ideally, virtual screening is included in an iterative cycle of prediction and experimental validation followed by rounds of refinement. If the final goal of virtual screening is to identify potent compounds, one of the main objectives of the first iteration cycle is to identify novel molecular scaffolds [132]. Similar to experimental screenings, virtual screening work flows are project-specific, tailored to the need of a particular target or biological context [133].

Virtual screening approaches can be classified into two major groups: structure-based and ligand-based methods. Structure-based approaches use the 3D structure of the target and ligand-based approaches utilize ligand information in light of structure–activity data derived from a set of known actives. Successful applications of virtual screening to identify bioactive compounds have been reviewed [3,7].

Traditionally, bioactive NPs with a promising therapeutic indication have been identified through random or fortuitous approaches. Thus, computational screening of NPs represents a valuable synergy for identifying bioactive NPs in a systematic manner. Indeed, virtual screening has been applied to screen small sets and large databases of NPs, giving rise to the identification of bioactive molecules [134–138]. Table 21.2 summarizes representative examples of virtual screening approaches applied to NPs. Figure 21.4 shows the corresponding chemical structures of the NPs uncovered by virtual screening summarized in Table 21.2. The reader is referred to a number of reviews that show the progress in the virtual screening of NPs [13,20,140–144].

Notably, a unique collection of NPs can be used in virtual screening through the Drug Discovery Portal (DDP; available at www.ddp.strath.ac.uk) [145]. The DDP developed a database that contains purified NPs obtained from plant, soil, or marine sources, synthetic compounds of some of which were inspired by NPs. Molecules stored in this database are available in research groups from various countries including Scotland (where DDP is based), Australia, France, and the United States. The DDP also collects biological targets that are being screened in academic laboratories. The goal of this initiative is to match a chemist's compound to a biological assay using computational techniques and then validate the computational predictions in the biological assay that is available [20].

Virtual screenings of NP databases for later experimental validation have been reported. For example, 197,201 compounds in the UNPD (vide supra) were docked with 332 target proteins relevant to approved drugs. Based on a docking-score-weighted prediction model, the most promising NPs with potential bioactivity were identified [118]. Virtual screenings of NPs for emerging and challenging targets are discussed below.

21.5.2 Target Fishing and Reverse Pharmacognosy

As discussed above, the goal of virtual screening is to identify new ligands for known targets. The inverse approach, i.e., identifying new targets for known ligands, is called "target fishing" [122] or inverse screening. Thus, in the context of chemogenomics, virtual screening is associated with ligand screening and target fishing is related to ligand profiling [121]. Similar

TABLE 21.2 Examples of Recent Applications of Virtual Screening to Identify Bioactive NPs[a]

Computational approach(es)	Key findings	References
Docking-based virtual screening of an in-house collection with 4000 NPs with matrix metalloproteinases (MMPs).	19 NPs with diverse structures are identified as MMP inhibitors. The most potent inhibitor (compound **5**) also represses MMP-2 and active MMP-9 expression in MDA-MB-231 cancer cells and suppresses the migration of MDA-MB-231 in a wound healing assay.	[135]
QSAR based on molecular topology and virtual screening.	Calcein, a natural dye, showed potent inhibitory activity against interleukin-6 and therefore is potentially effective in ulcerative colitis.	[137]
Inverse virtual screening of a small library of phenolic NPs with a panel of 163 targets involved in the genesis and progression of cancer.	Xanthohumol and isoxanthohumol showed inhibitory activity on PDK1 and PKC protein kinases in in vitro assays.	[139]
Structure-based virtual screening of an in-house library of 1430 NPs and their derivatives with a novel target for anti-HIV therapy.	18 hits were identified that bind at the interface of HIV-1 integrase and human LEDGF/p75. The two most potent inhibitors had IC_{50} values at 0.32 and 0.26 μM, respectively. NPD170 had the highest antiviral activity with an EC_{50} of 1.81 μM.	[134]
Structure-based virtual screening of an in-house database with more than 4000 NPs with two estrogen receptor (ER) modulators.	Eleven compounds were identified as ER modulators: 3 agonists and 8 antagonists. The most potent antagonist (compound **4**) had an EC_{50} value of 2.55 and 4.68 μM for ERα and ERβ, respectively.	[138]
Docking-based virtual screening of 216 diverse NPs with acetylcholinesterase as potential leads for Alzheimer disease.	One of the top-ranked compounds, **NDGA** had an IC_{50} of 46.2 μM. In silico studies of NDGA were conducted to design compounds with improved CNS activity.	[136]

QSAR, quantitative structure–activity relationships.
[a]*Chemical structures of selected hit compounds are in Figure 21.4.*

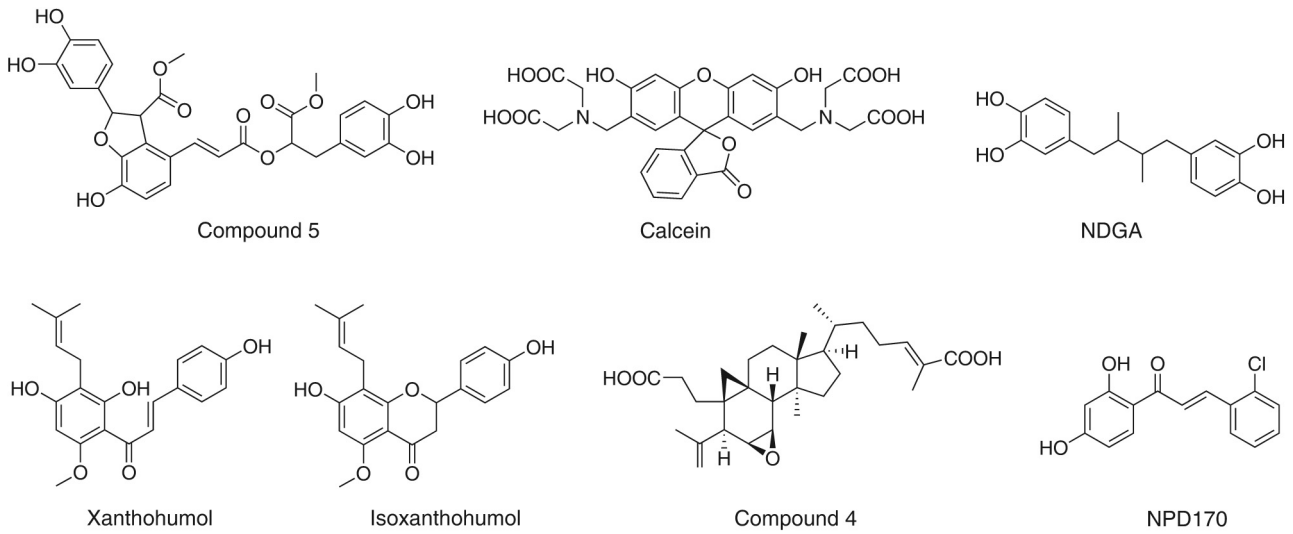


FIGURE 21.4 Chemical structures of exemplary NPs identified from virtual screening followed by experimental validation. See Table 21.2 for details. NPs, natural products.

to virtual screening, depending on the experimental information available, target fishing can be performed using structure-based methods, e.g., inverse docking, or ligand-based methods, e.g., similarity searching [146]. Ideally the combination of both approaches can be used if enough structural and SAR data are available. This point is emphasized by Yue et al., who have discussed progress on the target profiling of NPs using experimental (genomics and proteomics) and computational approaches [147]. In that review, Yue et al. point out the convenience of integrating various methods such as inverse docking (docking compounds across different targets), mapping ligand–target profiling space, and network analysis.

A notable example of a ligand-based method that can greatly benefit target fishing of NPs is the similarity ensemble approach (SEA) [20,148]. SEA is a statistical ligand-based design approach in which the structures of a series of ligands with known targets are used to train a model capable of predicting the poly pharmacological profile of other molecules. This approach was employed to predict the binding profiles of 3600 known drugs or compounds developed as potential drugs based on hundreds of thousands of bioactive molecules and their known targets. Two of three high-confidence predictions were retrospectively shown to be accurate [149].

A second remarkable example of a ligand-based method that can benefit NP research is prediction of activity spectra for substances (PASS) [150]. PASS predicts simultaneously more than 500 biological activities, including pharmacological main and side effects, mechanisms of action, mutagenicity, carcinogenicity, teratogenicity, and embryotoxicity. PASS is based on a regression approach applied to noncongeneric chemical series. The developers of this approach have expanded the capabilities of the PASS algorithm to predict the sites of metabolites for xenobiotics [151]. Lagunin et al. have discussed in detail the application of PASS to evaluate the multitarget profile of selected NPs [152].

Target fishing using computational approaches is increasingly being used to uncover biological activities of NPs. An example has been published by Gianluigi et al., who conducted inverse docking of 10 antioxidant phenolic NPs with 163 molecular targets involved in cancer progression. Xanthohumol and isoxanthohumol showed activity with the protein kinases PDK1 and PKC [139]. In a separate work, Carregal et al. developed a protocol combining molecular docking, refinement by molecular dynamics simulations, and quantum mechanics/molecular mechanics to determine the pharmacological receptors for NPs isolated from Cerrado species in Brazil [153]. Other applications of target fishing for NPs are reviewed elsewhere [13]. More recently, Wolber and Rollinger discussed the application of virtual screening and target fishing for NPs using 3D pharmacophores [154].

As part of the FoodInformatics symposia held at the 245th American Chemical Society National Meeting in 2013 [155] (vide infra), Quoc-Tuan Do explained the principles of reverse pharmacognosy [156], emphasizing the many roles of chemoinformatic approaches, including inverse screening, to accelerate the identification of the bioactive compounds of an organism. Quoc-Tuan discussed two successful and published examples of reverse pharmacognosy using Selnergy™, a platform developed in Greenpharma to predict, based on docking, interaction energies of a ligand with a target protein [157,158].

21.6 NPs AS LEADS FOR CHALLENGING AND EMERGING TARGETS

The overall unique structural diversity, structural features, and molecular complexity of NPs make them attractive for interrogating molecular targets that represent significant challenges using traditional small molecules typically used for common targets. Similarly, NPs represent a very attractive approach to address emerging molecular targets for which the "relevant" chemical space is not clearly defined yet. This section illustrates these points by focusing on the role of NPs in the pursuit of modulators of PPIs and of the emerging epigenetic targets.

21.6.1 NPs as Compounds for Modulating Protein–Protein Interactions

Several cellular functions are regulated by multiprotein complexes that are controlled by PPIs between protein subunits. It is also well known that human diseases can be caused by abnormal PPIs. Therefore PPI modulators, either inhibitors or stabilizing agents, are attractive in drug discovery [159]. Despite significant progress made toward the modulation of PPIs, these are still difficult to target with small molecules because of the structural characteristics of the protein–protein interfaces. For example: (1) in several cases, the contact surfaces involved in PPIs are large (~1500–3000 $Å^2$) compared with those involved in protein–small-molecule interactions (~300–1000 $Å^2$); (2) in general, the contact surfaces between proteins are flat as opposed to the types of pockets and grooves found in typical surfaces of proteins bound to small molecules; (3) in contrast to typical proteins of pharmaceutical relevance, PPIs lack endogenous small molecules that can be used as a reference or starting point to design modulators [160]. Despite these challenges, several strategies are being followed that

have succeeded in bringing compounds to clinical trials. Sperandio et al. have reviewed general strategies to design libraries focused on PPIs [161].

In an insightful review, Wells and McClendon reflect that the fact that many HTS efforts have yielded PPI hits with moderate potency is heavily influenced by historical reasons: common screening libraries of small molecules contain chemotypes dominated by past drug discovery focused on classical targets, e.g., G-protein-coupled receptors and protein kinases. Moreover, the authors of that review point out that PPI inhibitors reaching clinical trials have MW between 500 and 900 Da, with K_i values less than 1 μM [160], being clear exceptions to the traditional medicinally relevant chemical space (which contains molecules with MW less than 500 Da). Taking all these considerations together, it was then proposed that the structural features of NPs represent excellent candidates for PPI modulation [161,162]. It has been hypothesized that, in general, the rigidity of PPI inhibitors is required for good binding affinity and target selectivity. Some NPs have the desired profile of having a rigid molecular framework [159].

Several NPs have pharmacological activity as PPI blockers. Examples include FK506, rapamycin, and ascomycin, a family of closely related polyketide natural products derived from soil actinomycetes that have large different cellular effects via binding of the FK506-binding protein immunophilin and modulation of the PPIs involved in the signal transduction pathways of T cell activation and growth. Other examples are paclitaxel, epothilone A, and discodermolide, which affect mitosis by modulating the PPIs implicated in tubulin polymerization [162]. Rolitetracycline is an inhibitor of the hypoxia-inducible factor-1 (HIF-1) pathway, which is a key regulator of angiogenic and glucose metabolic processes and is used by tumor cells for both survival and growth. Most of the HIF-1 inhibitors are either NPs or synthetic compounds based on NPs. Chetomin is an NP that also interferes with the HIF-1 signaling pathway. Although further development of rolitetracycline and chetomin was hampered by a lack of activity in cell-based assays and toxicity in animal models, respectively, the chemical scaffolds of both compounds represent a unique architecture to develop distinct scaffolds [159].

To accelerate the identification of lead molecules that modulate PPIs, computational approaches are being developed to predict modes of PPIs as well as hot spots at the protein interface. Progress in the development of these computational methods is presented in an excellent review by Bienstock [163]. These techniques can be conveniently applied to NPs. For example, once hot spots have been identified for a given protein–protein interface, pharmacophore filtering can be applied using NP databases discussed earlier in this chapter.

21.6.2 NPs as Lead Compounds for DNA Methyltransferase Inhibitors

Emerging molecular targets such as DNA methyltransferases (DNMTs) and other epigenetic enzymes [164] are becoming attractive targets for the treatment of cancer and several other diseases. Among the epigenetic targets, DNMTs are a family of enzymes that catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine to the carbon-5 position of cytosine residues leading to an epigenetic modification [165]. The human genome encodes four distinct DNMTs: DNMT1, DNMT2, DNMT3A, and DNMT3B. Of these, DNMT1 and DNMT3B constitute the major activities. It has been demonstrated that inhibition of DNMT activity can lead to demethylation and reactivation of epigenetically silenced tumor suppressor genes [166]. Thus, DNA methylation represents a central mechanism for mediating epigenetic gene regulation, and the development of DNMT inhibitors provides novel opportunities for cancer therapy [167]. Inhibition of DNA methylation has also emerged as a promising strategy for the treatment of immunodeficiency and brain disorders [168,169]. However, DNMT inhibitors currently in clinical use, 5-azacytidine and 5-aza-2′-deoxycytidine, are nonselective cytosine analogues with significant cytotoxic side effects. Different approaches are being followed to identify distinct non-nucleoside DNMT inhibitors; these methods include chemical synthesis of lead compounds [170,171], docking-based virtual screening of chemical databases from various sources, and similarity searching of databases of approved drugs. Similarity searching, which is a ligand-based approach, led to the identification of olsalazine, an anti-inflammatory drug approved for the treatment of ulcerative colitis, as a novel DNA hypomethylating agent [172]. This case represents an example of a synergy by which computational approaches accelerate drug repurposing.

Because environmental exposures are usually assumed to have a major impact on the onset of abnormal DNA methylation patterns, a frequent uptake of DNA demethylating agents is believed to have a chemopreventive effect [173]. This could be achieved through the dietary uptake of natural product DNMT inhibitors [173]. Several examples of NPs with DNMT-inhibitory and/or hypomethylating activity have been reported. A classic example is (-)-epigallocatechin-3-gallate (EGCG), the main polyphenol compound from green tea. EGCG has been proposed to inhibit DNMT1 by blocking the active site of the enzyme and reactivating methylation-silenced genes in cancer cells [174]. Catechin and epicatechin, and the bioflavonoids quercetin, fisetin, and myricetin, are other tea polyphenols that have also been associated with

the inhibition of DNA methylation. Curcumin, the major component of the Indian curry spice turmeric, and parthenolide, the principal sesquiterpene lactone of feverfew, have also been reported to inhibit DNMT1 [175]. Psammaplin A and several other disulfide bromotyrosine derivatives isolated from the marine sponge *Pseudoceratina purpurea* have been described as potent inhibitors of DNMT1 [176,177]. Kuck et al. have reported nanaomycin A as a selective inhibitor of human DNMT3B [178]. Several other NPs reported as DNMT inhibitors or hypomethylating agents are reviewed elsewhere [173,179–181].

Nanaomycin A (Figure 21.5) is a quinone antibiotic isolated from a culture of *Streptomyces* that has been described as the first non-*S*-adenosyl-L-homocysteine (SAH) analogue acting as a DNMT3B-selective inhibitor that induces genomic demethylation. Nanaomycin A treatment reduced the global methylation levels in three cell lines and reactivated transcription of the RASSF1A tumor suppressor gene [178]. To explain at the molecular level the activity of this NP, nanaomycin A was docked with a homology model of the catalytic site of DNMT3B that was built based on the crystal structure of DNMT3A as a template [182]. Results of the docking study showed that the carboxylic acid, hydroxyl group, and adjacent carbonyl oxygen atoms are predicted to form an extensive hydrogen bond network with the side chains of arginine amino acids. The study also suggested that the hydroxyl group of nanaomycin A makes a hydrogen bond with the side chain of a glutamic acid residue (Figure 21.5). Similar hydrogen bond interactions with the equivalent glutamic acid and arginine residues that participate in the mechanism of methylation were not observed in the docking models obtained with DNMT1. These results provided a possible structural explanation for the enzyme selectivity of nanaomycin A for DNMT3B. Based on the binding model of nanaomycin A with DNMT3B, along with the experimental and theoretical evidence of the reaction between quinones and cysteine-rich proteins, the catalytic cysteines were hypothesized to perform a nucleophilic Michael 1,4 addition to the α,β-unsaturated carbonyl system of nanaomycin A. A similar plausible reaction was not observed in the binding model with DNMT1, which supported the experimental selectivity toward DNMT3B [178].

The occurrence of DNMT inhibitors in dietary products highlights the relevance of identifying additional inhibitors of natural origin [173]. The systematic search for active compounds in NP databases can thus be the basis for further support of the use of complementary and alternative medicine products for inhibition of DNA methylation. The systematic search can be greatly facilitated by the application of computational approaches. Indeed, in silico methods have been broadly used to propose the mechanism of action of DNMT inhibitors at the molecular level, to screen compound libraries to identify novel inhibitors, and to guide the lead optimization efforts using structure-based techniques [183,184]. For example, Yoo et al. reported docking models of a number of NPs with DNMT1. The docking models of the NPs, along with the predicted binding models of small-molecule DNMT inhibitors of different origins were the basis for developing a pharmacophore model [185,186]. Such model was subsequently used to help explain, at the molecular level, the activity of trimethylaurintricarboxylic acid [187].

The chemical spaces of two NP collections, including compounds from the TCM database, were compared to the property space of a DNMT-focused library, approved drugs, and synthetic commercial compounds. It was concluded that the DNMT-focused library and the two NP databases have molecules with properties similar to those of approved drugs [188].

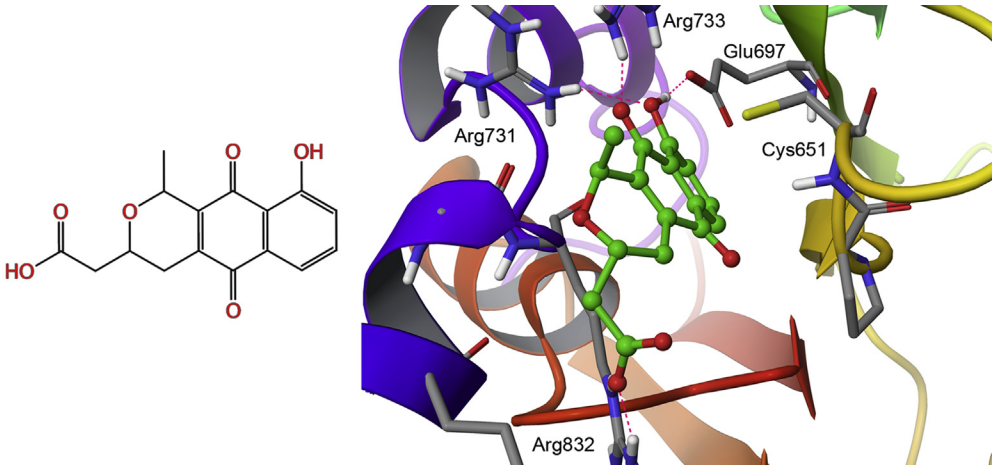


FIGURE 21.5 Docking model of the NP nanaomycin A with the catalytic site of human DNMT3B. NP, natural product.

As reviewed above, several bioactive compounds of natural origin have been discovered fortuitously. However, these findings have spurred follow-up studies to systematically uncover bioactive compounds with the aid of computational approaches. As part of an effort to identify novel inhibitors of DNMT of natural origin, the authors reported the virtual screening of a large collection of NPs from the ZINC database. Starting from 89,000 compounds, a subset of approximately 14,000 molecules was selected that was subjected to a multistep docking approach using three docking algorithms. Fifty-eight compound consensus hits with a docking score that was similar to or better than that of the reference molecule were selected and moved forward to experimental validation, which is ongoing at the time of writing. Notably, one of the consensus hits has reported DNMT1 inhibitory activity [173]. It is anticipated that computational approaches will continue to be used to develop DNMT inhibitors as promising epigenetic drugs.

21.7 UNCOVERING BIOACTIVITIES OF NPs OF DIETARY ORIGIN

For centuries, people from various cultures have used herbal remedies to try to maintain or improve their health (www.nlm.nih.gov/medlineplus/herbalmedicine.html). Despite the fact that plant extracts in the form of teas, decoctions, or tinctures may contain complex mixtures of compounds, they are broadly used in a safe and effective manner. In fact, plant-based extracts with defined composition, e.g., botanicals or phytopharmaceuticals, are registered for clinical use as dietary supplements or drugs, depending on the regulations of the country in which they are used. However, as commented above, one of the reasons pharmaceutical companies are reluctant to make large-scale use of NPs is because of the complexity of the mixtures of compounds in NP extracts [20]. Therefore, for many years it has been of interest to isolate, identify, and purify the bioactive components of plant extracts.

Chemoinformatics and molecular modeling approaches have been used to systematically identify bioactive components. In this context, *foodinformatics* is an emerging research field that is focused on uncovering health-related benefits of food components using chemoinformatic and other computational approaches [155,189]. Geldenhuys et al. have published an insightful review of the role of NPs from dietary sources as lead compounds for virtual screening and structure-based drug design. These authors put particular emphasis on compounds such as resveratrol, curcumin, caffeine, and genistein [144].

As part of a program to characterize natural extracts and identify their active compounds and their mode of action, Guasch et al. identified, from 11 extracts with known antidiabetic activity, 12 molecules as potential partial agonists of peroxisome proliferator-activated receptor γ. To that end, the authors used a validated virtual screening protocol based on a combination of pharmacophore modeling, docking, and shape-based similarity searching [190].

More recently Medina-Franco et al. [189] compared the physicochemical properties of NP databases with chemical structures in the public domain with more than 2000 food materials in the FEMA "Generally Recognized as Safe" (GRAS) list [189]. The authors concluded that NP databases from different sources have distinct distributions of physicochemical properties and structural diversity in support of previous conclusions derived from the scaffold analysis of the same databases [78]. The study also concluded that the GRAS chemicals analyzed in that work (discrete chemical entities only) have a high structural diversity, comparable to the high structural diversity of NPs and other reference libraries [189].

To identify potential bioactivity among the food flavoring components in the FEMA GRAS list, Martinez-Mayorga et al. carried out ligand-based virtual screening for compounds with structures similar to those of approved antidepressant drugs [191]. The virtual screening was performed by means of fingerprint-based similarity searching using the MACCS keys and the Tanimoto coefficient. Hit compounds in the FEMA GRAS list were selected as the compounds most similar (ranked with the highest similarity values) to any of 32 approved antidepressant drugs. Selected compounds represented the "nearest neighbors" of the approved antidepressants. Results indicated that valproic acid was the antidepressant most similar to GRAS compounds. Following the rationale that the inhibition of histone deacetylase-1 (HDAC1) could be associated with the efficacy of valproic acid in the treatment of bipolar disorder, Martinez-Mayorga et al. screened the GRAS compounds most similar to valproic acid for HDAC1 inhibition. The GRAS chemicals nonanoic acid and 2-decenoic acid inhibited HDAC1 at the micromolar level, with a potency comparable to that of valproic acid. It was pointed out that GRAS compounds are not expected to exhibit strong enzymatic inhibitory effects at the concentrations typically employed in flavor formulations designed for use in foods and beverages. However, as shown in that work, GRAS chemicals were able to bind to a relevant therapeutic target. Additional studies on bioavailability, toxicity at higher concentrations, and off-target effects are warranted. That study also exemplified the feasibility of exploring the FEMA GRAS flavoring list using computational

methods as a potential source of biologically active molecules. In addition, the study demonstrated that similarity searching followed by experimental evaluation can be used for rapid identification of GRAS chemicals with potential bioactivity [191].

21.8 CONCLUDING REMARKS

Historically, NPs have made outstanding contributions to drug discovery, providing bioactive molecules that have reached patients for clinical applications or that have inspired and stimulated the development of a significant portion of today's pharmaceuticals. Moreover, since ancient times NPs have been broadly employed as medicines, dietary products, and nutritional supplements. The development of compounds that can be used safely to deliver the desirable clinical effect(s) is a complex problem that demands the synergistic combination of major and complementary approaches. As such, the discovery and development of NPs as lead compounds for drug discovery can be accelerated by the rational application of computational methods. As reviewed in this chapter, computational methods play many roles in NP-based drug discovery. A broad set of computational strategies that are tailored to the specific project needs and information on the system available are increasingly making contributions to drug discovery campaigns based on NPs. Of note, the computational methods themselves are far from perfect and are constantly subject to improvement and refinement. It is also important to stress that by no means are computational strategies intended to erase traditional NP research. Instead, computational methods are meant to further improve common practices that have been successful over the years. On the other hand, there is a false expectation that computational methods alone are capable of designing by themselves molecules that will reach the market, i.e., the false notions of "computer-to-bedside" or "hit-one-button drug discovery." In this chapter we intended to make clear that computational approaches are part of a multidisciplinary and collective effort in which experimental strategies play a fundamental role.

Computational methods have clear applications in NP-based drug discovery that include but are not limited to:

- Filtering compound databases to select subsets of compounds for experimental screening;
- Systematic screening of compound data sets with few or many NPs;
- Measuring the physicochemical profile of NPs so that they can be compared in a consistent manner with compound collections from different sources (e.g., synthetic libraries) or performing interlibrary comparisons of various types of NP databases;
- Organizing and mining molecular databases;
- Quantifying the structural diversity and complexity of NP collections;
- Characterizing the coverage of the chemical space;
- Characterizing SARs systematically;
- Designing synthetic analogues of NPs using structure- or ligand-based approaches or both;
- Identifying and optimizing leads for "difficult" and emerging molecular targets;
- Deconvoluting bioactive mixtures;
- Uncovering possible therapeutic and health-related applications of NPs of dietary origin.

Over the years, around the world, diverse research groups have investigated and developed NPs and synthetic compounds inspired by NPs. Thousands of NPs and NP-like compounds are available and many compounds are assembled in compound databases. The molecular complexity, physicochemical profile, and structural diversity of such compound collections (commercial, public, or in-house data sets) can be readily assessed using chemoinformatic approaches.

The distinct structural characteristics of NPs offer a great promise for identifying compounds for use against molecular targets that are difficult to tackle using typical small-molecule combinatorial libraries or emerging molecular targets. Indeed, it has been acknowledged that "new classes of molecular targets may need new chemical scaffolds." In this context, molecular modeling and chemoinformatics provide relevant information to characterize the structures of the difficult and emerging targets and help find the NPs that satisfy the requirements for binding.

It is anticipated that the synergistic combination of two well-established approaches, NP-based drug discovery and computer-aided drug design, will continue evolving and delivering therapeutic compounds or molecules with health-related benefits to the best interest of the patients.

Acknowledgments

We thank Dr. Karina Martínez-Mayorga, Dr. Gerald M. Maggiora, and Dr. Nathalie Meurice for fruitful discussions and critical reading of the manuscript.

References

[1] Ritchie TJ, McLay IM. Should medicinal chemists do molecular modelling? Drug Discovery Today 2012;17:534–7.
[2] Yuriev E, Ramsland PA. Latest developments in molecular docking: 2010–2011 in review. J Mol Recognit 2013;26:215–39.
[3] Bello M, Martinez-Archundia M, Correa-Basurto J. Automated docking for novel drug discovery. Expert Opin Drug Discovery 2013;8:821–34.

[4] Scior T, Medina-Franco JL, Do QT, Martínez-Mayorga K, Yunes Rojas JA, Bernard P. How to recognize and workaround pitfalls in QSAR studies: a critical review. Curr Med Chem 2009;16: 4297–313.
[5] Cherkasov A, Muratov EN, Fourches D, Varnek A, Baskin II, Cronin M, et al. QSAR modeling: where have you been? where are you going to? J Med Chem 2014;57:4977–5010.
[6] Scior T, Bender A, Tresadern G, Medina-Franco JL, Martínez-Mayorga K, Langer T, et al. Recognizing pitfalls in virtual screening: a critical review. J Chem Inf Model 2012;52: 867–81.
[7] Lavecchia A, Di Giovanni C. Virtual screening strategies in drug discovery: a critical review. Curr Med Chem 2013;20: 2839–60.
[8] Durrant J, McCammon JA. Molecular dynamics simulations and drug discovery. BMC Biol 2011;9:71.
[9] Sanders MPA, Barbosa AJM, Zarzycka B, Nicolaes GAF, Klomp JPG, de Vlieg J, et al. Comparative analysis of pharmacophore screening tools. J Chem Inf Model 2012;52:1607–20.
[10] Duffy BC, Zhu L, Decornez H, Kitchen DB. Early phase drug discovery: cheminformatics and computational techniques in identifying lead series. Bioorg Med Chem 2012;20:5324–42.
[11] Pfisterer PH, Wolber G, Efferth T, Rollinger JM, Stuppner H. Natural products in structure-assisted design of molecular cancer therapeutics. Curr Pharm Des 2010;16:1718–41.
[12] Barlow DJ, Buriani A, Ehrman T, Bosisio E, Eberini I, Hylands PJ. In-silico studies in chinese herbal medicines' research: evaluation of in-silico methodologies and phytochemical data sources, and a review of research to date. J Ethnopharmacol 2012;140: 526–34.
[13] Medina-Franco JL. Advances in computational approaches for drug discovery based on natural products. Rev Latinoam Quim 2013;41:95–110.
[14] Cragg GM, Grothaus PG, Newman DJ. New horizons for old drugs and drug leads. J Nat Prod 2014;77:703–23.
[15] Li JW-H, Vederas JC. Drug discovery and natural products: end of an era or an endless frontier? Science 2009;325:161–5.
[16] Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 2012;75: 311–35.
[17] Schmitt EK, Moore CM, Krastel P, Petersen F. Natural products as catalysts for innovation: a pharmaceutical industry perspective. Curr Opin Chem Biol 2011;15:497–504.
[18] Harvey AL. Natural products in drug discovery. Drug Discovery Today 2008;13:894–901.
[19] Bohlin L, Göransson U, Alsmark C, Wedén C, Backlund A. Natural products in modern life science. Phytochem Rev 2010;9: 279–301.
[20] Harvey AL, Clark RL, Mackay SP, Johnston BF. Current strategies for drug discovery through natural products. Expert Opin Drug Discovery 2010;5:559–68.
[21] Rosén J, Gottfries J, Muresan S, Backlund A, Oprea TI. Novel chemical space exploration via natural products. J Med Chem 2009;52:1953–62.
[22] Kombarov R, Altieri A, Genis D, Kirpichenok M, Kochubey V, Rakitina N, et al. Biocores: identification of a drug/natural product-based privileged structural motif for small-molecule lead discovery. Mol Diversity 2010;14:193–200.
[23] Newman DJ. Natural products as leads to potential drugs: an old process or the new hope for drug discovery? J Med Chem 2008; 51:2589–99.
[24] Boldi AM. Libraries from natural product-like scaffolds. Curr Opin Chem Biol 2004;8:281–6.
[25] Medina-Franco JL, Giulianotti MA, Welmaker GS, Houghten RA. Shifting from the single to the multitarget paradigm in drug discovery. Drug Discovery Today 2013;18:495–501.
[26] Medina-Franco JL, Martinez-Mayorga K, Meurice N. Balancing novelty with confined chemical space in modern drug discovery. Expert Opin Drug Discovery 2014;9:151–65.
[27] Medina-Franco JL. Chemoinformatic characterization of the chemical space and molecular diversity of compound libraries. In: Andrea T, editor. Diversity-oriented synthesis: Basics and applications in organic synthesis, drug discovery, and chemical biology. Hoboken (New Jersey): John Wiley & Sons, Inc.; 2013. p. 325–52.
[28] Medina-Franco JL. Interrogating novel areas of chemical space for drug discovery using chemoinformatics. Drug Dev Res 2012;73:430–8.
[29] Engel T. Basic overview of chemoinformatics. J Chem Inf Model 2006;46:2267–77.
[30] Willett P. Chemoinformatics: a history. WIREs Comput Mol Sci 2011;1:46–56.
[31] Singh N, Guha R, Giulianotti MA, Pinilla C, Houghten RA, Medina-Franco JL. Chemoinformatic analysis of combi natorial libraries, drugs, natural products, and molecular libraries small molecule repository. J Chem Inf Model 2009;49: 1010–24.
[32] Medina-Franco JL, Martínez-Mayorga K, Giulianotti MA, Houghten RA, Pinilla C. Visualization of the chemical space in drug discovery. Curr Comput-Aided Drug Des 2008;4: 322–33.
[33] Virshup AM, Contreras-García J, Wipf P, Yang W, Beratan DN. Stochastic voyages into uncharted chemical space produce a representative library of all possible drug-like compounds. J Am Chem Soc 2013;135:7296–303.
[34] Bohanec S, Zupan J. Structure generation of constitutional isomers from structural fragments. J Chem Inf Comput Sci 1991; 31:531–40.
[35] Pearlman RS, Smith KM. Novel software tools for chemical diversity. Perspect Drug Discovery Des 1998;9–11:339–53.
[36] Sheridan RP, Kearsley SK. Why do we need so many chemical similarity search methods? Drug Discovery Today 2002;7: 903–11.
[37] Ruddigkeit L, Blum LC, Reymond J-L. Visualization and virtual screening of the chemical universe database gdb-17. J Chem Inf Model 2013;53:56–65.
[38] Owen JR, Nabney IT, Medina-Franco JL, López-Vallejo F. Visualization of molecular fingerprints. J Chem Inf Model 2011;51: 1552–63.
[39] Rabal O, Oyarzabal J. Biologically relevant chemical space navigator: from patent and structure–activity relationship analysis to library acquisition and design. J Chem Inf Model 2012;52: 3123–37.
[40] Wetzel S, Schuffenhauer A, Roggo S, Ertl P, Waldmann H. Cheminformatic analysis of natural products and their chemical space. Chimia 2007;61:355–60.
[41] Shultz MD. Setting expectations in molecular optimizations: strengths and limitations of commonly used composite parameters. Bioorg Med Chem Lett 2013;23:5980–91.
[42] Yusof I, Segall MD. Considering the impact drug-like properties have on the chance of success. Drug Discovery Today 2013;18: 659–66.
[43] Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Delivery Rev 1997;23:3–25.
[44] Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW, Kopple KD. Molecular properties that influence the oral bioavailability of drug candidates. J Med Chem 2002;45:2615–23.
[45] Hopkins AL, Keseru GM, Leeson PD, Rees DC, Reynolds CH. The role of ligand efficiency metrics in drug discovery. Nat Rev Drug Discovery 2014;13:105–21.

[46] Feher M, Schmidt JM. Property distributions: differences between drugs, natural products, and molecules from combinatorial chemistry. J Chem Inf Comput Sci 2003;43:218–27.
[47] Shelat AA, Guy RK. The interdependence between screening methods and screening libraries. Curr Opin Chem Biol 2007;11:244–51.
[48] Kong D-X, Li X-J, Zhang H-Y. Where is the hope for drug discovery? Let history tell the future. Drug Discovery Today 2009;14:115–9.
[49] Koehn FE, Carter GT. The evolving role of natural products in drug discovery. Nat Rev Drug Discov 2005;4:206–20.
[50] Ganesan A. The impact of natural products upon modern drug discovery. Curr Opin Chem Biol 2008;12:306–17.
[51] Irwin JJ, Shoichet BK. ZINC - a free database of commercially available compounds for virtual screening. J Chem Inf Model 2005;45:177–82.
[52] Lee ML, Schneider G. Scaffold architecture and pharmacophoric properties of natural products and trade drugs: application in the design of natural product-based combinatorial libraries. J Comb Chem 2001;3:284–9.
[53] Chen CY-C. TCM database@Taiwan: the world's largest traditional chinese medicine database for drug screening in silico. PLoS One 2011;6:e15939.
[54] López-Vallejo F, Giulianotti MA, Houghten RA, Medina-Franco JL. Expanding the medicinally relevant chemical space with compound libraries. Drug Discovery Today 2012;17:718–26.
[55] O'Connell KMG, Galloway WRJD, Spring DR. The basics of diversity-oriented synthesis. In: Andrea T, editor. Diversity-oriented synthesis: Basics and applications in organic synthesis, drug discovery, and chemical biology. Hoboken (New Jersey): John Wiley & Sons, Inc.; 2013. p. 1–26.
[56] Clemons PA, Wilson JA, Dancik V, Muller S, Carrinski HA, Wagner BK, et al. Quantifying structure and performance diversity for sets of small molecules comprising small-molecule screening collections. Proc Natl Acad Sci USA 2011;108:6817–22.
[57] Manallack DT, Prankerd RJ, Nassta GC, Ursu O, Oprea TI, Chalmers DK. A chemogenomic analysis of ionization constants-implications for drug discovery. ChemMedChem 2013;8:242–55.
[58] Manallack DT, Dennis ML, Kelly MR, Prankerd RJ, Yuriev E, Chalmers DK. The acid/base profile of the human metabolome and natural products. Mol Inf 2013;32:505–15.
[59] Yongye AB, Medina-Franco JL. Systematic characterization of structure–activity relationships and ADMET compliance: a case study. Drug Discovery Today 2013;18:732–9.
[60] Larsson J, Gottfries J, Muresan S, Backlund A. ChemGPS-NP: Tuned for navigation in biologically relevant chemical space. J Nat Prod 2007;70:789–94.
[61] Rosen J, Lovgren A, Kogej T, Muresan S, Gottfries J, Backlund A. ChemGPS-NP$_{Web}$: chemical space navigation online. J Comput-Aided Mol Des 2009;23:253–9.
[62] Larsson J, Gottfries J, Bohlin L, Backlund A. Expanding the ChemGPS chemical space with natural products. J Nat Prod 2005;68:985–91.
[63] Oprea TI, Gottfries J. Chemography: the art of navigating in chemical space. J Comb Chem 2001;3:157–66.
[64] Medina-Franco JL, Waddell J. Towards the bioassay activity landscape modeling in compound databases. J Mex Chem Soc 2012;56:163–8.
[65] Brown N, Jacoby E. On scaffolds and hopping in medicinal chemistry. Mini-Rev Med Chem 2006;6:1217–29.
[66] Schuffenhauer A, Varin T. Rule-based classification of chemical structures by scaffold. Mol Inf 2011;30:646–64.
[67] Schneider G, Neidhart W, Giller T, Schmid G. Scaffold-hopping by topological pharmacophore search: a contribution to virtual screening. Angew Chem Int Ed 1999;38:2894–6.
[68] Evans BE, Rittle KE, Bock MG, DiPardo RM, Freidinger RM, Whitter WL, et al. Methods for drug discovery: development of potent, selective, orally effective cholecystokinin antagonists. J Med Chem 1988;31:2235–46.
[69] Mason JS, Morize I, Menard PR, Cheney DL, Hulme C, Labaudiniere RF. New 4-point pharmacophore method for molecular similarity and diversity applications: overview of the method and applications, including a novel approach to the design of combinatorial libraries containing privileged substructures. J Med Chem 1999;42:3251–64.
[70] Xu Y, Johnson M. Algorithm for naming molecular equivalence classes represented by labeled pseudographs. J Chem Inf Comput Sci 2001;41:181–5.
[71] Xu YJ, Johnson M. Using molecular equivalence numbers to visually explore structural features that distinguish chemical libraries. J Chem Inf Comput Sci 2002;42:912–26.
[72] Bemis GW, Murcko MA. The properties of known drugs. 1. Molecular frameworks. J Med Chem 1996;39:2887–93.
[73] Medina-Franco JL, Petit J, Maggiora GM. Hierarchical strategy for identifying active chemotype classes in compound databases. Chem Biol Drug Des 2006;67:395–408.
[74] López-Vallejo F, Peppard TL, Medina-Franco JL, Martínez-Mayorga K. Computational methods for the discovery of mood disorder therapies. Expert Opin Drug Discovery 2011;6:1227–45.
[75] López-Vallejo F, Castillo R, Yépez-Mulia L, Medina-Franco JL. Benzotriazoles and indazoles are scaffolds with biological activity against *Entamoeba histolytica*. J Biomol Screening 2011;16:862–8.
[76] Villar HO, Hansen MR, Kho R. Substructural analysis in drug discovery. Curr Comput-Aided Drug Design 2007;3:59–67.
[77] Medina-Franco JL, Martínez-Mayorga K, Bender A, Scior T. Scaffold diversity analysis of compound data sets using an entropy-based measure. QSAR Comb Sci 2009;28:1551–60.
[78] Yongye AB, Waddell J, Medina-Franco JL. Molecular scaffold analysis of natural products databases in the public domain. Chem Biol Drug Des 2012;80:717–24.
[79] Koch MA, Schuffenhauer A, Scheck M, Wetzel S, Casaulta M, Odermatt A, et al. Charting biologically relevant chemical space: a structural classification of natural products (SCONP). Proc Natl Acad Sci USA 2005;102:17272–7.
[80] Ertl P, Roggo S, Schuffenhauer A. Natural product-likeness score and its application for prioritization of compound libraries. J Chem Inf Model 2008;48:68–74.
[81] Chen H, Engkvist O, Blomberg N, Li J. A comparative analysis of the molecular topologies for drugs, clinical candidates, natural products, human metabolites and general bioactive compounds. Med Chem Comm 2012;3:312–21.
[82] Leach AR, Gillet VJ. An introduction to chemoinformatics. Dordrecht (The Netherlands): Kluwer Academic Publishers; 2003.
[83] Shanmugasundaram V, Maggiora GM, Lajiness MS. Hit-directed nearest-neighbor searching. J Med Chem 2005;48:240–8.
[84] Medina-Franco JL, Martínez-Mayorga K, Bender A, Marín RM, Giulianotti MA, Pinilla C, et al. Characterization of activity landscapes using 2D and 3D similarity methods: consensus activity cliffs. J Chem Inf Model 2009;49:477–91.
[85] Willett P. Similarity-based virtual screening using 2D fingerprints. Drug Discovery Today 2006;11:1046–53.
[86] Feher M. Consensus scoring for protein-ligand interactions. Drug Discovery Today 2006;11:421–8.
[87] Yongye A, Byler K, Santos R, Martínez-Mayorga K, Maggiora GM, Medina-Franco JL. Consensus models of activity landscapes with multiple chemical, conformer and property representations. J Chem Inf Model 2011;51:1259–70.
[88] Medina-Franco JL, Yongye AB, López-Vallejo F. Consensus models of activity landscapes. In: Matthias D, Kurt V, Danail B,

editors. Statistical modeling of molecular descriptors in QSAR/QSPR. Weinheim, Germany: Wiley-VCH; 2012. p. 307–26.
[89] Chu C-W, Holliday JD, Willett P. Combining multiple classifications of chemical structures using consensus clustering. Bioorg Med Chem 2012;20:5366–71.
[90] Pérez-Villanueva J, Santos R, Hernández-Campos A, Giulianotti MA, Castillo R, Medina-Franco JL. Towards a systematic characterization of the antiprotozoal activity landscape of benzimidazole derivatives. Bioorg Med Chem 2010;18:7380–91.
[91] Jaccard P. Etude comparative de la distribution florale dans une portion des alpes et des jura. Bull Soc Vaudoise Sci Nat 1901;37:547–79.
[92] Willett P, Barnard JM, Downs GM. Chemical similarity searching. J Chem Inf Comput Sci 1998;38:983–96.
[93] Lovering F, Bikker J, Humblet C. Escape from flatland: Increasing saturation as an approach to improving clinical success. J Med Chem 2009;52:6752–6.
[94] Clemons PA, Bodycombe NE, Carrinski HA, Wilson JA, Shamji AF, Wagner BK, et al. Small molecules of different origins have distinct distributions of structural complexity that correlate with protein-binding profiles. Proc Natl Acad Sci USA 2010;107:18787–92.
[95] Bertz SH. The 1st general index of molecular complexity. J Am Chem Soc 1981;103:3599–601.
[96] Barone R, Chanon M. A new and simple approach to chemical complexity. Application to the synthesis of natural products. J Chem Inf Comput Sci 2001;41:269–72.
[97] Allu TK, Oprea TI. Rapid evaluation of synthetic and molecular complexity for in silico chemistry. J Chem Inf Model 2005;45:1237–43.
[98] Schuffenhauer A, Brown N, Selzer P, Ertl P, Jacoby E. Relationships between molecular complexity, biological activity, and structural diversity. J Chem Inf Model 2006;46:525–35.
[99] Miller MA. Chemical database techniques in drug discovery. Nat Rev Drug Discov 2002;1:220–7.
[100] Reymond J-L, van Deursen R, Blum LC, Ruddigkeit L. Chemical space as a source for new drugs. Med Chem Comm 2010;1:30–8.
[101] Nguyen KT, Syed S, Urwyler S, Bertrand S, Bertrand D, Reymond JL. Discovery of NMDA glycine site inhibitors from the chemical universe database GDB. ChemMedChem 2008;3:1520–4.
[102] Nguyen KT, Luethi E, Syed S, Urwyler S, Bertrand S, Bertrand D, et al. 3-(aminomethyl)piperazine-2,5-dione as a novel NMDA glycine site inhibitor from the chemical universe database GDB. Bioorg Med Chem Lett 2009;19:3832–5.
[103] Scior T, Bernard P, Medina-Franco JL, Maggiora GM. Large compound databases for structure-activity relationships studies in drug discovery. Mini-Rev Med Chem 2007;7:851–60.
[104] Bender A. Databases compound bioactivities go public. Nat Chem Biol 2010;6:309.
[105] Barbosa AJM, Del Rio A. Freely accessible databases of commercial compounds for high- throughput virtual screenings. Curr Top Med Chem 2012;12:866–77.
[106] López-Vallejo F, Nefzi A, Bender A, Owen JR, Nabney IT, Houghten RA, et al. Increased diversity of libraries from libraries: chemoinformatic analysis of bis-diazacyclic libraries. Chem Biol Drug Des 2011;77:328–42.
[107] Fullbeck M, Michalsky E, Dunkel M, Preissner R. Natural products: sources and databases. Nat Prod Rep 2006;23:347–56.
[108] Haustedt LO, Mang C, Siems K, Schiewe H. Rational approaches to natural-product-based drug design. Curr Opin Drug Discovery Dev 2006;9:445–62.
[109] Grabowski K, Baringhaus K-H, Schneider G. Scaffold diversity of natural products: Inspiration for combinatorial library design. Nat Prod Rep 2008;25:892–904.
[110] Over B, Wetzel S, Grutter C, Nakai Y, Renner S, Rauh D, et al. Natural-product-derived fragments for fragment-based ligand discovery. Nat Chem 2013;5:21–8.
[111] Ntie-Kang F, Zofou D, Babiaka SB, Meudom R, Scharfe M, Lifongo LL, et al. AfroDb: a select highly potent and diverse natural product library from african medicinal plants. PLoS One 2013;8:e78085.
[112] Dunkel M, Fullbeck M, Neumann S, Preissner R. Supernatural: a searchable database of available natural compounds. Nucleic Acids Res 2006;34:D678–83.
[113] Kang H, Tang K, Liu Q, Sun Y, Huang Q, Zhu R, et al. HIM-herbal ingredients in-vivo metabolism database. J Cheminf 2013;5:28.
[114] Lei J, Zhou J. A marine natural product database. J Chem Inf Comput Sci 2002;42:742–8.
[115] Valli M, dos Santos RN, Figueira LD, Nakajima CH, Castro-Gamboa I, et al. Development of a natural products database from the biodiversity of Brazil. J Nat Prod 2013;76:439–44.
[116] Tsai T-Y, Chang K-W, Chen C. Iscreen: world's first cloud-computing web server for virtual screening and de novo drug design based on TCM database@Taiwan. J Comput-Aided Mol Des 2011;25:525–31.
[117] Chen K-Y, Chang S-S, Chen CY-C. In silico identification of potent pancreatic triacylglycerol lipase inhibitors from traditional chinese medicine. PLoS One 2012;7:e43932.
[118] Gu J, Gui Y, Chen L, Yuan G, Lu H-Z, Xu X. Use of natural products as chemical library for drug discovery and network pharmacology. PLoS One 2013;8:e62839.
[119] Ntie-Kang F, Onguene PA, Scharfe M, Owono LCO, Megnassan E, Mbaze LM, et al. ConMedNP: a natural product library from central african medicinal plants for drug discovery. RSC Adv 2014;4:409–19.
[120] Ntie-Kang F, Mbah JA, Mbaze LM, Lifongo LL, Scharfe M, Hanna JN, et al. CamMedNP: Building the cameroonian 3d structural natural products database for virtual screening. BMC Complementary Altern Med 2013;13:88.
[121] Jacoby E. Computational chemogenomics. Wiley Interdiscip Rev: Comput Mol Sci 2011;1:57–67.
[122] Rognan D. Structure-based approaches to target fishing and ligand profiling. Mol Inf 2010;29:176–87.
[123] Bajorath J. A perspective on computational chemogenomics. Mol Inf 2013;32:1025–8.
[124] Rognan D. Towards the next generation of computational chemogenomics tools. Mol Inf 2013;32:1029–34.
[125] Mullard A. 2013 FDA drug approvals. Nat Rev Drug Discov 2014;13:85–9.
[126] Yongye AB, Medina-Franco JL. Data mining of protein-binding profiling data identifies structural modifications that distinguish selective and promiscuous compounds. J Chem Inf Model 2012;52:2454–61.
[127] Dimova D, Hu Y, Bajorath J. Matched molecular pair analysis of small molecule microarray data identifies promiscuity cliffs and reveals molecular origins of extreme compound promiscuity. J Med Chem 2012;55:10220–8.
[128] Yongye AB, Medina-Franco JL. Toward an efficient approach to identify molecular scaffolds possessing selective or promiscuous compounds. Chem Biol Drug Des 2013;82:367–75.
[129] Dossetter AG, Griffen EJ, Leach AG. Matched molecular pair analysis in drug discovery. Drug Discovery Today 2013;18:724–31.
[130] Klebe G. Virtual ligand screening: strategies, perspectives and limitations. Drug Discovery Today 2006;11:580–94.
[131] Heikamp K, Bajorath J. The future of virtual compound screening. Chem Biol Drug Des 2013;81:33–40.
[132] Muegge I. Synergies of virtual screening approaches. Mini-Rev Med Chem 2008;8:927–33.

[133] Clark DE. What has virtual screening ever done for drug discovery? Expert Opin Drug Discovery 2008;3:841–51.
[134] Hu GP, Li X, Zhang X, Li YZ, Ma L, Yang LM, et al. Discovery of inhibitors to block interactions of HIV-1 integrase with human LEDGF/p75 via structure-based virtual screening and bioassays. J Med Chem 2012;55:10108–17.
[135] Wang LY, Li X, Zhang SD, Lu WQ, Liao S, Liu XF, et al. Natural products as a gold mine for selective matrix metalloproteinases inhibitors. Bioorg Med Chem 2012;20:4164–71.
[136] Remya C, Dileep KV, Tintu I, Variyar EJ, Sadasivan C. In vitro inhibitory profile of NDGA against AChE and its in silico structural modifications based on ADME profile. J Mol Model 2013; 19:1179–94.
[137] Galvez-Llompart M, Recio Iglesias M d C, Galvez J, Garcia-Domenech R. Novel potential agents for ulcerative colitis by molecular topology: suppression of IL-6 production in Caco-2 and RAW 264.7 cell lines. Mol Diversity 2013;17:573–93.
[138] Cao X, Jiang J, Zhang S, Zhu L, Zou J, Diao Y, et al. Discovery of natural estrogen receptor modulators with structure-based virtual screening. Bioorg Med Chem Lett 2013;23:3329–33.
[139] Lauro G, Masullo M, Piacente S, Riccio R, Bifulco G. Inverse virtual screening allows the discovery of the biological activity of natural compounds. Bioorg Med Chem 2012;20:3596–602.
[140] Schuster D, Wolber G. Identification of bioactive natural products by pharmacophore-based virtual screening. Curr Pharm Des 2010;16:1666–81.
[141] Ehrman TM, Barlow DJ, Hylands PJ. Phytochemical informatics and virtual screening of herbs used in chinese medicine. Curr Pharm Des 2010;16:1785–98.
[142] Shen JH, Xu XY, Cheng F, Liu H, Luo XM, Shen JK, et al. Virtual screening on natural products for discovering active compounds and target information. Curr Med Chem 2003;10:2327–42.
[143] Ma DL, Chan DSH, Leung CH. Molecular docking for virtual screening of natural product databases. Chem Sci 2011;2: 1656–65.
[144] Geldenhuys WJ, Bishayee A, Darvesh AS, Carroll RT. Natural products of dietary origin as lead compounds in virtual screening and drug design. Curr Pharm Biotechnol 2012;13: 117–24.
[145] Clark RL, Johnston BF, Mackay SP, Breslin CJ, Robertson MN, Harvey AL. The drug discovery portal: a resource to enhance drug discovery from academia. Drug Discovery Today 2010;15: 679–83.
[146] Nettles JH, Jenkins JL, Bender A, Deng Z, Davies JW, Glick M. Bridging chemical and biological space: "Target fishing" using 2D and 3D molecular descriptors. J Med Chem 2006;49:6802–10.
[147] Yue R, Shan L, Yang X, Zhang W. Approaches to target profiling of natural products. Curr Med Chem 2012;19:3841–55.
[148] Keiser MJ, Roth BL, Armbruster BN, Ernsberger P, Irwin JJ, Shoichet BK. Relating protein pharmacology by ligand chemistry. Nat Biotech 2007;25:197–206.
[149] Keiser MJ, Setola V, Irwin JJ, Laggner C, Abbas AI, Hufeisen SJ, et al. Predicting new molecular targets for known drugs. Nature 2009;462:175–82.
[150] Lagunin A, Stepanchikova A, Filimonov D, Poroikov V. PASS: prediction of activity spectra for biologically active substances. Bioinformatics 2000;16:747–8.
[151] Rudik AV, Dmitriev AV, Lagunin AA, Filimonov DA, Poroikov VV. Metabolism site prediction based on xenobiotic structural formulas and PASS prediction algorithm. J Chem Inf Model 2014:498–507.
[152] Lagunin A, Filimonov D, Poroikov V. Multi-targeted natural products evaluation based on biological activity prediction with PASS. Curr Pharm Des 2010;16:1703–17.
[153] Carregal AP, Comar M, Alves SN, de Siqueira JM, Lima LA, Taranto AG. Inverse virtual screening studies of selected natural compounds from cerrado. Int J Quantum Chem 2012;112: 3333–40.
[154] Gerhard W, Judith MR. Virtual screening and target fishing for natural products using 3D pharmacophores. In: Jacoby E, editor. Computational chemogenomics. Florida, (United States): Tylor & Francis Group; 2013. p. 117–39.
[155] Martínez-Mayorga K, Medina-Franco JL, Organizers. Symposium: Foodinformatics: applications of chemical information to food chemistry. Division of Chemical Information. 245th ACS National Meeting. New Orleans, LI, United States; 2013.
[156] Blondeau S, Do QT, Scior T, Bernard P, Morin-Allory L. Reverse pharmacognosy: another way to harness the generosity of nature. Curr Pharm Des 2010;16:1682–96.
[157] Do QT, Lamy C, Renimel I, Sauvan N, Andre P, Himbert F, et al. Reverse pharmacognosy: Identifying biological properties for plants by means of their molecule constituents: application to meranzin. Planta Med 2007;73:1235–40.
[158] Bernard P, Dufresne-Favetta C, Favetta P, Do QT, Himbert F, Zubrzycki S, et al. Application of drug repositioning strategy to TOFISOPAM. Curr Med Chem 2008;15:3196–203.
[159] Zinzalla G, Thurston DE. Targeting protein-protein interactions for therapeutic intervention: a challenge for the future. Future Med Chem 2009;1:65–93.
[160] Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces. Nature 2007;450: 1001–9.
[161] Sperandio O, Reynes CH, Camproux AC, Villoutreix BO. Rationalizing the chemical space of protein-protein interaction inhibitors. Drug Discovery Today 2010;15:220–9.
[162] Whitty A, Kumaravel G. Between a rock and a hard place? Nat Chem Biol 2006;2:112–8.
[163] Bienstock RJ. Computational drug design targeting protein-protein interactions. Curr Pharm Des 2012;18:1240–54.
[164] Knapp S, Weinmann H. Small-molecule modulators for epigenetics targets. ChemMedChem 2013;8:1885–91.
[165] Rius M, Lyko F. Epigenetic cancer therapy: Rationales, targets and drugs. Oncogene 2012;31:4257–65.
[166] Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004;429: 457–63.
[167] Svedruzic ZM. Mammalian cytosine DNA methyltransferase Dnmt1: enzymatic mechanism, novel mechanism-based inhibitors, and RNA-directed DNA methylation. Curr Med Chem 2008;15:92–106.
[168] Miller CA, Gavin CF, White JA, Parrish RR, Honasoge A, Yancey CR, et al. Cortical DNA methylation maintains remote memory. Nat Neurosci 2010;13:664–6.
[169] Zawia NH, Lahiri DK, Cardozo-Pelaez F. Epigenetics, oxidative stress, and Alzheimer disease. Free Radical Biol Med 2009;46: 1241–9.
[170] Castellano S, Kuck D, Viviano M, Yoo J, López-Vallejo F, Conti P, et al. Synthesis and biochemical evaluation of δ2-isoxazoline derivatives as DNA methyltransferase 1 inhibitors. J Med Chem 2011;54:7663–77.
[171] Rilova E, Erdmann A, Gros C, Masson V, Aussagues Y, Poughon-Cassabois V, et al. Design, synthesis and biological evaluation of 4-amino-n-(4-aminophenyl)benzamide analogues of quinoline-based SGI-1027 as inhibitors of DNA methylation. ChemMedChem 2014;9:590–601.
[172] Méndez-Lucio O, Tran J, Medina-Franco JL, Meurice N, Muller M. Towards drug repurposing in epigenetics: olsalazine as a novel hypomethylating compound active in a cellular context. ChemMedChem 2014;9:560–5.
[173] Medina-Franco JL, López-Vallejo F, Kuck D, Lyko F. Natural products as DNA methyltransferase inhibitors: a computer-aided discovery approach. Mol Diversity 2011;15:293–304.

[174] Lee WJ, Shim JY, Zhu BT. Mechanisms for the inhibition of DNA methyltransferases by tea catechins and bioflavonoids. Mol Pharmacol 2005;68:1018–30.
[175] Liu ZF, Xie ZL, Jones W, Pavlovicz RE, Liu SJ, Yu JH, et al. Curcumin is a potent DNA hypomethylation agent. Bioorg Med Chem Lett 2009;19:706–9.
[176] Pina IC, Gautschi JT, Wang GYS, Sanders ML, Schmitz FJ, France D, et al. Psammaplins from the sponge *pseudoceratina purpurea*: Inhibition of both histone deacetylase and DNA methyltransferase. J Org Chem 2003;68:3866–73.
[177] Pereira R, Benedetti R, Perez-Rodriguez S, Nebbioso A, Garcia-Rodriguez J, Carafa V, et al. Indole-derived psammaplin a analogues as epigenetic modulators with multiple inhibitory activities. J Med Chem 2012;55:9467–91.
[178] Kuck D, Caulfield T, Lyko F, Medina-Franco JL. Nanaomycin a selectively inhibits DNMT3B and reactivates silenced tumor suppressor genes in human cancer cells. Mol Cancer Ther 2010;9:3015–23.
[179] Hauser AT, Jung M. Targeting epigenetic mechanisms: potential of natural products in cancer chemoprevention. Planta Med 2008;74:1593–601.
[180] Li Y, Tollefsbol TO. Impact on DNA methylation in cancer prevention and therapy by bioactive dietary components. Curr Med Chem 2010;17:2141–51.
[181] Cherblanc FL, Davidson RWM, Di Fruscia P, Srimongkolpithak N, Fuchter MJ. Perspectives on natural product epigenetic modulators in chemical biology and medicine. Nat Prod Rep 2013;30:605–24.
[182] Jia D, Jurkowska RZ, Zhang X, Jeltsch A, Cheng X. Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. Nature 2007;449:248–51.
[183] Medina-Franco JL, Caulfield T. Advances in the computational development of DNA methyltransferase inhibitors. Drug Discovery Today 2011;16:418–25.
[184] Yoo J, Medina-Franco JL. Inhibitors of DNA methyltransferases: Insights from computational studies. Curr Med Chem 2012;19:3475–87.
[185] Yoo J, Medina-Franco JL. Homology modeling, docking, and structure-based pharmacophore of inhibitors of DNA methyltransferase. J Comput-Aided Mol Des 2011;25:555–67.
[186] Yoo J, Kim JH, Robertson KD, Medina-Franco JL. Molecular modeling of inhibitors of human DNA methyltransferase with a crystal structure: discovery of a novel DNMT1 inhibitor. Adv Protein Chem Struct Biol 2012;87:219–47.
[187] Yoo J, Medina-Franco JL. Trimethylaurintricarboxylic acid inhibits human DNA methyltransferase 1: Insights from enzymatic and molecular modeling studies. J Mol Model 2012;18:1583–9.
[188] Yoo J, Medina-Franco JL. Chemoinformatic approaches for inhibitors of DNA methyltransferases: comprehensive characterization of screening libraries. Comput Mol Biosci 2011;1:7–16.
[189] Medina-Franco JL, Martínez-Mayorga K, Peppard TL, Del Rio A. Chemoinformatic analysis of GRAS (Generally Recognized as Safe) flavor chemicals and natural products. PLoS One 2012;7:e50798.
[190] Guasch L, Sala E, Castell-Auvi A, Cedo L, Liedl KR, Wolber G, et al. Identification of PPARgamma partial agonists of natural origin (i): development of a virtual screening procedure and in vitro validation. PLoS One 2012;7:e50816.
[191] Martinez-Mayorga K, Peppard TL, López-Vallejo F, Yongye AB, Medina-Franco JL. Systematic mining of generally recognized as safe (GRAS) flavor chemicals for bioactive compounds. J Agric Food Chem 2013;61:7507–14.

LIST OF ABBREVIATIONS

2D Two-dimensional
3D Three-dimensional
DDP Drug Discovery Portal
DNMT DNA methyltransferase
DOS Diversity-oriented synthesis
EGCG (-)-Epigallocatechin-3-gallate
FDA Federal Drug Administration
GDB Generated Database of Chemical Space
GpiDAPH3 Graph-based three-point pharmacophore
GRAS Generally Recognized as Safe
HBA Hydrogen bond acceptor
HBD Hydrogen bond donor
HDAC1 Histone deacetylase-1
HTS High-throughput screening
MACCS Molecular Access System
MLSMR Molecular Libraries Small Molecule Repository
MW Molecular weight
NMR Nuclear magnetic resonance
NPs Natural products
PASS Prediction of activity spectra for substances
PPIs Protein–protein interactions
PSA Polar surface area
QSAR Quantitative structure–activity relationship
RB Rotatable bond
SCONP Structural classification of natural products
SEA Similarity ensemble approach
SAH *S*-adenosyl-L-homocysteine
SAM *S*-adenosyl-L-methionine
Slog P Octanol/water partition coefficient
SPID Structure–promiscuity index difference
TCM Traditional Chinese Medicine
TGD Typed graph distance
TPSA Topological polar surface area
UNPD Universal Natural Products Database

This page intentionally left blank

CHAPTER

22

Infrared Spectroscopic Technologies for the Quality Control of Herbal Medicines

Christian Huck

Institute of Analytical Chemistry and Radiochemistry, CCB – Center for Chemistry and Biomedicine, Leopold-Franzens University, Innsbruck, Austria

OUTLINE

22.1 INTRODUCTION

Herbal medicine is the oldest and most widely used form of medical treatment in the world, and enjoys increasing popularity due to its health-promoting properties [1]. Medicinal and herbal plant properties are related to individual ingredients usually found in the parts-per-million (ppm) and/or parts-per-billion (ppb) range [1]. During the recent decade, the pharmaceutical industry has initiated sophisticated plant screening programs, applying biochemical high-throughput techniques to find new drugs with distinct properties (e.g., anticancer, antibacterial, and/or antiviral properties) [2].

Traditionally, separation techniques including thin-layer chromatography (TLC), liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE) hyphenated to mass spectrometry (MS) were employed for the identification, quantification, and structural elucidation of selected compounds being present or deriving from different plant matrices [1,2]. These analytical techniques have been found useful in

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00022-2

phytochemical and physiological studies, enabling recording a fingerprint and/or identifying single active compounds [3].

Spectroscopic analytical techniques using the *near-infrared (NIR)* wavelength region of the electromagnetic spectrum have been used in the food industry to monitor and evaluate on one side the composition and on the other side the quality of foods [4]. Even though the NIR region was the first known nonvisible part of the electromagnetic spectrum and was discovered in 1800 by Herschel, it was not until the 1950s that the first applications of NIR spectroscopy for analytical chemistry were developed. Since the 1960s, there has been a steady increase in the use of NIR spectroscopy, with the most dramatic growth in the last 25 years.

NIR spectroscopy is characterized by low molar absorptivity and scattering. In the beginning, the NIR region was regarded as having little potential for analytical work. In recent times, it has become one of the most promising techniques for molecular spectroscopy. Affordable and powerful computers have further supported the implementation of applications in several fields, including medical, textile, polymer, and pharmaceutical applications [5].

Vibrational spectroscopic imaging has become an essential tool for tissue analyses in life science, and can support NIR spectroscopic studies in a synergistic manner, because it allows depiction of the spatial distribution of potent ingredients. It is a modern analytical technique enabling the detection and characterization of molecular components of plant tissue samples down to a resolution of approximately 1.2 μm, applying NIR and mid-infrared (MIR) spectroscopy [6]. It is based on the absorption of Infrared (IR) radiation by vibrational transitions in covalent bonds, and enables global analysis of samples, with resolution close to the cellular level. An advantage of vibrational spectroscopic imaging is that it can acquire local molecular expression profiles while maintaining the topographic integrity of the tissue by avoiding time-consuming extraction, purification, and/or separation steps, respectively. With this nondestructive analytical method, it is possible to obtain, on one hand, qualitative and quantitative information on heterogeneous samples, and on the other hand, unique chemimorphological information about the tissue status on the other hand. This characteristic of vibrational spectroscopic imaging represents an extremely important benefit for further interpretation of the present current tissue status.

This contribution highlights the fundamental principles of NIR and imaging spectroscopy, its applicability, regulatory issues, advantages, synergistic combination, and limitations in herbal medicine research.

22.2 TECHNICAL PRINCIPLES

22.2.1 NIR Spectroscopy

IR radiation is the region of the electromagnetic spectrum between the visible (VIS) and the microwave wavelength [7]. In NIR spectroscopy, excitation of molecules is accomplished in a wavelength range between 750 and 2500 nm (Figure 22.1), corresponding to a wavenumber range between 4.000 and 13.000/cm [8]. C−H, C−O, C═O, N−H, and O−H functional groups are excited to perform stretching-, deformation- and scissor-vibrations. In comparison to the MIR region, where only fundamental vibrations ("signatures") can be observed, overtones and combinations can be found in the NIR region containing a manifold of information compared to MIR [9]. The result is often a crowded

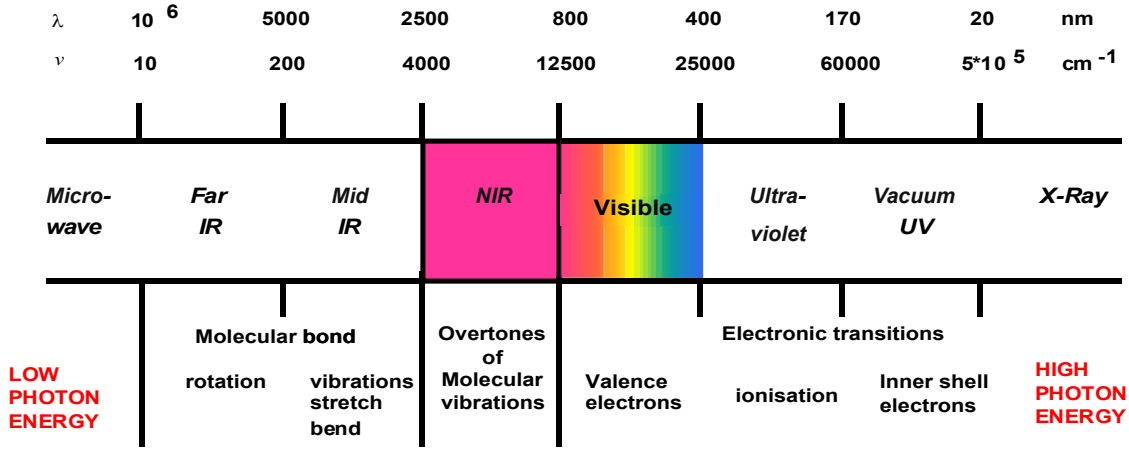


FIGURE 22.1 Electromagnetic spectrum. IR, infrared; NIR, near-infrared.

spectrum with overlapping peaks. Although NIR-intensities are 10 to1000 times lower than for MIR, highly sensitive spectrometers can be built through several means including the use of efficient detectors. The light recorded by the detector contains compositional information, which can be unraveled by a computer to report multiple analyses almost instantaneously. NIR spectroscopy can provide simultaneous, rapid, and nondestructive qualitative and quantitative analysis of major components in many organic substances [10].

22.2.2 Model of the Harmonic and An-Harmonic Oscillator

The physical principle describing the observed effects in both the MIR and NIR regions is the model of the harmonic and an-harmonic oscillator. According to the inset in Figure 22.2, the reduced mass μ performs vibrations with the frequency ν_{osc}. In MIR, this vibration follows the equation for the harmonic oscillator, whereas in NIR, the equation for the an-harmonic oscillator is valid, describing the excitation into higher energy states. Chemometrics, a mathematical, statistical, multivariate analytical (MVA) tool, is applied for further treatment of recorded spectra.

22.2.3 Instrumentation and Sample Preparation

An NIR spectrophotometer consists of a light source (e.g., tungsten halogen lamp), sample presentation accessories, monochromator, detector, and optical components including lenses, collimators, beam splitters, integrating spheres, and optical fibers (Figure 22.3) [11].

One of the most frequently cited benefits of analysis by NIR spectroscopy is that little or no sample preparation prior to analysis is required. In principle, transparent materials such as liquid extracts can be analyzed by transmission or transflection, solid materials like tissue by diffusive reflection and/or interactance mode (Figure 22.4) [11]. Spectrophotometers are conveniently classified into dispersive and nondispersive instruments. For example, in a dispersive filter instrument, the monochromator is a wheel holding a number of absorption or interference filters, with the disadvantage of limited resolution [11]. In a scanning monochromator instrument, a grating or a prism is used to separate the individual frequencies of the radiation. In Fourier transform (FT) spectrophotometers, interferometers are used to generate modulated light, and time domain signal of the light reflected or transmitted by the sample can be converted into a spectrum

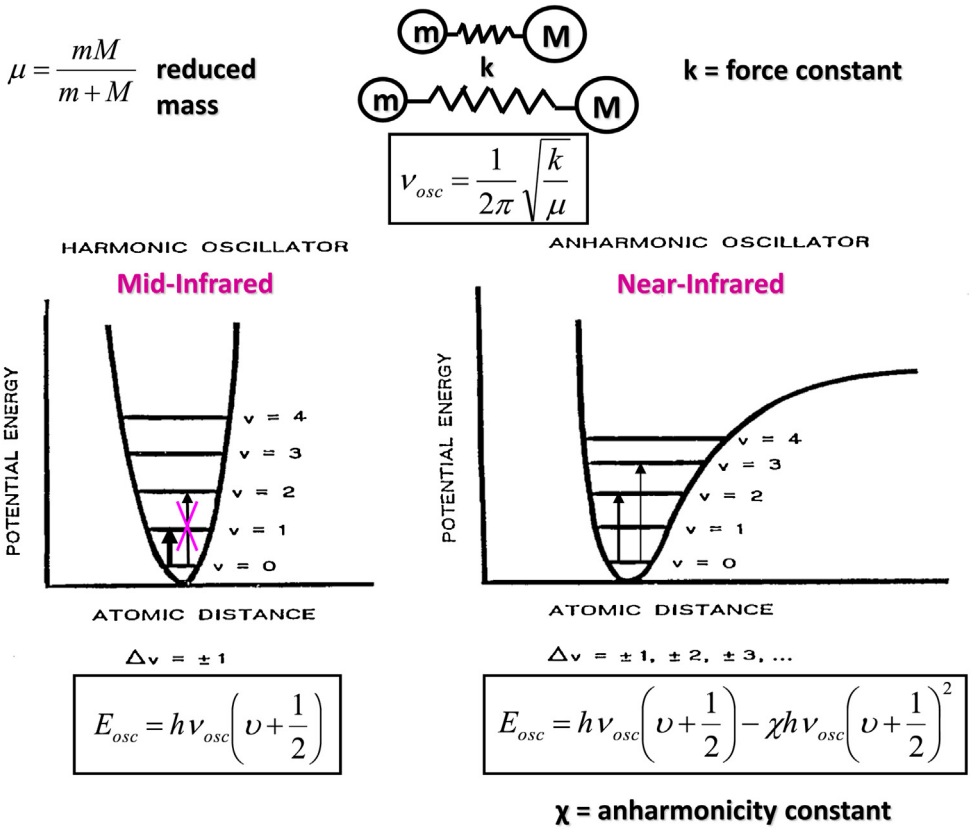


FIGURE 22.2 Model of the harmonic and an-harmonic oscillator.

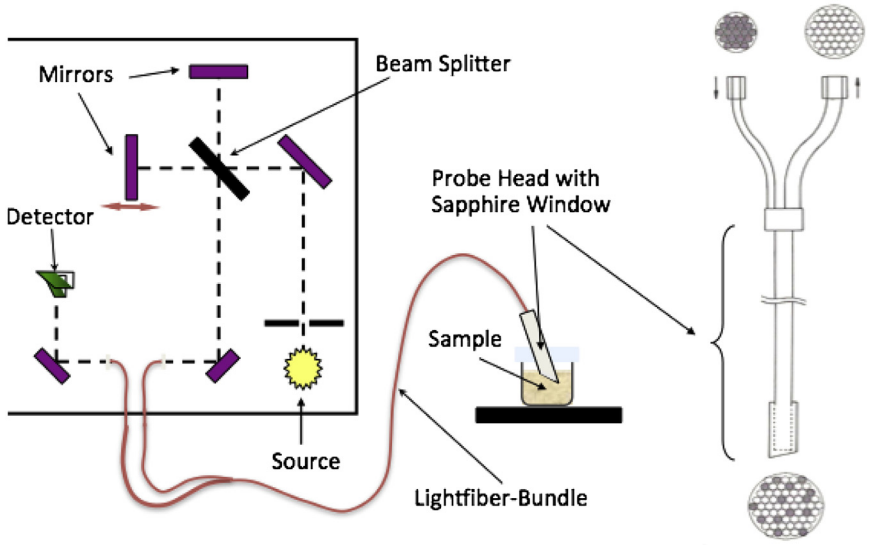


FIGURE 22.3 Measurement set-up in near-infrared spectroscopy.

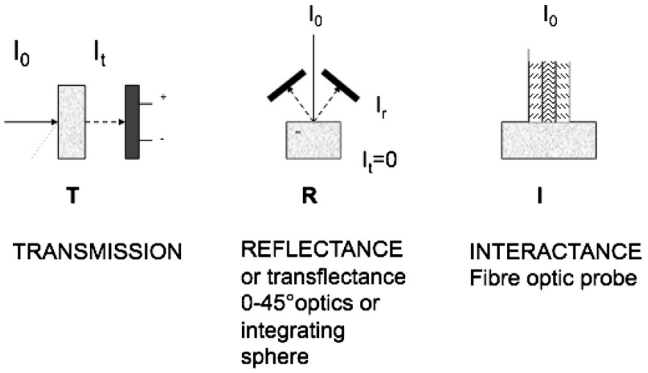


FIGURE 22.4 Measurement modes.

via a fast transformation [12]. In most cases, a Michelson or polarization interferometer is used.

Photodiode array (PDA) spectrophotometers consisting of a fixed grating that focuses the dispersed radiation onto an array of silicon (Si, 350–1100 nm) or indium gallium arsenide (InGaAs, 1100–2500 nm) offer the advantage of high acquisition speed (between 50 ms and a few milliseconds). As alternatives, laser-based systems that do not require a monochromator or acousto-optic tuneable filter instruments using a diffraction-based optical band-pass filter can be used [13].

22.3 IR IMAGING SPECTROSCOPY

The first IR microscopes were built during the 1940s and 1950s, but these microscopes were slow. It was in the 1990s that the subsequent development of microprocessor-controlled motorized stages made raster scan mapping convenient. Enhanced spatial resolution, which is enabled by the substitution of synchrotron radiation for a thermal source, was a significant instrumental advance. The introduction of focal plane array (FPA) systems, initially uncooled InGaAs, for near-IR by Mascott and Lewis [14] occurred in 1994, while the subsequent development for mid-IR, using a mercury cadmium telluride (MCT) array by Lewis and Levine [15] resulted in a very rapid image generation. These developments allowed simultaneous measurements and significantly increased data acquisition rates.

22.3.1 Imaging Microscopy

Fourier transform IR (FTIR) spectroscopic microscopy can be considered as the coupling of a microscope to an IR spectrometer. From the physical point of view, diffraction, refraction, reflection, and absorption effects are playing a considerable role. In principle, an FTIR microscope is similar to its optical analog, and consists of four main parts: (1) light source (single polychromatic thermal source), (2) splitter ((FT), tunable filter and diffraction grating), (3) detector (photon detectors, lead sulfide (PbS) detectors, indium antimonide (InSb) detectors, uncooled InGaAs and HgCdTeor MCT detectors), and (4) optics (fitted to a microscope) [16] (Figure 22.5). Advantages of FTIR spectroscopic microscopes are microspatial chemical mapping or imaging of complex heterogeneous samples with a resolution down to approximately 1.2 μm, high sensitivity, high selectivity, rapid data acquisition, simple sample preparation, fully automated examination, and computer-enhanced visualization.

22.3.2 Measurement Techniques

During the last couple of years, hyperspectral imaging systems have become popular [16]. A multispectral

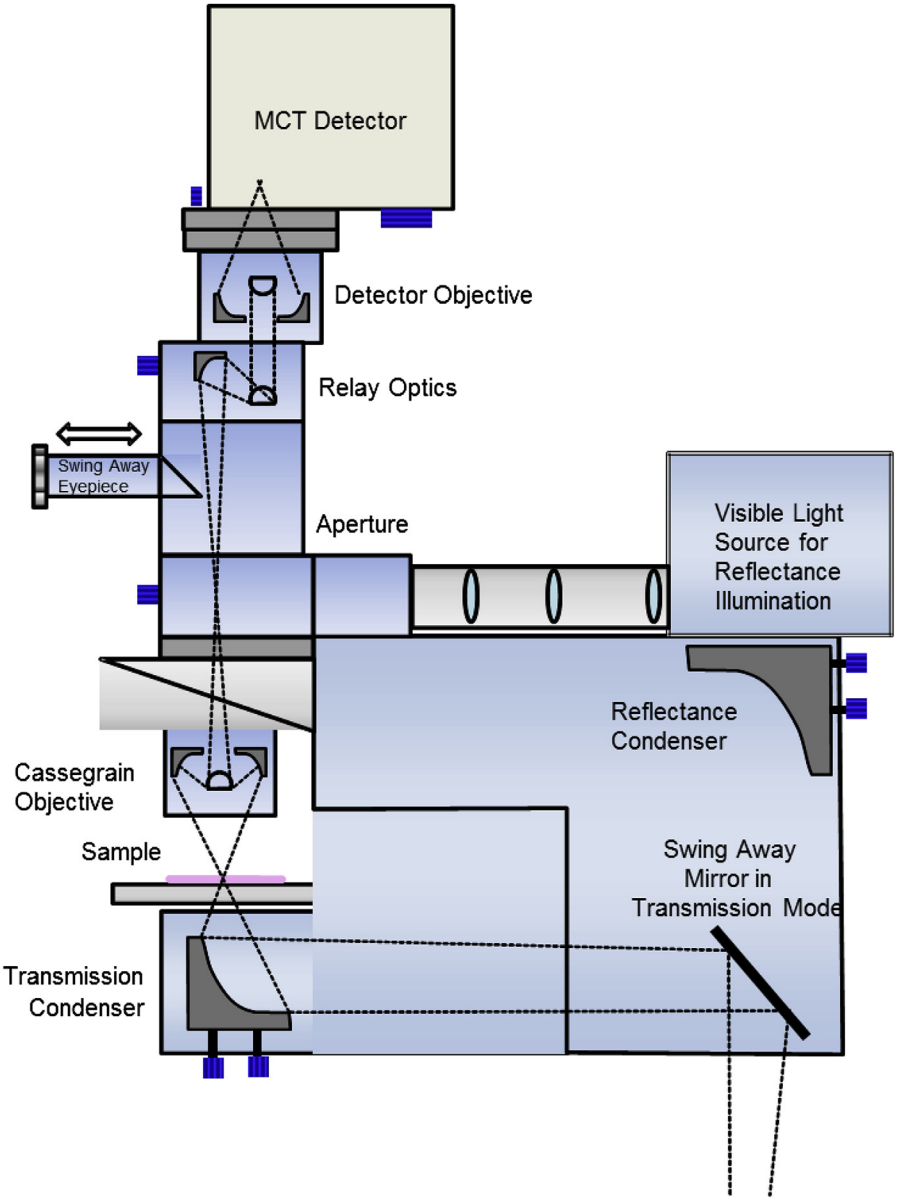


FIGURE 22.5 Schematic view of a mid-infrared reflective microscope, which can operate in either reflectance or transmission mode. MCT, mercury cadmium telluride.

(a few wavelengths) or hyperspectral (a continuous range of wavelengths) cube is recorded, consisting of spectra recorded at every 2-D spatial position (Figure 22.6). The cube is recorded by stepwise moving of the object of interest under the camera by means of an actuator, while at each step a line is scanned. In latest developments, FPA detectors are employed (MCT). In combination with attenuated total reflection (ATR) spectroscopy, the maximal resolution of 1.2 μm can be reached [10,16].

In quantitative analysis, the amount of absorbed radiation is dependent upon the Lambert–Beer law of the concentration c of the sample, the thickness d of the sample, and its molar extinction coefficient ε.

$$E = \log\frac{I_0}{I} = \varepsilon * d * c \tag{22.1}$$

I intensity of the transmitted light I_0 intensity of the incident light c concentration of the absorbing substance (unit: mol/dm^3 or mol/L) ε decimal extinction coefficient (unit: mol$^{-1}\cdot$dm^2) d thickness of the irradiated body (unit: cm)

22.3.3 Data Recording

There are two possible experimental measurement set-ups in FTIR microscopy: FTIR mapping and FTIR imaging. In FTIR mapping, the IR spectra of the sample

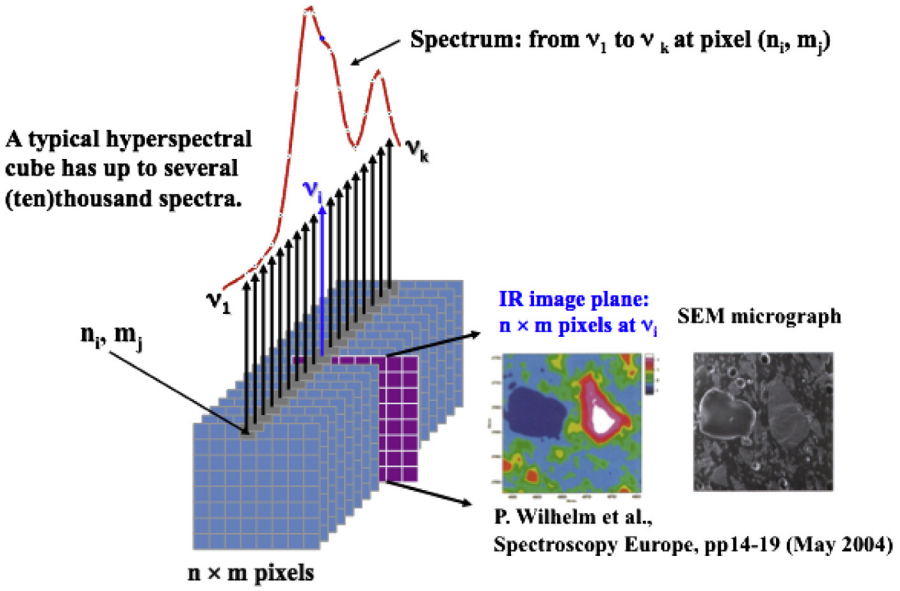


FIGURE 22.6 Hyperspectral cube. IR, infrared.

are collected sequentially at predefined spatial coordinates. This sampling technique offers a convenient, fast, and inexpensive route for analysis of static samples. It allows information about the spatial distribution of the chemical species within the sample to be obtained. The operator can choose between three different types of FTIR mapping techniques:

1. *Point mapping* provides several different areas of a sample to be analyzed consecutively
2. *Line mapping* defines a series of spectra obtained along one dimension, where chemical changes that occur along this dimension are investigated
3. *Area mapping* defines a series of spectra to be collected in two dimensions

In FTIR imaging, the whole area of interest is sampled simultaneously and allows a large number of spectra to be acquired with fine spatial detail over an area [17]. However, obtained imaging data are challenging, and a key issue is how to extract relevant information from the huge amount of data. In FTIR microscopy, one can choose between two different sampling techniques: transmission (IR beam passes through the sample) and reflection (IR beam reflects from the sample surface). In transmission measurements, the IR beam passes through the sample and the transmitted light is recorded by a detector, which is placed behind the sample following the law of Lambert–Beer. Transmission analysis requires the sample to be partly transparent. Samples can be placed on substrates transparent to the wavelength range of the probing radiation (e.g., MIR) such as calcium fluoride (CaF_2), barium fluoride (BaF_2) or zinc selenide (ZnSe) [18].

In reflection measurements, the IR beam reflects from the sample surface and the reflected light is recorded by a detector. This sampling technique allows rapid examination of the distribution of organic compounds on a complex surface of solid samples. There are two reflection measurement sampling techniques in FTIR microscopy:

1. Diffuse Reflection (DR) and
2. ATR

In diffuse refection measurements, the incoming radiation interacts with the sample and is scattered by interaction with the particles. A fraction of this light is reflected by the sample and recorded by the detector. In the NIR range, DR is widely used for the image analysis of thick nontransparent samples in various noninvasive applications (e.g., food industry and pharmaceuticals) [19].

In ATR measurements, the IR radiation enters a prism made of a high refractive index IR transmitting material, and is totally internally reflected. This reflectance creates an evanescent wave. The wave extends beyond the surface of the crystal into the sample held in intimate contact with the crystal and in regions of the IR spectrum where the sample absorbs energy. With this sampling technique, the IR beam typically penetrates from 0.5 to 2.0 μm into the sample. Main advantages of ATR imaging measurements are minimal or no sample preparation. Additionally, samples with high water contents can be analysed more efficiently than in conventional transmission mode. For detailed information about detector theory, technology, and current developments, the interested reader is referred to the cited literature.

Data processing techniques for imaging data will be discussed in the next chapter.

22.4 CHEMOMETRICS INCLUDING DATA PREPROCESSING

22.4.1 Chemometrics in NIR Spectroscopy

The NIR spectrum is represented by a huge number of partially overlapping overtones and combination vibrations, therefore appears to be much more complicated than the MIR spectrum. Additionally, scattering effects, instrumental noise, and/or sample inhomogeneities can occur. Therefore, it is in many cases impossible to correctly assign the corresponding vibration bands. For this reason, multivariate statistical analysis (MVA) is a powerful mathematical tool enabling the extraction of the required information from the spectrum [20].

The most frequently applied chemometrical procedures include principal component analysis (PCA) for reducing the number of variables facilitating both qualitative and quantitative analysis. Data pretreatment minimizes inhomogeneities originating from the recording of the spectra, and enables elimination of baseline shifts. Normalization algorithms can eliminate differences in intensity caused by different sample positioning. Diffusion and/or unexpected particle size effects can be compensated by multiplicative scatter correction (MSC). Performing the first or second derivative of the original spectrum can reduce spectral noise. Calibration development can mathematically describe the covariation between certain variables or find a mathematical function (regression model) by which the values of the dependent variables are calculated from values of the measured variables [21]. The calibration procedure of the NIR spectrometer can be summarized in five steps: (1) choice of a representative sample set; (2) recording of the NIR spectra; (3) measurement of the reference values; (4) multivariate modelling to generate a relationship between the recorded spectra and the reference values; and (5) validation of the system. The most frequently used regression methods comprise principal component regression (PCR) and partial least squares regression (PLSR), discriminant analysis (DA) and artificial neural networks (ANN) [6].

The choice of the highest suitable regression model is based on the calculation of the following values:

1. BIAS, i.e., the average deviation between the predicted values (y_n) and the actual values (x_n), in the calibration set, should be close to zero.

$$\mathrm{BIAS} = \frac{1}{N}\sum (x_n - y_n) \tag{22.2}$$

2. PRESS (predicted residual error sum square) is the sum of the square of the deviation between predicted and reference values. The PRESS value of the validation set should be as small as possible and similar to that of the calibration set.

$$\mathrm{PRESS} = \sum (x_n - y_n)^2 \tag{22.3}$$

3. Standard error of estimation (SEE), i.e., the standard deviation of the differences between reference values and NIRS results in the calibration set.

$$\mathrm{SEE} = \sqrt{\frac{1}{N}\sum (x_n - y_n - \mathrm{BIAS})^2} \tag{22.4}$$

4. Standard error of prediction (SEP), i.e., the counterpart for the test set samples. SEE and SEP should be as small as possible.

$$\mathrm{SEP} = \sqrt{\frac{1}{N}\sum (x_n - y_n - \mathrm{BIAS})^2} \tag{22.5}$$

5. The correlation coefficient (R^2) should approach 1.

The analysis of IR imaging data sets includes denoising, baseline correction, normalization, suppression of anomalous pixels, image compression, and univariate and multivariate analysis [16]. Available software programs improve the quality of information extracted from the large data sets, reduce the dimensionality to more practical levels, allow different imaging data sets to be aligned and compared, and address data management, including determination of statistical significance and relative abundance between particular chemical species. It has been reported that data analysis of IR imaging results consists of several steps:

- Demonstration of a constituent by taking a slice of the image on a particular relevant wavenumber, which gives selective information for the particular compound. Resulting distribution images can be easily interpreted with minimal computation effort and allow the observation of certain spatial features in the image. This simple method (known as univariate analysis) can only provide partial representation of the obtained imaging data and likely makes comparisons of several data sets impossible [18].
- Reduction of the complexity of IR imaging datasets with PCA, which transforms the original coordinate system defined by peak intensities to a coordinate system that better explains the variance in the dataset.
- Unsupervised classification such as hierarchical clustering, k-means (KM) clustering, and fuzzy C-means (FCM) clustering use all of the information contained in the hyperspectral image, where unlabeled IR spectral data can be separated into different clusters based on their characteristics in an unsupervised way.

22.4.2 Chemometrics in Imaging Spectroscopy

To analyze the spectral and spatial information contained in an image, various techniques also known as multivariate image analysis (MIA) have been introduced. In contrast to univariate image analysis, MIA uses all of the information contained in the hyperspectral image. Unlabeled IR spectral data can be separated into different clusters based on their characteristics in an unsupervised way. Clustering, also called unsupervised classification of FTIR microscopic data, can be performed such that spectra within the same cluster are as similar as possible and spectra in different clusters are as dissimilar as possible where different types of cells may be separated within biological tissue. There are many clustering techniques that have been applied for hyperspectral images, such as hierarchical clustering, KM clustering, and FCM clustering, which increase the information content of FTIR imaging data dramatically [18]. For detailed information about statistical classification, the interested reader is referred to the cited literature.

22.5 AD- AND DISADVANTAGES OF NIR AND IMAGING SPECTROSCOPY

The ad- and disadvantages of NIR spectroscopy are summarized in the following.

Advantages:
- Noninvasive measurement (sample can be used for other purposes after the measurement)
- Simultaneous determination of several parameters (the information is all packed within the spectrum)
- Chemical and physical properties can be determined in parallel (e.g., ingredients and solvent composition)

Disadvantages:
- Calibration takes a long time, requiring the consultancy of a reference method
- The lower limit of detection (LOD) can normally be found in the lower percentage range

The advantages and disadvantages of imaging spectroscopy are summarized in the following.

Advantages:
- Noninvasive measurement
- Huge potential for spectral interpretation
- Sensitive

Disadvantages:
- Time consuming (measurement takes several hours)
- Complicated sample preparation before measurement

22.6 QUANTITATIVE ANALYSIS OF SECONDARY METABOLITES

In this chapter, we discuss the potential of NIR spectroscopy for the quantitative characterization of herbal medicine and its constituents, including secondary metabolites and leading compounds. The following section summarizes the potential of NIR spectroscopy for the classification of the origin of natural products and verification of authenticity.

22.6.1 Phenolic Compounds

NIR spectroscopy has been applied to determine the content of total polyphenols, catechins, and others [22], [23]. Furthermore, attempts to analyze the antioxidative [24], antimicrobial [25], antiviral [26], anti-inflammatory, analgesic, antipyretic, and vasodilatory effects have been described [27].

Green tea: The quantitative analysis of the epigallocatechin (EGC) gallate, epicatechin (EC), and trolox equivalent antioxidant capacity (TEAC) in green tea (*Camelia sinensis* L.) leaves was described. As a reference for the control of the total phenolic content in green tea, Folin-Ciocalteu (FC) was used, resulting in an RMSECV of 0.75 g/g for a calibration range of 15.84−24.39 g/g [28]. For the quantitation of total polyphenol content high-performance liquid chromatography (HPLC) was applied. Chen and coworkers compared the three algorithms partial least square (PLS), interval PLS (iPLS), and synergy interval PLS (siPLS) to predict the total polyphenol content [29]. The siPLS model performed best, with an RMSEP of 0.7327 (range: 15.84−24.39%) using five PLS factors. The same work group reported PLS calibrations to determine the contents of the main catechins. Using 75 samples, EGC (0.14%, range: 2.4−5.4%), EC (0.017%, range: 0.1−0.4%), epigallocatechingallate (EGCG) (0.38%, range: 7.7−14.1%), and epicatechingallate (ECG) (0.12%, range: 1.8−3.7%) were calibrated using 10−14 PLS factors. Luypaert et al. [30] reported on a NIR spectroscopy method in combination with PLS algorithms to predict caffeine, EGCG, EC, and total antioxidant capacity. Zhang and coworkers [31] predict the total antioxidant capacity in green tea using PCR regression with test set validation, with 100 samples in the calibration set and 23 in the validation set.

Grape skins: Phenolic compounds in grape skins and intact grapes during ripening were analyzed by Ferrer-Gellego et al. [32]. Anthocyanins, phenolic acids, flavonols, flavanols, and total phenolic compounds were quantified with standard ratio of performance to deviations (RPDs) ranging from 4.4−13.6 applying partial least square (PLS) (sample set of 56). The same workgroup also worked on calibration models to determine flavanols in grape seeds [33].

Blueberries: Total phenols, total flavonoids, total anthocyanins, and ascorbatein blueberries (*Vaccinium corymbosum*) were investigated using a system to determine total soluble solid (TSS), applying NIR and MIR spectroscopy comparing cross validation and test set validation, respectively [34].

Kava: In the South Pacific, kava (*Piper methysticum*) has been traditionally used for thousands of years for relaxation without the loss of mental alertness. Kava has become a part of the herbal pharmacopoeia throughout the United States and Europe because of its anxiolytic properties [35]. A PLS calibration model for kavalactones (desmethoxyangonin, dihydrokavain, yangonin, kavain, dihydromethysticin, methysticin and total kalvalactones) using a maximum of 7 PLS factors was developed by Gautz and coworkers [35]. The SEPs were 0.20% (range: 0.08–2.35%) for desmethoxyangonin, 0.31% (range: 0.10–3.33%) for dihydrokavain, 0.47% (range: 0.08–3.02%) for yangonin, 0.21% (range: 0.11–3.02%) for kavain, 0.15% (range: 0.08–2.58%) for dihydromethysticin, 0.19% (range: 0.09–2.70%) for methysticin and 1.05% (range: 0.54–14.68%) for total kalvalactones.

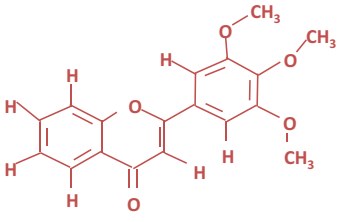
Honeybush: Mangiferin and hesperidin in dried green honeybush (*Cyclopia genistoides*) were analyzed by Joubert et al. [36]. PLS calibrations were calculated using 160 samples with four and six PLS factors, resulting in SEPs of 0.46% (range: 0.70–7.21%) for mangiferin and 0.38% (range: 0.64–4.80%) for hesperidin. RPDs were 1.96 (mangiferin) and 1.90 (hesperidin).

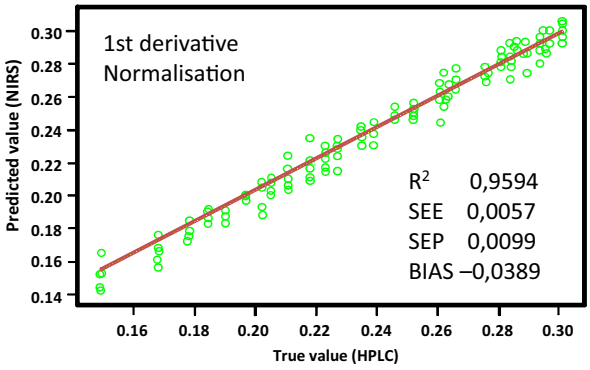
Rooibos: An NIR spectroscopy method in combination with PLS to predict total polyphenols, aspalathin, nothofagin, and dihydrochaclone in dried green rooibos (*Aspalathus linearis*) and aspalathin in water extracts was reported by Manley et al. [37]. The addition of dried rooibos extract powder to some of the samples helped to increase the aspalathin and nothofagin content.

Magnolia officinalis: An NIR spectroscopy method for quantification of phenolic compounds in *M. officinalis* was reported by Yu and coworkers [38]. PLS, mPLS, and PCR algorithms were compared, with mPLS performing best, with reaching correlation coefficients of 0.97 for the calibration and 0.95 for validation.

Primula: A method for controlling the flavonoid content in *Primulae veris flos* was developed by Huck et al. This herbal medicine is used as an expectorant related to its anti-inflammatory properties for the treatment of sinusitis. For the control of the *Primulae veris flos* content, the leading compound 3′,4′,5′-trimethoxyflavone was determined by reversed-phase liquid chromatography (RP-LC) as a reference method. The ethanol/water ratio was controlled simultaneously with the same system with a correlation coefficient of 0.99,530 for water (reference method: Karl-Fischer titration), and a coefficient of 0.99,701 for ethanol (reference method: GC). Validation and results of real samples showed that the robustness and reproducibility of the NIR spectroscopy model for the determination of the 3′,4′,5′-trimethoxyflavone, water, and ethanol content is high (Figure 22.7) [39]. For the identification of *Primulae veris flos* and quantitation of the leading compound, NIR spectroscopy was applied as a detector in TLC [39].

Schoenbichler et al. described a study using NIR and attenuated total-reflectance IR (ATR-IR) spectroscopy in hyphenation with PLSR to determine the antioxidant





Charge	Area (µV*sec)	Primula (g/100g)	Conc. LC (ng/µl)	NIR-MW (ng/µl)	Water (%)	EtOH (%)
91102064 28.10.91	70490	0,493	0,219	0,219	80,38	15,7
91112182 05.11.91	71740	0,500	0,222	0,210	80,83	15,2
91112191 08.11.91	61346	0,442	0,197	0,230	80,25	15,7
91112302 22.11.91	66799	0,472	0,210	0,186	81,33	14,5
91112311 28.11.91	81551	0,555	0,245	0,193	79,69	15,7

FIGURE 22.7 Quantitative analysis of 3′,4′,5′-trimethoxyflavone, water, and ethanol content in a liquid plant extract. SEE, standard error of estimation; SEP, standard error of prediction.

capacity of *Primulae flos cum calycibus* samples [39]. FC, ferric ion reducing antioxidant power (FRAP), 2,2-diphenyl-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and cupric reducing antioxidant capacity (CUPRAC) assays were performed as reference methods. Different spectra pretreatments such as standard normal variate (SNV), first or second derivative were applied to remove scattering effects. The ability of the two spectroscopic techniques to replace the five assays was evaluated and compared. In general, NIR demonstrated advantages over ATR-IR spectroscopy, and had the best results for the ABTS assay (R^2: 0.94, RPD_{corr}: 4.66; test set validation). Also, ATR-IR spectroscopy revealed the best prediction power for the ABTS assay (R^2: 0.94, RPD_{corr}: 4.10; test set validation). The feasibility of vibrational spectroscopy as a fast and simple tool to replace wet chemistry assays for the measurement of the antioxidant capacity of *Primulae flos cum calycibus* samples was demonstrated [39].

St. John's wort: St. John's wort extract is used for treatment of skin injuries, burns, neuralgia, for its bacteriostatic and bactericide activity, and as a treatment for mild to moderate depression. The pharmacological mechanism by which this extract works as an antidepressant is still not discovered. Both hypericin and hyperforin play an important role as standards in the phytopharmaceutical industry. For the investigation of St. John's Wort and its ingredients, different analytical procedures have been established, including IR imaging spectroscopy, UV spectroscopy, fluorescence microscopy, TLC, LC, LC coupled with mass spectrometry (LC-MS), and CE. Prior to spectroscopic analysis via NIR spectroscopy, a reference method based on LC, LC-MS, and CE was established [40]. In the following, 320 spectra of 80 extracts were recorded in transflection mode using light fiber optics over a wavelength range from 4008 to 9996/cm with a resolution of 12/cm at 23 °C and an optical pathway of 1 mm. The most intensive band in the first derivative spectrum belonged to the vibration of the second overtone of the carbonyl group (5352/cm), followed by C−H stretch and C−H deformation vibration, the −OH vibration (4440/cm) and the $-CH_2$ overtone (5742/cm). Five primary factors were necessary to reach the best calibration equation. The robustness of the established NIR spectroscopy model is high, which is demonstrated in the similarity of results for SEE and SEP: 0.52 and 0.50 μg m/L and 0.64 and 0.71 μg m/L for hypericin and hyperforin, respectively (Table 22.1).

Rice grain: Total phenolic and flavonoid contents as well as antioxidant capacity of rice grain was discussed by Zhang et al. applying NIR spectroscopy in combination with multivariate data analysis [41]. The PLS and mPLS algorithms were compared, delivering similar low SEP and correlation coefficients above 0.84 for total phenolics and antioxidant capacity.

Honghua oil: Wu and coworkers [42] reported about MIR and NIR spectroscopic methods for quantitative analysis of the three marker components, α-pinene, methyl salicylate, and eugenol, to assess the quality of honghua oil, a traditional Chinese medicine (TCM) oil preparation, consisting of several plant essential oils.

Bamboo leaves: Flavonoids and phenolic acids in extracts of bamboo leaves were found by Lu et al. [43]. NIR spectroscopy was combined with PLS and least-squares support vector machine (SVM) to establish the corresponding models.

Snow lotus: Chen and coworkers [44] applied NIR spectroscopy to measure the total flavone content in snow lotus (*Saussurea involucrata*) using iPLS with genetic algorithm (iPLS-GA).

Alfalfa: Gonzalez-Martin [45] applied NIR spectroscopy to determine tocopherols in alfalfa. Using mPLS calibration models for 60 fresh and dehydrated samples, SECVs of 0.37 mg/100 g for α-tocopherol (range: 0.55−5.16) and 0.027 for (β + γ)-tocopherol (range: 0.07−0.48) were achieved.

Radix puerariae: Lau et al. [46] determined the puerarin, daidzin, and total flavonoids in *R. puerariae* by calculating PLS regression models. Correlation coefficients in the range 0.939−0.970 were reached using a maximum of five PLS factors.

Ginkgo biloba: Zhou and coworkers [47] reported about NIR spectroscopy in combination with iPLS to determine quercetin in extracts of *G. biloba*.

Cannabis: An NIR spectroscopy method to discriminate between tetrahydrocannabinol (THC)-rich and hemp forms of cannabis was reported by Wilson et al. [48].

Scutellariae radix: This plant, also known as huangqin, is of high pharmacological interest due to the contained flavonoids. About 30 flavonoids were identified and quantified in *Radix scutellariae*, using different analytical techniques such as CE [49], gas chromatography, TLC, ion-pair HPLC, HPLC with UV spectroscopy, high-speed counter-current chromatography (HSCCC), HPLC, and HPLC coupled with MS [50]. The main flavonoids contained in the plant are: wogonin and baicalin; the latter was found in higher amount. Various scientific publications investigated several pharmacological effects of baicalin and its aglycone baicalin as an antitumor agent [51] that can inhibit cancer cell growth or induce apoptosis in breast, prostatic cell lines, and act as anti-inflammatory, antioxidant, and free radical scavenger, as well as an antiviral (HIV), anti-SARS coronavirus agent [52]. Huang and coworkers [53] determined baicalin and total flavonoids in Radix scutellariae measuring 61 samples in DR mode with

TABLE 22.1 Summarized Quality Parameters of the Performed PLSR Calibrations for the Determination of St. John's Wort Ingredients by NIR Compared to MIR-ATR Spectroscopy [61]

	MIR-ATR				NIR			
	Hyperforin	**Hypericin**	**Rutoside**	**Hyperoside**	**Hypertorin**	**Hypericin**	**Rutoside**	**Hyperoside**
Pretreatments	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**
Normalization	1.29	0.00655	1.58	0.249	1.41	0.00418	0.976	0.141
	6	5	3	5	5	7	4	6
	0.914	0.679	0.61	0.757	0.895	0.7	0.856	0.912
	2.75	1.58	1.57	1.96	2.52	2.47	2.54	3.46
SNV	1.24	0.00624	1.54	0.253	1.4	0.0035	0.935	0.133
	7	6	3	4	6	5	4	9
	0.923	0.67	0.631	0.749	0.893	0.82	0.863	0.901
	2.87	1.66	1.61	1.93	2.54	2.95	2.66	3.67
MSG	1.25	0.00629	1.558	0.252	1.53	0.00343	0.976	0.186
	7	6	3	4	4	5	4	6
	0.921	0.667	0.624	0.751	0.856	0.8	0.851	0.829
	2.84	1.64	1.59	1.94	2.32	3.01	2.54	2.62
1st derivative	1.12	0.00794	1.46	0.173	1.39	0.00328	1.55	0.145
	7	6	3	5	3	6	3	4
	0.921	0.454	0.729	0.86	0.874	0.881	0.675	0.915
	3.17	1.30	1.70	2.82	2.56	3.15	1.60	3.37
2nd derivative	1.27	0.00695	1.29	0.169	1.51	0.00565	1.37	0.139
	6	3	7	3	6	6	7	5
	0.91	0.692	0.771	0.887	0.87	0.754	0.706	0.926
	2.80	1.49	1.92	2.89	2.35	1.83	1.81	3.51
3rd derivative	1.50	0.00678	1.34	0.211	1.43	0.00506	1.38	0.115
	3	4	6	3	5	4	6	5
	0.861	0.702	0.729	0.81	0.898	0.788	0.703	0.947
	2.37	1.52	1.85	2.31	2.49	2.04	1.80	4.25
Norm. + 1st der.	1.30	0.00764	1.35	0.17	1.36	0.00402	1.47	0.164
	4	5	3	5	4	4	3	3
	0.915	0.684	0.779	0.846	0.879	0.827	0.711	0.895
	2.73	1.35	1.84	2.87	2.61	2.57	1.69	2.98
SNV + 1st der.	1.19	0.00762	1.35	1.51	1.50	0.0041	1.33	0.186
	5	5	3	4	7	4	7	4
	0.917	0.676	0.779	0.866	0.857	0.763	0.718	0.865
	2.99	1.36	1.84	0.32	2.37	2.52	1.87	2.62
MSG + 1st der.	1.19	0.00754	1.36	0.15	1.51	0.00411	1.32	0.185

Continued

TABLE 22.1 Summarized Quality Parameters of the Performed PLSR Calibrations for the Determination of St. John's Wort Ingredients by NIR Compared to MIR-ATR Spectroscopy [61]—cont'd

	MIR-ATR				NIR			
	Hyperforin	**Hypericin**	**Rutoside**	**Hyperoside**	**Hypertorin**	**Hypericin**	**Rutoside**	**Hyperoside**
Pretreatments	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**	**SEP [%] PCs r^2(val.) RPD**
	5	5	3	4	7	4	6	4
	0.917	0.678	0.777	0.866	0.856	0.757	0.724	0.867
	2.99	1.37	1.83	3.25	2.35	2.51	1.88	2.64
Norm. + 2nd der.	1.36	0.00689	1.02	0.17	1.45	0.00765	1.33	0.168
	6	4	6	4	4	2	6	3
	0.921	0.708	0.925	0.879	0.865	0.528	0.718	0.898
	2.61	1.50	2.43	2.87	2.45	1.35	1.87	2.91
SNV + 2nd der.	1.36	0.00692	0.979	0.159	1.58	0.00309	1.25	0.177
	6	4	6	4	6	6	7	3
	0.915	0.707	0.859	0.894	0.87	0.895	0.751	0.895
	2.61	1.49	2.54	3.07	2.25	3.35	1.99	2.76
MSG + 2nd der.	1.35	0.00689	0.983	0.158	1.59	0.00299	1.25	0.18
	6	4	6	4	6	6	7	3
	0.915	0.71	0.858	0.895	0.868	0.901	0.753	0.892
	2.63	1.50	2.53	3.09	2.24	3.46	1.99	2.71
Norm. + 3rd der.	1.24	0.00602	1.16	0.157	1.46	0.00594	1.37	0.202
	7	5	6	4	5	2	5	2
	0.909	0.753	0.816	0.885	0.901	0.623	0.698	0.882
	2.87	1.72	2.14	3.11	2.43	1.74	1.81	2.42
SNV + 3rd der.	1.21	0.00589	1.13	0.153	1.445	0.00411	1.33	0.122
	7	5	6	4	5	6	6	4
	0.909	0.763	0.815	0.892	0.902	0.813	0.722	0.947
	2.94	1.75	2.20	3.19	2.46	2.51	1.87	4.00
MSG + 3rd der.	1.21	0.00585	1.14	0.151	1.45	0.00411	1.33	0.12
	7	5	6	4	5	6	6	4
	0.909	0.764	0.815	0.895	0.902	0.813	0.72	0.948
	2.94	1.77	2.18	3.23	2.45	2.51	1.87	4.07

SEP, standard error of prediction; SNV standard normal variate; NIR, near-infrared; MIR, mid-infrared; ATR, attenuated total reflection.

baicalin contents ranging from 12.24% to 21.34% and total flavonoid contents ranging from 16.08% to 26.52%. PLS calibration showed correlation coefficients of 0.902 for baicalin and 0.952 for total flavonoids.

22.6.2 Glycoside Compounds

Buckwheat: Tartary buckwheat (*Fagopyrum tartaricum*) was analyzed by Yang and coworkers [54]. They established PLS regression models to quantify the rutin and D-chiro-inositol (DCI) content.

Verbena officinalis: ATR-IR and NIR diffuse reflectance spectroscopy (NIR) in hyphenation with multivariate analysis was used to quantify verbenalin and verbascoside in *V. officinalis* by Schoenbichler et al. [55]. A new HPLC method as a reference was established and validated, being highly suitable as a reference method for calibrating the IR models. For both, vibrational spectroscopic methods test set and cross validation were performed. Different data pretreatments like SNV, first and second derivative were applied to remove systematic errors and were evaluated. Quality parameters obtained for the test set validation revealed that ATR-IR (verbenalin: $R^2 = 0.94$, RPD = 4.23; verbascoside: $R^2 = 0.93$, RPD = 3.63) has advantages over NIR (verbenalin: $R^2 = 0.91$, RPD = 3.75; verbascoside: $R^2 = 0.80$, RPD = 2.35) in the given application.

22.6.3 Ginsenosides

Yap et al. performed simultaneous quantification of ginsenosides Rb1, Rb2, Rc, Re, Rd, Rg1, Ro *m*-Rb1-*m*-Rb2, *m*-Rd, and *m*-Rc in American ginseng. Among the calibration equations for the 11 individual ginsenosides, those of RB1, Re, and *m*-Rb1 showed the lowest relative standard deviation using HPLC as a reference method [56].

22.6.3.1 Glucosinolates

Indian mustard: 2700 winter Indian mustard seeds were analyzed using MPLS as regression method with reported SEP values of 15.65 for glucosinolates [57].

Brassica: The determination of glucosinolates in *Brassica* species [22], including *Brassica napus* L. [58], *pabularia*, *oleracea*, and *juncea* was reported.

22.6.3.2 Essential Oils

Honghua oil: Honghua oil, a TCM oil preparation, is a mixture of several plant essential oils. Gas chromatographic (GC) investigation of 48 commercially available oils was carried out to establish PLS calibrations for the three marker components α-pinene, methyl salicylate, and eugenol with SEP values of 1.55, 0.957, and 0.389%, respectively [42].

22.7 QUALITATIVE ANALYSIS: CLASSIFICATION, DISCRIMINATION AND/OR AUTHENTICATION

NIR spectroscopy shows great potential to use the generated fingerprint spectrum for classification, discrimination, and/or authentication queries [59]. Furthermore, it shows high potential for classifying the origin of natural products and detecting adulteration.

Tea plant: 293 samples from field experiments were analyzed by Xiaoli and Yong [60] and in the following wavelet transformation (WT), PCA and ANN were used to classify the tea samples. The ANN models developed gave good classification accuracy up to 77.3% for the varieties analyzed.

For the fast analytical judgment of green, black, and oolong tea, the combination of NIR spectroscopy with SVM was reported [29]. In this attempt, spectral features of each category can be used to differentiate in the NIR region the three tea varieties. The best classification rates were up to 90, 100, and 93.33%, using the calibration set and 90, 100, and 95% using the validation set, respectively.

St. John's wort: Huck-Pezzei et al. [61] established a procedure to discriminate between pharmaceutical formulations containing either *Hypericum perforatum* or *Hypericum hirsutum* originating from China.

Scutellariae radix: The suitability of NIR to distinguish between 27 cultivated and 22 wild samples collected from nine different regions in China was investigated. Spectral differences between wild and cultivated plants were enhanced after second derivative preprocessing. The most intense band in each spectrum could be assigned to the second overtone of the carbonyl group (5352/cm), followed by the CH stretch and CH deformation vibration (7212/cm), the OH vibration (4440/cm), the CH_2 (5742/cm), and the CH_3 overtone (5808/cm). Furthermore, PCA was used to develop a cluster model for qualitative analysis of wild and cultivated Radix scutellariae (not shown). The total variance explained by the first principal component was 81%. In this study, the best results were obtained when models were based on the 4200−7716/cm spectral region. Second derivative is obviously superior to other pretreatments.

Tanreqing: Tanreqing injection is a widely used patent drug in China. It is made from five kinds of TCM extracts, namely: Radix Scutellariae, *Forsythia suspense*, *Flos lonicerae*, bear gall powder, and *Cornu gorais*. It was used chiefly in treating infection of the upper respiratory tract and serious influenza, and also has satisfactory efficacy in treating severe acute respiratory syndrome (SARS) and A/H1N1 flu. In its manufacturing process, several kinds of intermediates need to be analyzed to ensure that the operation runs steadily. A qualitative model to monitor the quality of

produced batches according to process analytical technology (PAT) guidelines recommended by the United States Food and Drug Administration (FDA) was established by Wenlong et al. [62].

Saffron: An NIR spectroscopy method to determine the chemical composition and geographical origin of 111 samples from the main producing countries, Iran, Greece, and Spain was reported by Zalacain [63]. Compared to UV-VIS and HPLC as reference methods, NIR spectroscopy was found to be superior, following the ISO 3632 Technical Specification Normative.

22.8 IR IMAGING SPECTROSCOPY STUDIES

The goals of plant IR spectroscopic imaging are to determine the severity of plant diseases, detect defects and contaminations, and determine the distribution of certain chemical components such as cellulose, hemicelluloses, lignin, lipids, proteins, and DNA [64]. An approach to understanding the function and the biochemical composition of plant tissue is one aim of imaging studies. IR spectroscopic imaging and mapping technologies have proven to be powerful options for generating new information on plant tissue compositions and alterations. The use of FTIR imaging to evaluate differences in the chemical composition of the *Urtica dioica* root tissue was described by Pallua et al. [64]. Tissue specimens were cut on a microtome at 5-μm thickness and mounted onto CaF_2-slides. FTIR microscopic measurements were performed on an FTIR microscope equipped with a liquid nitrogen-cooled MCT 16-element linear array detector at atmospheric conditions. The samples were measured in MIR transmission mode with a nominal lateral resolution of 25 × 25 μm per pixel for each spot. Absorbance spectra were recorded in the range from 4.000/cm to 750/cm with a spectral resolution of 4/cm with two co-added scans at 1.0 cm/s step size. After measurement, the samples were analyzed by spectra, univariate, and cluster analyses. The analyses were performed using Spectrum software (PerkinElmer, England), Spectrum IMAGE software (PerkinElmer, England) and CytoSpec software package (http://www.cytospec.com, Germany). For the interpretation and calibration of the system, the FTIR-images were correlated with the histomorphological information. With this method, it was shown that it is possible to image different types of ingredients in the root applying a resolution of 25 × 25 μm.

Results presented in Figure 22.8 clearly illustrate the capability of spectroscopic imaging to accurately reproduce tissue histology of *U. dioica* root tissue. The optical image shows different tissue types: plerom tissue, periblem tissue, and rhizoderm tissue. The chemical map of the absorption at 1662–1645/cm, which is commonly attributed to amid-I proteins, represents a homogeneous distribution in all tissue types. This FTIR imaging result clearly demonstrated a very high absorption of this band in the periblem and rhizodermis

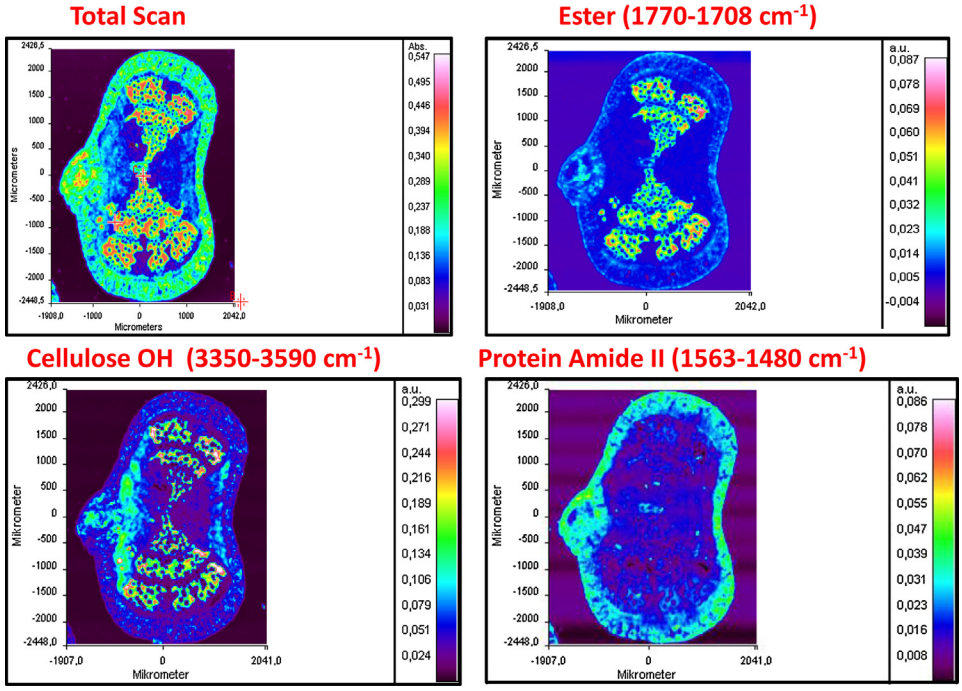


FIGURE 22.8 IR imaging results of *Urtica dioica* samples. IR, infrared.

region, and indicates that these tissue types produce a high amount of amid-II proteins. A chemical map was generated by integrating the area under the band absorption at 1138−988/cm, which is commonly attributed to carbohydrates, nucleic acids, and phospholipids. The chemical map of the absorption at 1160−1140/cm correlated well with the morphology of the plerom region, and indicates that this tissue type produces a high amount of the mentioned ingredients. These observations led to the fact that the plerom tissue produces a high amount of carbohydrates, nucleic acids, and phospholipids compared to periblem or rhizoderm tissue. However, this form of processing cannot do specific correlations with morphological and histological features. Therefore, different cluster analyses are performed to fully characterize the range of spectral variations through the tissue section. Therefore, FCM and KM clustering are the most appropriate methods of choice.

22.9 REGULATORY ISSUES

In 2012, the European Medicine Agency published guidelines on the use of NIR spectroscopy by the pharmaceutical industry and the data requirements for new submissions and variations (EMEA/CHMP/CVMP/QWP/17,760/2009 Rev2; http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/02/WC500122769.pdf). This guideline describes the regulatory requirements for marketing authorization applications and variation applications submitted for medicinal products for human or veterinary use, which include the use of NIR spectroscopy. NIR spectroscopy is described in the European Pharmacopoeia; however, a single reference to the Ph.Eur. general chapter on NIR spectroscopy (Ph.Eur. 2.2.40) as a sole description for the NIR spectroscopic procedure is insufficient to support the use of such a procedure in marketing authorization applications or variation submissions.

This guideline outlines the requirements for applications in which NIR spectroscopy is used for qualitative and quantitative analysis or where it is used as a PAT for monitoring and controlling drug substance synthesis and finished product manufacturing processes.

Acknowledgments

The authors are grateful to Eurasia-Pacific Uninet (EPU) (Salzburg, Austria), the Ministry for Science and Research and the Ministry for Health, Family and Youth (Vienna, Austria) (Novel Analytical Tools for Quality Control in Traditional Chinese Medicine, Project No. 80855) for financial support.

References

[1] Krüger H, Schulz H. Analytical techniques for medicinal and aromatic plants. Stewart Postharvest Rev 2007;3:12.
[2] Schulz H. Analysis of coffee, Tea, Cocoa, Tobacco, Spices, medicinal and aromatic plants, and related products. Roberts CWJRIJ, editor. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America; 2004.
[3] Stecher G, Huck CW, Stöggl WM, Bonn GK. Phytoanalysis: a challenge in phytomics. TrAC Trends Anal Chem 2003;22:1−14.
[4] Williams PC, Norris KH, Sobering DC. Determination of protein and moisture in wheat and barley by near-infrared transmission. J Agric Food Chem 1985;33:239−44.
[5] Blanco M, Coello J, Iturriaga H, Maspoch S, de la Pezuela C. Near-infrared spectroscopy in the pharmaceutical industry. Analyst 1998;123:135R−50R.
[6] Pallua JD, Recheis W, Pöder R, Pfaller K, Pezzei C, Hahn H, et al. Morphological and tissue characterization of the medicinal fungus *Hericium coralloides* by a structural and molecular imaging platform. Analyst 2011;137:1584−95.
[7] McClure WF. 204 years of near infrared technology: 1800−2003. J Near infrared Spectrosc 2003;11:487−518.
[8] Herschel W. Experiments on the refrangibility of the invisible rays of the Sun. Philos Trans R Soc London 1800;90:284−92.
[9] Barton F. Theory and principles of near infrared spectroscopy. Spectrosc Eur 2002;14:12−8.
[10] Osborne BG, Fearn T, Hindle PH. Practical NIR spectroscopy with applications in food and beverage analysis. Longman Scientific and Technical; 1993.
[11] Williams P, Norris K. Near-infrared technology in the agricultural and food industries. American Association of Cereal Chemists, Inc.; 1987.
[12] Faix O. In: Lin SY, Dence CW, editors. Methods in lignin chemistry. Berlin, Heidelberg: Springer Berlin Heidelberg; 1992.
[13] Nicolaï BM, Beullens K, Bobelyn E, Peirs A, Saeys W, Theron KI, et al. Nondestructive measurement of fruit and vegetable quality by means of NIR spectroscopy: a review. Postharvest Biol Technol 2007;46:99−118.
[14] Lewis EN, Treado PJ, Reeder RC, Story GM, Dowrey AE, Marcott C, et al. Fourier transform spectroscopic imaging using an infrared focal-plane array detector. Anal Chem 1995;67:3377−81.
[15] Lewis EN, Levin IW. Real-time, mid-infrared spectroscopic imaging microscopy using indium antimonide focal-plane array detection. Appl Spectrosc 1995;49:672−8.
[16] Salzer R, Siesler HW. Infrared and Raman spectroscopic imaging. Wiley-VCH; 2009.
[17] Pezzei C, Pallua JD, Schaefer G, Seifarth C, Huck-Pezzei V, Bittner LK, et al. Characterization of normal and malignant prostate tissue by Fourier transform infrared microspectroscopy. Mol Biosyst 2010;6:2287−95.
[18] Pallua DJ, Pezzei C, Schaefer G, Zelger B, Brunner A, Kloss-Brandstaetter A, et al. Advanced vibrational spectro scopic imaging of human tissue in life science. Curr Proteomics 2012;9:11.
[19] Gowen A, Odonell C, Cullen P, Downey G, Frias J. Hyperspectral imaging − an emerging process analytical tool for food quality and safety control. Trends Food Sci Technol 2007;18:590−8.
[20] Blanco M, Villarroya I. NIR spectroscopy: a rapid-response analytical tool. TrAC Trends Anal Chem 2002;21:240−50.
[21] Siebert KJ. Chemometrics in brewing-a review. J Am Soc Brew Chem 2001;59:147−56.
[22] Cozzolino D. Near infrared spectroscopy in natural products analysis. Planta Med 2009;75:746−56.

[23] Bittner L, Schönbichler S, Bonn G, Huck C. Near infrared spectroscopy (NIRS) as a tool to analyze phenolic compounds in plants. Curr Anal Chem 2013;9:417–23.
[24] Perron NR, Brumaghim JL. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. Cell Biochem Biophys 2009;53:75–100.
[25] Rauha J-P, Remes S, Heinonen M, Hopia A, Kähkönen M, Kujala T, et al. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. Int J Food Microbiol 2000;56:3–12.
[26] Vijayan P, Raghu C, Ashok G, Dhanaraj SA, Suresh B. Antiviral activity of medicinal plants of Nilgiris. Indian J Med Res 2004;120: 24–9.
[27] Padilla E, Ruiz E, Redondo S, Gordillo-Moscoso A, Slowing K, Tejerina T. Relationship between vasodilation capacity and phenolic content of Spanish wines. Eur J Pharmacol 2005;517: 84–91.
[28] Quansheng C, Jiewen Z, Muhua L, Jianrong C, Jianhua L. Determination of total polyphenols content in green tea using FT-NIR spectroscopy and different PLS algorithms. J Pharm Biomed Anal 2008;46:568–73.
[29] Chen Q, Zhao J, Fang CH, Wang D. Feasibility study on identification of green, black and Oolong teas using near-infrared reflectance spectroscopy based on support vector machine (SVM). Spectrochim Acta Part A 2007;66:568–74.
[30] Luypaert J, Zhang MH, Massart DL. Feasibility study for the use of near infrared spectroscopy in the qualitative and quantitative analysis of green tea, *Camellia sinensis* (L.). Anal Chim Acta 2003;478:303–12.
[31] Zhang MH, Luypaert J, Fernández Pierna JA, Xu QS, Massart DL. Determination of total antioxidant capacity in green tea by near-infrared spectroscopy and multivariate calibration. Talanta 2004; 62:25–35.
[32] Ferrer-Gallego R, Hernández-Hierro JM, Rivas-Gonzalo JC, Escribano-Bailón MT. Determination of phenolic compounds of grape skins during ripening by NIR spectroscopy. LWT – Food Sci Technol 2011;44:847–53.
[33] Ferrer-Gallego R, Hernández-Hierro JM, Rivas-Gonzalo JC, Escribano-Bailón MT. Feasibility study on the use of near infrared spectroscopy to determine flavanols in grape seeds. Talanta 2010; 82:1778–83.
[34] Sinelli N, Spinardi A, Di Egidio V, Mignani I, Casiraghi E. Evaluation of quality and nutraceutical content of blueberries (*Vaccinium corymbosum* L.) by near and mid-infrared spectroscopy. Postharvest Biol Technol 2008;50:31–6.
[35] Gautz LD, Kaufusi P, Jackson MC, Bittenbender HC, Tang C-S. Determination of kavalactones in dried kava (*Piper methysticum*) powder using near-infrared reflectance spectroscopy and partial least-squares regression. J Agric Food Chem 2006;54:6147–52.
[36] Joubert E, Manley M, Botha M. Use of NIRS for quantification of mangiferin and hesperidin contents of dried green honeybush (*Cyclopia genistoides*) plant material. J Agric Food Chem 2006;54: 5279–83.
[37] Joubert E, Manley M, Botha M. Evaluation of spectrophotometric methods for screening of green rooibos (*Aspalathus linearis*) and green honeybush (*Cyclopia genistoides*) extracts for high levels of Bio-active compounds. Phytochem Anal 2008;19:169–78.
[38] Yu C-Y. Quantification of phenolic compound in *Magnolia officinalis* herb by near infrared reflectance spectroscopy. J Zhejiang For Coll 2007;05.
[39] Schönbichler SA, Falser GF, Hussain S, Bittner L, Abel G, Popp M, et al. Comparison of NIR and ATR-IR spectroscopy for the determination of the antioxidant capacity of *Primulae flos cum calycibus*. Anal Methods 2014;6:6343–51.
[40] Huck CW, Abel G, Popp M, Bonn GK. Comparative analysis of naphthodianthrone and phloroglucine derivatives in St. John's Wort extracts by near infrared spectroscopy, high-performance liquid chromatography and capillary electrophoresis. Anal Chim Acta 2006;580:223–30.
[41] Zhang C, Shen Y, Chen J, Xiao P, Bao J. Nondestructive prediction of total phenolics, flavonoid contents, and antioxidant capacity of rice grain using near-infrared spectroscopy. J Agric Food Chem 2008;56:8268–72.
[42] Wu Y-W, Sun S-Q, Zhou Q, Leung H-W. Fourier transform mid-infrared (MIR) and near-infrared (NIR) spectroscopy for rapid quality assessment of Chinese medicine preparation Honghua Oil. J Pharm Biomed Anal 2008;46:498–504.
[43] Lu B, Chen J, Huang W, Wu D, Xu W, Xie Q, et al. Determination of flavonoids and phenolic acids in the extract of bamboo leaves using near-infrared spectroscopy and multivariate calibration. Afr J Biotechnol 2011;10:8448–845.
[44] Chen Q, Jiang P, Zhao J. Measurement of total flavone content in snow lotus (*Saussurea involucrate*) using near infrared spectroscopy combined with interval PLS and genetic algorithm. Spectrochim Acta A Mol Biomol Spectrosc 2010;76:50–5.
[45] González-Martín I, Hernández-Hierro JM, Bustamante-Rangel M, Barros-Ferreiro N. Near-infrared spectroscopy (NIRS) reflectance technology for the determination of tocopherols in alfalfa. Anal Bioanal Chem 2006;386:1553–8.
[46] Lau C-C, Chan C-O, Chau F-T, Mok DK-W. Rapid analysis of *Radix puerariae* by near-infrared spectroscopy. J Chromatogr A 2009; 1216:2130–5.
[47] Zhou X, Xiang B, Wang Z, Zhang M. Determination of quercetin in extracts of *ginkgo biloba* l. Leaves by Near-Infrared reflectance spectroscopy based on interval partial Least-Squares (iPLS) model. Anal Lett 2007;40:3383–91.
[48] Wilson N, Heinrich M. The use of near infrared spectroscopy to discriminate between THC-rich and hemp forms of Cannabis. Planta Med 2006;72:260.
[49] Liu Y-M, Sheu S-J. Determination of the six major flavonoids in Scutellariae Radix by micellar electrokinetic capillary electrophoresis. Anal Chim Acta 1994;288:221–6.
[50] Horvath CR, Martos PA, Saxena PK. Identification and quantification of eight flavones in root and shoot tissues of the medicinal plant Huang-qin (Scutellaria baicalensis Georgi) using high-performance liquid chromatography with diode array and mass spectrometric detection. J Chromatogr A 2005; 1062:199–207.
[51] Motoo Y, Sawabu N. Antitumor effects of saikosaponins, baicalin and baicalein on human hepatoma cell lines. Cancer Lett 1994;86: 91–5.
[52] Cushnie TPT, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents 2005;26:343–56.
[53] Huang Q, Pan R, Wei J, Wu Y, Zhang L. Determination of baicalin and total flavonoids in *Radix scutellariae* by near infrared diffuse reflectance spectroscopy. Spectrosc Spectr Anal 2009;29:2425.
[54] Yang N, Ren G. Application of near-infrared reflectance spectroscopy to the evaluation of rutin and D-chiro-Inositol contents in tartary buckwheat. J Agric Food Chem 2008;56:761–4.
[55] Schönbichler SA, Bittner LKH, Pallua JD, Popp M, Abel G, Bonn GK, et al. Simultaneous quantification of verbenalin and verbascoside in *Verbena officinalis* by ATR-IR and NIR spectroscopy. J Pharm Biomed Anal 2013;84:97–102.
[56] Yap KY-L, Chan SY, Weng Chan Y, Sing Lim C. Overview on the analytical tools for quality control of natural product-based supplements: a case study of ginseng. Assay Drug Dev Technol 2005;3:683–99.

[57] Font R, Del Rio-Clestino M, Rosa E, Aires A, De Haro-Bailon A. Glucosinolate assessment in *Brassica oleracea* leaves by near-infrared spectroscopy. J Agric Sci 2005;143:65–73.
[58] Bala M, Singh M. Non destructive estimation of total phenol and crude fiber content in intact seeds of rapeseed–mustard using FTNIR. Ind Crops Prod 2013;42:357–62.
[59] Cordella C, Moussa I, Martel A-C, Sbirrazzuoli N, Lizzani-Cuvelier L. Recent developments in food characterization and adulteration detection: technique-oriented perspectives. J Agric Food Chem 2002;50:1751–64.
[60] He Y, Li X, Deng X. Discrimination of varieties of tea using near infrared spectroscopy by principal component analysis and BP model. J Food Eng 2007;79:1238–42.
[61] Huck-Pezzei VA, Bittner LK, Pallua JD, Sonderegger H, Abel G, Popp M, Bonn GK, Huck CW. A chromatographic and spectroscopic analytical platform for the characterization of St John's wort extract adulterations. Anal Methods 2013;6:616–28.
[62] Li W, Xing L, Fang L, Wang J, Qu H. Application of near infrared spectroscopy for rapid analysis of intermediates of Tanreqing injection. J Pharm Biomed Anal 2010;53:350–8.
[63] Zalacain A, Ordoudi SA, Díaz-Plaza EM, Carmona M, Blázquez I, Tsimidou MZ, et al. Near-infrared spectroscopy in saffron quality control: determination of chemical composition and geographical origin. J Agric Food Chem 2005;53: 9337–41.
[64] Pallua JD, Pezzei C, Huck-Pezzei VA, Schonbichler SK, Bittner LK, Bonn G, et al. Advances of infrared spectroscopic imaging and mapping technologies of plant material. Curr Bioact Compd 2011;7:12.

LIST OF ABBREVIATIONS

ATR Attenuated total reflection
BaF_2 Barium fluoride
CaF_2 Calcium fluoride
DR Diffuse reflection
FCM Fuzzy C-means clustering
FIR Far infrared
FPA Focal plane array
FT Fourier Transform
FTIR Fourier Transform Infrared
GC–MS Gas chromatography–Mass spectrometry
HCA Hierarchical cluster analysis
InSb Indium antimonide
InGaAs Indium gallium arsenide
KMC K-Means clustering
MALDI Matrix assisted laser desorption/ionization
MCT Mercury cadmium telluride
MIA Multivariate imaging analysis
MIR Mid-infrared
MS Mass spectrometry
NIR Near infrared
PbS Lead sulfide
PCA Principal component analysis
PLS Partial least square
RPD Ratio of performance to deviation
UV Ultraviolet
ZnSe Zinc selenide

This page intentionally left blank

CHAPTER

23

Antimicrobial Secondary Metabolites—Extraction, Isolation, Identification, and Bioassay

Mukhlesur Rahman

Medicine Research Group, School of Health, Sports and Bioscience, University of East London, Stratford Campus, London, UK

OUTLINE

23.1 INTRODUCTION

The concept of antibiotics originates from "antibiosis," which is considered as an association of two or more organisms with at least one being detrimental to the other(s). Thus, antibiosis reflects on the presence of the organism that is capable of producing harmful effects to the other organism(s). Although antibiotics are literally used to describe chemical compounds that are responsible for the inhibition of the growth of or the killing of bacteria with minimum or no harm to the host, a broader term "antimicrobial agents" is used to

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00023-4

include antibacterial, antifungal, antiprotozoal, and antiviral agents. Whatever the terms are used for a proper definition, the ultimate aim is to get rid of any kind of infections caused by microorganisms. Moreover, another term "antiinfective agent" is also used worldwide in antimicrobial drug discovery research. Antiinfective drug discovery research emphasizes on systematic approaches for the isolation and identification of lead compounds that might have the capability of inhibiting the growth of (static) or killing (cidal) a microorganism responsible for a particular type of infection. Bioassay-directed search for secondary metabolites from natural sources covering plants, microbes, marine organisms, and animals as well as synthesis of lead natural product analogs are the main focus of antiinfective drug discovery research.

Although the antibiotic era started with the accidental discovery of penicillin by Alexander Fleming in 1928, there has been evidence of using indigenous herbs for centuries for the treatment of infections without the necessity of having any scientific knowledge. Some of the obvious examples of herbs used for the treatment of infectious diseases are moldy soybean curd and honey. The ancient Chinese used moldy soybean curd for the treatment of boils and controlled foot infections by wearing sandals that were furry with mold [1]. In the middle ages, honey was used for the treatment of infections associated with postarrow wounds [2].

In 1877, Pasteur and Joubert noticed that *Anthrax bacilli* were killed when the culture became contaminated with other bacteria. In 1901, rabbits were protected from anthrax when a liquid culture of *Pseudomonas aeruginosa* was injected. Such activities were due to the production of metabolites by one organism that inhibited the growth of other organisms [1]. In the early 1900s, Paul Ehrlich, a Nobel Prize winner, for his contribution to immunology, further explored the concept of antibiosis [2]. This idea was well known as the magic bullet. In 1910, Ehrlich synthesized an arsenic-containing chemical compound known as salvarsan (Figure 23.1). This became the choice of medicine for the treatment of infectious diseases including syphilis and trypanosomiasis [2] until it was replaced by penicillin in 1945. In 1934, proflavine (Figure 23.1) was introduced, which was capable of fighting infections associated with deep surface wounds during World War II [2]. Because of their high toxicity, there was an urgent need for new antibiotics to combat infection during World War II. The discovery of prontosil in 1935 was a real breakthrough for the treatment of systematic infections [2]. Prontosil (Figure 23.1), a sulfur-containing prodrug, was used effectively until the availability of penicillin in early 1940s. Fleming grew the mold in a pure culture and noticed that it produced a substance that was beneficial against a number

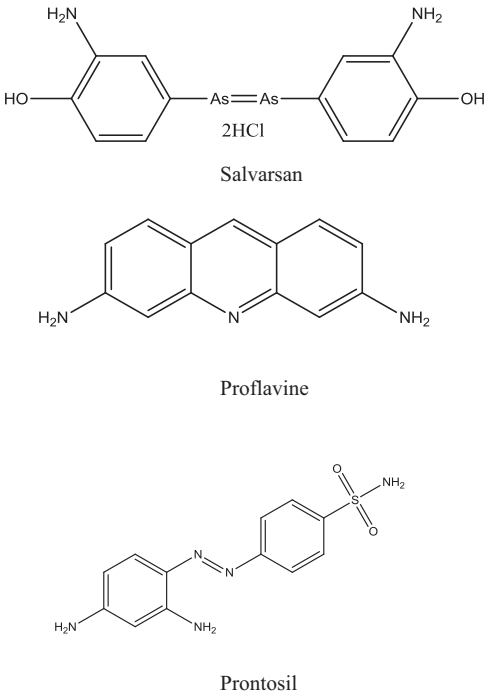
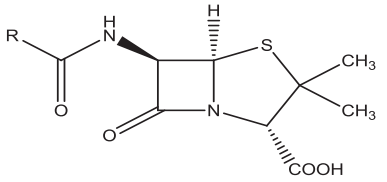


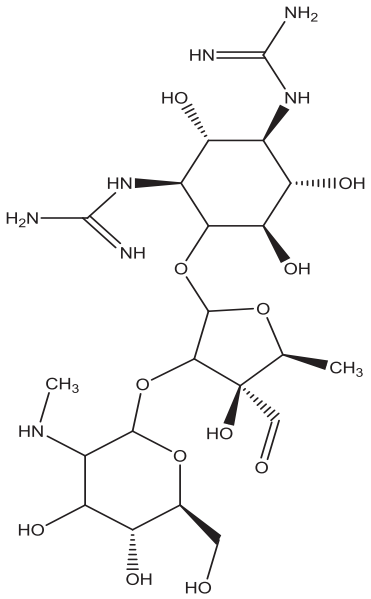
FIGURE 23.1 Structures of salvarsan, proflavine, and prontosil—antimicrobial agents used before the discovery of penicillin.

of diseases that were caused by bacteria. He identified the mold as *Penicillium notatum* and named the metabolite as "penicillin," which inhibited the growth of bacteria [3]. Penicillin (Figure 23.2), a fungal metabolite, became the choice of drug to fight bacterial infections during that period.

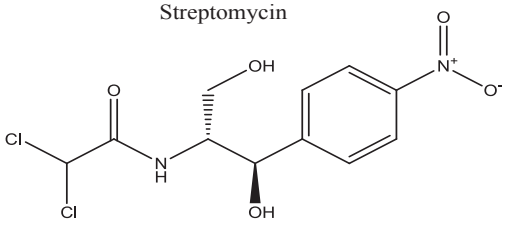
The outcome of such research led to the discovery of a number of natural antibiotics, such as streptomycin (Figure 23.2), from an actinomycete, *Streptomyces griseus* [4]. Chloramphenicol (Figure 23.2), an orally active broad spectrum antibiotic, was discovered in 1947 from *Streptomyces venezuelae*, another actinomycete from soil sample [5]. A golden actinomycete, *Streptomyces aureofaciens*, produced chlortetracycline [6], which was revealed to be a broad spectrum antibiotic with a therapeutic efficiency similar to that of chloramphenicol (Figure 23.2) but with no activity against tuberculosis. Erythromycin (Figure 23.2) [7], the first macrolide antibiotic, was isolated from a strain of *Streptomyces erythreus* in 1952, which was mainly used in the treatment of Gram-positive infections of the skin and respiratory tract. Vancomycin, a cyclic peptide antibiotic, with effectiveness against both aerobic and nonaerobic Gram-positive bacteria, was isolated from *Streptomyces orientalis* in 1955 [8], but its chemical structure was confirmed in 1981 [9]. The search for new antibiotics continued and led to the discovery of different classes of antibiotics including natural, semisynthetic, and synthetic compounds.



Penicillin (Penicillin G, R=C_6H_5-CH_2; Penicillin V, R=C_6H_5-O-CH_2)

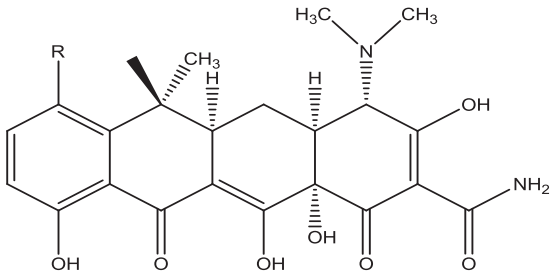


Streptomycin

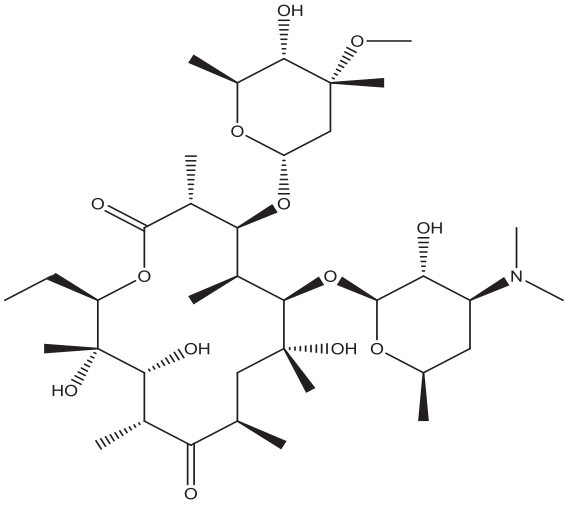


Chloramphenicol

FIGURE 23.2 Structures of some antibiotics—penicillin, streptomycin, chloramphenicol, tetracycline, and erythromycin.



Tetracyclin, R=H; Chlortetracyclin, R=Cl



Erythromycin

FIGURE 23.2 cont'd.

23.2 EXTRACTION AND ISOLATION OF METABOLITES FOR ANTIMICROBIAL POTENTIALS

The main natural source of antimicrobial agents is microbes. However, plants, marine organisms, and some animals are also important natural sources for producing antimicrobial secondary metabolites. Extraction is the one of the earlier stages for any kind of drug discovery programs from natural resources including plants, microbes, marine organisms, and animal toxins. A general term "biomass" is used to describe plants, microbes, marine organisms, and animal toxins. Extraction depends on the nature of biomass. When we focus on the antimicrobial agents from microbial source, the microorganism of interest is normally grown in large vessels containing an appropriate growth medium followed by incubation with continuous shaking at an appropriate temperature for a certain period of time. After completion of fermentation, the microbial cells are normally freeze dried and kept in appropriate containers and stored in the freezer until extraction. In the case of plants, we rely on the local knowledge for any type of drug discovery research. Traditional healers provide information about medicinal plants that are used or prescribed by them for the treatment of infectious diseases. Once such plant has been selected for a drug discovery research project, the first step is to get the plant identified by a field taxonomist followed by collection of a voucher specimen in a well-recognized herbarium. The next step is to grind the plant material into powder using a suitable blender or miller followed by extraction. The choice of the solvent is an important factor in the extraction, which depends on the nature of the chemicals present in the biomass. For example, nonpolar solvents, such as hexane or petroleum ether, can easily extract volatile components such as monoterpenes or sesquiterpenes, whereas polar solvents, such as water or methanol, favor the extraction of polar components such as glycosides. Several

methods have been developed for the extraction of biomass in drug discovery approaches from natural resources. Among these methods, cold extraction is preferred for biomass which might contain volatile components or heat-sensitive substances, whereas hot extraction using a Soxhlet apparatus offers the advantage of being a more efficient method but suffers from the drawback of its unsuitability for thermolabile substances. Supercritical fluid extraction, a relatively newer extraction method, applies the principle that some gases near the critical point behave like liquids and have solvating properties [10]. The most widely used supercritical fluid is carbon dioxide (CO_2), which replaces the use of nonpolar organic solvents, such as hexane and dichloromethane, for the extraction of lipophilic components. Addition of a modifier such as methanol to CO_2 enables the extraction of hydrophilic components from the biomass.

In bioassay-directed approaches, crude extracts are subjected to an initial screening for bioactivity (e.g., antibacterial and antifungal) followed by fractionation of active extracts by flash chromatography or vacuum liquid chromatography (VLC) [11,12], and further purification by suitable chromatographic techniques. Analytical thin layer chromatography (TLC) [13,14] is widely used for the initial screening of extracts and/or fractions and also to ensure the purity of compounds. To isolate and purify secondary metabolites from crude extracts, there are numerous chromatographic methods available that are used widely in both academic and industrial research in drug discovery [11–14]. Among these, gel filtration or size exclusion chromatography is very useful for the removal of pigments (i.e., chlorophyll) and the separation of components of crude extracts or VLC fractions based on molecular size, and Sephadex LH20 is used as the stationary phase in size exclusion chromatography. Although traditional column chromatography (CC) is also in use, solid phase extraction (SPE) using either normal or reversed phase silica (C-18) is well accepted for both fractionation and purification. High-performance liquid chromatography (HPLC) is a modern, sophisticated, and very powerful separation technique for the analysis and final purification of compounds. HPLC is used as the final chromatographic method for the purification of polar compounds from bioactive fractions that have been obtained by VLC, SPE, gel filtration, or CC.

23.3 IDENTIFICATION OF COMPOUNDS

Once the compounds have been isolated and purified by a combination of chromatographic techniques, a number of spectroscopic methods are employed for the confirmation of the structures of the isolated compounds. Although mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are key spectroscopic methods for the confirmation of structures, infrared (IR) and ultraviolet–visible (UV–Vis) spectra support the structural features by identifying the presence of certain functional groups (IR) and the degree of conjugation (UV) in the molecules, respectively.

23.3.1 UV–Vis Spectroscopy

UV–Vis spectra of the samples are recorded on a UV–Vis spectrophotometer. The samples are normally dissolved in methanol and are then scanned to know the λ_{max} and absorption coefficient in a particular concentration. Although UV spectroscopy is a very useful tool for the quantitative estimation of analytes or active pharmaceutical ingredients in the formulation, it has a very limited role in the structural elucidation of natural products. It only provides the indication of the degree of conjugation (chromophore) in the molecule.

23.3.2 IR Spectroscopy

IR spectra are usually recorded on a Fourier transform IR spectrophotometer using KBr discs or dry films. IR spectroscopy helps in structural elucidation by revealing the presence of certain functional groups (e.g., -OH, CO, -COOH, $-NH_2$, $-CONH_2$, and arenes) present in the molecules.

23.3.3 Mass Spectrometry

MS is an analytical technique that allows the measurement of the molecular weight of the compound. There are several ionization techniques available in MS including atmospheric pressure chemical ionization, chemical ionization, electron impact, electrospray ionization, fast atom bombardment, field desorption/field ionization, and matrix-assisted laser desorption ionization. In the case of small bioactive "organic molecules," it is possible to measure the molecular mass within an accuracy of $\leq$5 ppm by high-resolution electron impact mass spectra. The fragmentation patterns as exhibited in the mass spectra are useful in building the structure of an organic compound. Hyphenated techniques, such as liquid chromatography mass spectrometry and gas chromatography mass spectrometry, are widely used for the identification of known compounds.

23.3.4 Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy is extensively used to characterize isolated compounds. NMR is one of the most powerful

tools for structural assignments of organic compounds. The Pulse-Fourier-transform technique has greatly increased the efficiency and sensitivity of NMR spectra and has made it possible to obtain the spectra of low-abundance nuclei, particularly those of ^{13}C.

The 2D NMR techniques have facilitated the unambiguous assignment of the structures of organic compounds. The simplest method for obtaining an NMR spectrum by the Pulse-Fourier-transform technique involves the irradiation of sample with a single radiofrequency pulse of a few microsecond duration [15], which is normally short enough to excite all nuclei of a given magnetic isotope to result in an emission of signal from the excited nuclei. This signal is called free induction decay (FID), which is converted into an NMR spectrum by Fourier transformation. The key NMR techniques that are widely used for the structural elucidation of natural products have been outlined briefly in this section.

23.3.4.1 ^{1}H NMR Spectroscopy

This is the first and fundamental NMR experiment for the structural elucidation of organic compounds. The development of a highly sensitive instrument (400−800 MHz) allows the measurement of the ^{1}H NMR spectrum of a smaller amount of samples within a few minutes. Such a spectrum provides the following information:

1. the number of signals, that is, how many different types of protons are present in a molecule;
2. the chemical shifts, that is, the electronic environment of each set of protons;
3. the intensities of signals, that is, how many protons exist in each signal;
4. the splitting pattern of a signal, that is, the environment of a proton with respect to its neighbors.

23.3.4.2 Broad Band Decoupled ^{13}C NMR Spectroscopy

This is a quick method utilizing a very short relaxation time (<1 s). This technique is particularly useful for smaller amounts of samples and is the method of choice when any quaternary carbon peaks are missed in the *J*-modulated spectrum. But, all carbons (C, CH, CH_2, and CH_3) appear on the same side, which makes it difficult to differentiate among different carbon types present in the molecule.

23.3.4.3 J-Modulated ^{13}C NMR Spectroscopy

This technique (also known as the attached proton test) is also used for the study for ^{13}C NMR. In this experiment, resonances from ^{13}C nuclei of CH_3 and CH appear to be inverted with respect to those from CH_2 and quaternary carbons. This is achieved by simultaneously applying a proton 180° pulse with the carbon 180° pulse, by using a delay time of $\frac{1}{2}J$ seconds and by utilizing broad band decoupling while acquiring the FID [16]. The main advantage of this technique over the conventional Broad Band Decoupling ^{13}C spectrum is its simplicity to differentiate between C/CH_2 and CH/CH_3 carbons. However, it suffers from the disadvantage of having less sensitivity over broad band-decoupled ^{13}C NMR spectroscopy, and in some cases, quaternary carbons may disappear. However, quaternary carbons can be seen by increasing the relaxation delay to 6−10 s.

23.3.4.4 Distortionless Enhancement by Polarization Transfer-135

Distortionless enhancement by polarization transfer-135 spectra [17] differentiate methylene peaks from methyl and methine peaks. Quaternary carbons do not appear in this spectrum, and thus, they help to separate C and CH_2 peaks in a *J*-modulated experiment. This is therefore very useful in the structural elucidation of terpenoids [18] in confirming CH_2 peaks. As the carbon in a solvent is quaternary, it also disappears; it is thus essential to calibrate the spectrum by using the same solvent reference value or a carbon chemical shift from another ^{13}C (e.g., *J*-modulated) experiment.

23.3.4.5 Homonuclear Correlation Spectroscopy

Experiments in homonuclear correlation spectroscopy tell us how the different components of the same nuclei (^{1}H) are related to one another through scalar or spatial coupling. ^{1}H-^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and Nuclear Overhauser Enhancement Spectroscopy (NOESY) are the most commonly used homonuclear correlation spectroscopic experiments.

23.3.4.5.1 $^{1}H-^{1}H$ Correlation Spectroscopy

Among the 2D NMR experiments, the ^{1}H-^{1}H COSY experiment provides information about protons that are coupled to each other. Basically, the COSY experiment is the process of coherence transfer in which magnetization is transferred between coupled spins [19]. It consists of two 90° pulses separated by a time delay. After the second pulse, an exchange of magnetization owing to scalar coupling (coherence transfer) between spins may occur. Fourier transformation of the FID presents a COSY spectrum showing crosspeaks that connect the coupled nuclei.

The COSY-45 experiment consists of a 45° pulse in place of the second 90° pulse. Thus, a simplified spectrum is obtained by reduction of the crosspeaks within multiplets such as CH_2. Thus, it is useful in a complex spectrum in identifying the correlations that would otherwise be hidden in the clutter of peaks close to the diagonal [20]. The COSY-lr is another homonuclear 2D

NMR experiment using a relatively long delay and thereby enhances the relative intensity of crosspeaks from long range coupling [21]. Usually, the delay time is set at 0.3 s. The COSY experiment gives rise to coupling information among the protons that are directly coupled to other protons. Such coupling can be through two, three, and sometimes four bonds. The diagonal gives the correlations (couplings) of signals among themselves, while identical information is shown on either side of the diagonal. So, one side of the diagonal is required to be taken into consideration for interpretation.

23.3.4.5.2 $^{1}H-^{1}H$ Total Correlation Spectroscopy

Although TOCSY [22] is very similar to COSY in its basic principle, it provides information on the crosspeaks for all protons that are part of the same spin system. For example, the COSY spectra in the case of an ABCD system of four protons reveal crosspeaks for "A to B," "B to C," and "C to D," whereas the TOCSY spectra show an additional crosspeaks for "A to C," "A to D," and "B to D." This means that the TOCSY spectrum reveals a total correlation among the ^{1}H values within the same spin system. So TOCSY is very useful for the identification of natural peptide products as it reveals strong interactions among the spin systems of each amino acid.

23.3.4.5.3 $^{1}H-^{1}H$ Nuclear Overhauser Enhancement Spectroscopy

This is useful for nuclei that are close in spatial proximity within the molecule. It looks like a COSY spectrum, but the crosspeaks are now evidence of a through spatial dipolar interaction instead of a scalar coupling through bonds. In a 2D NOE (NOESY) experiment, the nuclei are excited at the same time and nonselective pulses are labeled by their precessional frequencies. This frequency labeling is equivalent to selectively exciting each of the nuclei and separately measures each pairwise interaction [23]. The NOESY experiment consists of three 90° pulses and requires one to incorporate a delay time known as mixing time before the third 90° pulse. The mixing time is very important in the NOESY experiment and depends upon the properties of the molecules under investigation. It is assumed that small molecules require a long mixing time (1−5 s), and large molecules require a shorter time (0.1−0.5 s). However, this is not true in all cases. In this study, a mixing time of 0.5 or 1 s was normally used. Sometimes, it was changed further depending upon the spectra desired.

23.3.4.6 Heteronuclear Correlation Spectroscopy

These 2D NMR experiments show how different species of nuclei, most commonly, ^{1}H and ^{13}C, are related through scalar coupling. The heteronuclear multiple quantum coherence, heteronuclear single quantum correlation, and HC-COBIDEC [24] experiments show ^{1}J or direct $^{1}H-^{13}C$ couplings, whereas long range (^{2}J, ^{3}J, etc.) heteronuclear ($^{1}H-X-^{13}C-^{13}C$) couplings are observed from heteronuclear multiple bond coherence (HMBC) experiments [25].

23.3.4.6.1 Heteronuclear Multiple Quantum Coherence

Heteronuclear multiple quantum coherence (HMQC) is also known as heteronuclear single quantum correlation (HSQC). This heteronuclear 2D experiment shows crosspeaks that reveal the direct (^{1}J) correlation between ^{1}H and ^{13}C nuclei. In this experiment, the ^{1}H spectrum is plotted on the *x*-axis, and ^{13}C spectrum is plotted on the *y*-axis, whereas there is no spectrum on the diagonal. As the ^{1}H NMR spectrum can directly be correlated to ^{13}C NMR in the HMQC, this experiment is very useful in assigning both ^{1}H and ^{13}C values and in thereby facilitating structural elucidation. Both HMQC and HSQC allow the correlation to be made between ^{1}H and ^{13}C nuclei by using a pulse sequence with a delay time set to $\frac{1}{2}J$ seconds. These experiments suffer from a drawback of noise artifacts arising from intense protons peaks such as methyl groups.

23.3.4.6.2 Heteronuclear Multiple Bond Coherence

HMBC spectroscopy correlates ^{1}H and ^{13}C nuclei through two, three, or sometimes four bonds. Normally, a variable delay time $\left(\frac{1}{2}J\right)$ is set for 70 msec, which optimizes ^{3}J crosspeaks that are more prominent than those for ^{2}J. However, it is possible to make ^{2}J crosspeaks more prominent and even ^{4}J peaks visible by changing the value of delay time. Sometimes, in HMBC experiments, direct C-H couplings appear with a magnitude of J_{CH} being the equal distance between crosspeaks (the so-called ^{13}C satellite). The HMBC experiment provides a wealth of structural information that is particularly helpful in the structural elucidation of complex compounds. It plays a key role by connecting various fragments of a big molecule through their carbon−proton coupling via two, three, or more bonds and thereby confirms the structure of the molecule. As in HMQC, HMBC spectra exhibit ^{1}H spectra on the x-axis and ^{13}C on the *y*-axis, and there are no spectra on the diagonal.

23.4 BIOASSAY: METHODS COMMONLY USED FOR THE SCREENING OF ANTIMICROBIAL ACTIVITY

Among the commonly used techniques for the screening of extracts, fractions, and compounds for antimicrobial properties are disc diffusion, bioautography,

and broth dilution (also known microdilution titer) assay. These methods have been used for many years in research to find antimicrobial compounds from both natural and synthetic sources. However, recent drug discovery approaches utilize high-throughput screening (HTS) to find lead compounds by screening hundreds of samples in several in vitro testing methods within a short time.

23.4.1 Disc Diffusion Method

The disc diffusion method [26] involves pouring a sterile nutrient broth medium into a petridish in a laminar flow cabinet. Once the medium has settled down into a completely flat surface, the test organism is seeded over the solidified medium. The material to be tested is dissolved in small amounts of organic solvent and applied to a paper disc. The solvent is allowed to evaporate in the laminar flow cabinet. The disc is then placed on the petridish and allowed to incubate overnight at 37 °C. The antimicrobial activity is noted by measuring the diameter of zones of inhibition in millimeters. This technique is easy to perform and relatively inexpensive. No special equipment is needed. However, it suffers from the disadvantage of the inability of nonpolar compounds to diffuse from the disc to the broth.

23.4.2 Bioautography

Bioautography [27] is particularly useful to find out active compounds in a crude extract or fractions. The extract or fraction is spotted on a TLC plate and allowed to run using a suitable mobile phase that offers a reasonable resolution of the compounds. Once the TLC has been developed, the compounds on the TLC plate are marked. The TLC plate is then placed in contact with an agar plate seeded with an organism followed by incubation overnight at 37 °C. The activity is expressed in terms of the zone of inhibition. This method requires using special microbiological equipment. It also requires overnight incubation to get the results.

23.4.3 Broth Dilution or Microdilution Titer Assay

Broth dilution or microdilution titer assay is a quantitative method that is widely used in antimicrobial research as it offers the measurement of minimum inhibitory concentrations (MICs) of test compounds. This method requires 96 (12X8) well plates. In this method, an adequate amount (say 100 μL) of Muller Hinton broth is dispensed into wells of columns 1–11 using a multichannel pipette. The samples are prepared by dissolving extracts or compounds in water or in dimethyl sulfoxide (DMSO) depending on their solubility. The samples are further diluted with Muller Hinton broth to ensure a lower content (<4%) of DMSO. The antibiotic is prepared in a similar way. Samples and antibiotic (say 100 μL) are then dispensed into the wells of column 1, which are mixed thoroughly by using a multichannel pipette filler followed by transferring its half of its content (100 μL) to the second column and so on up to column 10 and then column 12 leaving column 11 as the growth control. Bacterial suspensions (100 μL) containing standard numbers of bacteria are added into the columns 1–11 of 96-well plates and allowed to incubate overnight at 37 °C. The MIC is recorded as the lowest concentration where no viability is observed in the wells of 96-microwell plates after incubation. A color indicator such as resazurin [28] or methyl thiazolyldiphenyl-tetrazolium bromide (MTT) [29] when added reacts with bacteria and changes the color from pink (resazurin) or yellow (MTT) to dark blue. Thus, such color changes in the 96-well plates confirm the measurement of MIC of compounds. This technique is cheap, very easy to set up, and rapid and it requires small amounts of samples. So it is suitable for the testing of phytochemicals.

23.4.4 High-Throughput Screening

HTS, a recent method of in vitro testing using robotics and miniaturization, allows one to test thousands of samples of a very small volume in order to identify potential lead compounds in drug discovery. In general, this involves the screening of a large number (up to several thousands) of compounds against several targets using an automatic system. Typically, several thousands of compounds with tiny amounts can be tested at a time against 30–50 biochemical tests.

Along with other in vitro tests, HTS for antimicrobial testing has been well adapted to find lead compounds with potential antibacterial activity. Although many of the current in vitro assays for drug testing against *Mycobacterium tuberculosis* are not well adapted for HTS [30], Ananthan and coworkers [31] have recently described HTS for antituberculosis testing. They have used the Alamar blue assay [32] for the 384-well plate format screening of a 100,997 compound library for antitubercular activity against *M. tuberculosis* on a BSL3-contained HTS platform. Recently, Schmitt and coauthors [33] have outlined HTS using nL-reactor to search for antimicrobial peptides. During the experiment, they have used nL-sized reaction vessels for both peptide production and antimicrobial screening in a single step at a rate of 10^5 variants per day. This method involves the use of large particle flow cytometry and fluorescently labeled cells to find out potential lead antimicrobial agents.

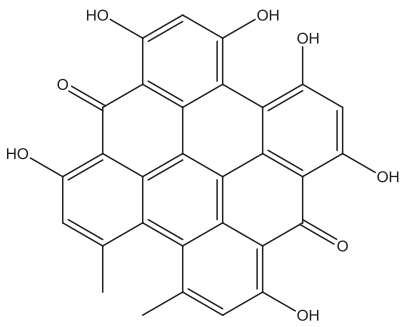
Although HTS offers the advantages of finding hits with activity even in the range of 30 to 1 nM, it suffers the drawback of generating many false positive hits. So there is a high failure rate between the number of hits and the number of authentic lead compounds.

23.5 ANTIMICROBIAL SECONDARY METABOLITES

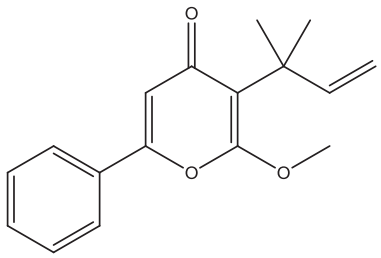
23.5.1 Plant-Derived Antimicrobial Secondary Metabolites

Plants have been well reported for the production of lead biologically active secondary metabolites including anticancer agents such as taxol [34]; antimalarial drugs, such as artemisinin [35]; narcotic analgesic drug, such as morphine [2]; and cardioactive agents, such as digoxin [36]. This natural resource has been underexploited for the search of lead antimicrobial metabolites although there are numerous reports of plants being used as antimicrobial agents in traditional Ayurvedic medicine [37], Chinese medicine [38], and even in western Herbal medicine [39] as plants are capable of acting against bacteria and fungi because of their self-protection strategy in their environment [40]. To find out 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 of both academic institutes and pharmaceutical industries in recent years because of the development of resistance to existing antibiotics. Research focused on various medicinal plants has led to the isolation and identification of a wide range of compounds with structural diversity. This section covers some plant-derived compounds with potential antimicrobial activity.

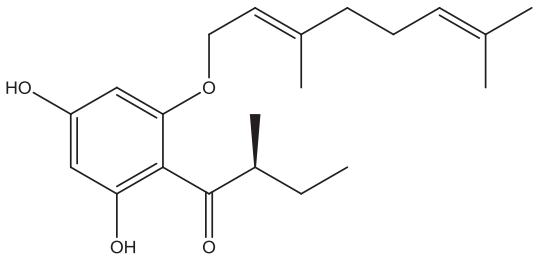
St John's Wort (*Hypericum perforatum*), a medicinal plant being used widely as an antidepressant in herbal medicine, has been reported to produce a major antibacterial metabolite called hypericin (Figure 23.3) with MIC values of 0.1 μg/mL against methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant variants [41]. Professor Simon Gibbons [42] has done extensive work on the genus *Hypericum*, by exploring this genus as a lead source of antibacterial secondary metabolites with potential activity against *S. aureus*. Bioassay-guided research on some *Hypericum* species led to the isolation of new acylphloroglucinols [29,43,44], norlignans [45], and xanthone [45] with potential activity against *S. aureus*. An acylphloglucinol, (*S*)-4,6-dihydroxy-2-*O*-(3″,7″-dimethyl-2″,6″-octadienyl)-1-(2′-methylbutanoyl)benzene (trivial name olympicin A; Figure 23.3), reported from *Hypericum olympicum*, revealed very potent (MICs of 1 μg/mL) activity against



Hypericin



Hyperinone A



Olympicin A

FIGURE 23.3 Hypericin, hyperinone A, and olympicin A—antibacterial compounds from *Hypericum*.

some clinical isolates of multidrug-resistant (MDR) and MRSA [43]. Hyperenone A (Figure 23.3), a constituent of *Hypericum acmosepalum* [44], revealed antibacterial activity against *Staphylococcus aureus* and *M. tuberculosis* and also inhibited the adenosine triphosphate-dependent MurE ligase of *M. tuberculosis*, a crucial enzyme for peptidoglycan biosynthesis.

Aromatic medicinal plants, such as cinnamon, clove, cilantro, coriander, fennel, oregano, peppermint, rosemary, sage, thyme, and lavender, have been well documented for producing essential oils. Essential oils are well known to inhibit the growth of microorganisms [46]. Cinnamon oil and clove oil are considered as natural preservatives and flavoring substances that are not harmful when consumed in food products. The essential oil constituents present in cinnamon, *Cinnamomum*

zeylandicum, and clove, *Syzgium 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 (PDA) 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* for a period of up to 21 days [47]. Both oils have also been documented for the inhibition of the growth of yeasts [48]. Another report by Suksrikarm [49] demonstrated that cinnamon oil and clove oil could separately have the capability of inhibiting 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. According to Soliman and Badeaa [50], 500 ppm of cinnamon oil was capable of inhibiting the growth of *A. flavus*, *A. parasiticus*, *A. ochraceus*, and *Fusarium moniliforme* on PDA. Another report by August [51] stated that high concentrations of cinnamon oil and clove oil could also be capable of inhibiting the asexual spores of fungi. Eugenol (75–85%; Figure 23.4), eugenol acetate (8–15%), and β-carryophyllene are the main components of clove oil [52], whereas cinnamon oil's key component is *trans*-cinnamaldehyde (Figure 23.4). Eugenol has been used widely in perfumeries as flavoring agents, and also as its analgesic, local anesthetic, antiinflammatory, and antibacterial agents [53,54]. It is used in the form of a paste or mixture as dental cement, filler, and restorative material local antiseptic and anesthetic in dentistry. Clove has also been reported to have strong antibacterial activity against *S. aureus* (MIC = 0.4–2.5 μL/mL), *Escherichia coli* (MIC = 0.4–2.5 μL/mL), and *Listeria monocytogenes* (MIC = 0.3 μL/mL) [55].

The antibacterial activity of sage, *Salvia officinalis*, has been reported against *S. aureus* (MIC = 0.75–10 μL/mL), *E. coli* (MIC = 3.5–5 μL/mL), and *Salmonella typhimurium* (MIC = 10–20 μL/mL) [55,56], which have been accounted for the presence of monoterpenes such as camphor (6–15%), α-pinene (4–5%), β-pinene (2–10%), 1,8-cineole (6–14%), and α-tujone (20–42%) ([57]; Figure 23.4). The main chemical constituents of thyme are thymol (10–64%) [58], carvacrol (2–11%) [59], and terpinene (2–31%) [57]; (Figure 23.4). The antibacterial activity of these essential oils in thyme, *Thymus vulgaris*, was reported against *S. aureus* (MIC = 0.2–2.5 μL/mL) [55], *E. coli* (MIC = 0.45–1.25 μL/mL) [55], and *S. typhimurium* (MIC = 0.45–20 μLmL) [56]. Rosemary is well known for its essential oil components such as α-pinene (2–25%), bornyl acetate (0–17%), camphor (2–14%), and 1,8-cineole (3–89%) ([60]; Figure 23.4). The antibacterial activity of Rosemary has been reported against *S. aureus* (MIC = 0.4–10 μL/mL), *E. coli* (MIC = 4.5–10 μL/mL), and *S. typhimurium* (MIC > 20 μL/mL) [56]. Carvacrol (up to 80%), thymol (up to 64%), terpinene (2–52%), and cymene (up to 52%) (Figure 23.4) are major essential oil components of oregano, *Origanum vulgare*, which have been reported to exhibit antibacterial activities against *E. coli* (MIC = 0.5–1.2 μL/mL), *S. aureus* (MIC = 0.5–1.2 μL/mL), and *S. typhimurium* (MIC = 1.2 μL/mL) [62].

Octanordammaranes, mansumbinone, and 3,4-*seco*-mansumbinoic acid, together with sesquiterpenes, β-elemene and T-cadinol (Figure 23.5), have been isolated from the oleo resin of myrrh *Commiphora molmol* (Engl.). The MICs of the above compounds were reported to be in the range of 4–256 μg/mL against a number of *S. aureus* strains (SA1199B, ATCC25923, XU212, RN4220, and EMRSA15) [61]. The highest activity was observed by the *seco*-A-ring octanordammarane with an MIC of 4 μg/mL (norfloxacin with an MIC of 32 μg/mL) against SA1199B, an MDR strain that overexpresses the NorA efflux transporter. The crude extract of the oleo resin of *C. molmol* displayed a weak response to potentiate the activity of ciprofloxacin and tetracycline against a variety of *Salmonella enterica* serovar Typhimurium strains, one *S. aureus* isolate and two *Klebsiella pneumoniae* strains. Additionally, 3,4-*seco*-mansumbinoic acid 3,4-*seco*-mansumbinoic acid displayed weak potentiation of ciprofloxacin and tetracycline activity against strains of *S. enterica* serovar Typhimurium SL1344 and L10 [61]. A mixture of furanodiene-6-one and methoxyfuranoguaia-9-ene-8-one reported from myrrh showed antibacterial and antifungal activities against standard pathogenic strains of *E. coli*, *S. aureus*, *P. aeruginosa*, and *Candida albicans*, with an MIC of 0.18–2.8 μg/mL [62]. The abietane diterpenes, horminone, and 7-acetylhorminone, isolated from *Salvia blepharochlaena*, were reported to exhibit antistaphylococcal activity with MICs of 1.5–10 μg/mL [63].

Phenolic compounds are well known for antimicrobial activities. Nor-lignan isolated from a wound-healing Brazilian plant, *Styrax ferrugineus*, has potential activity against *S. aureus* with an MIC of 10 μg/mL compared to that of chloramphenicol (5 μg/mL) [64]. Simple flavones [65] such as apigenin and luteolin (Figure 23.6) showed good antibacterial activity against *S. aureus* including some MRSA strains with MICs of 3.9–62.5 μg/mL. Rahman and Gray [66] reported a new flavanone, (−)-(2S)-5,3′-dihydroxy-4′-methoxy-6″,6″-dimethylchromeno-(7,8,2″,3″)-flavanone (Figure 23.6), and known compounds including alkaloids, coumarins, flavanones, lignans, and triterpenes from the stem bark of *Feronia limonia*, a rutaceous plant used in Ayurveda for the treatment of indigestion and minor bowel infection in children [67]. The antimicrobial activities against bacteria (Gram-positive and

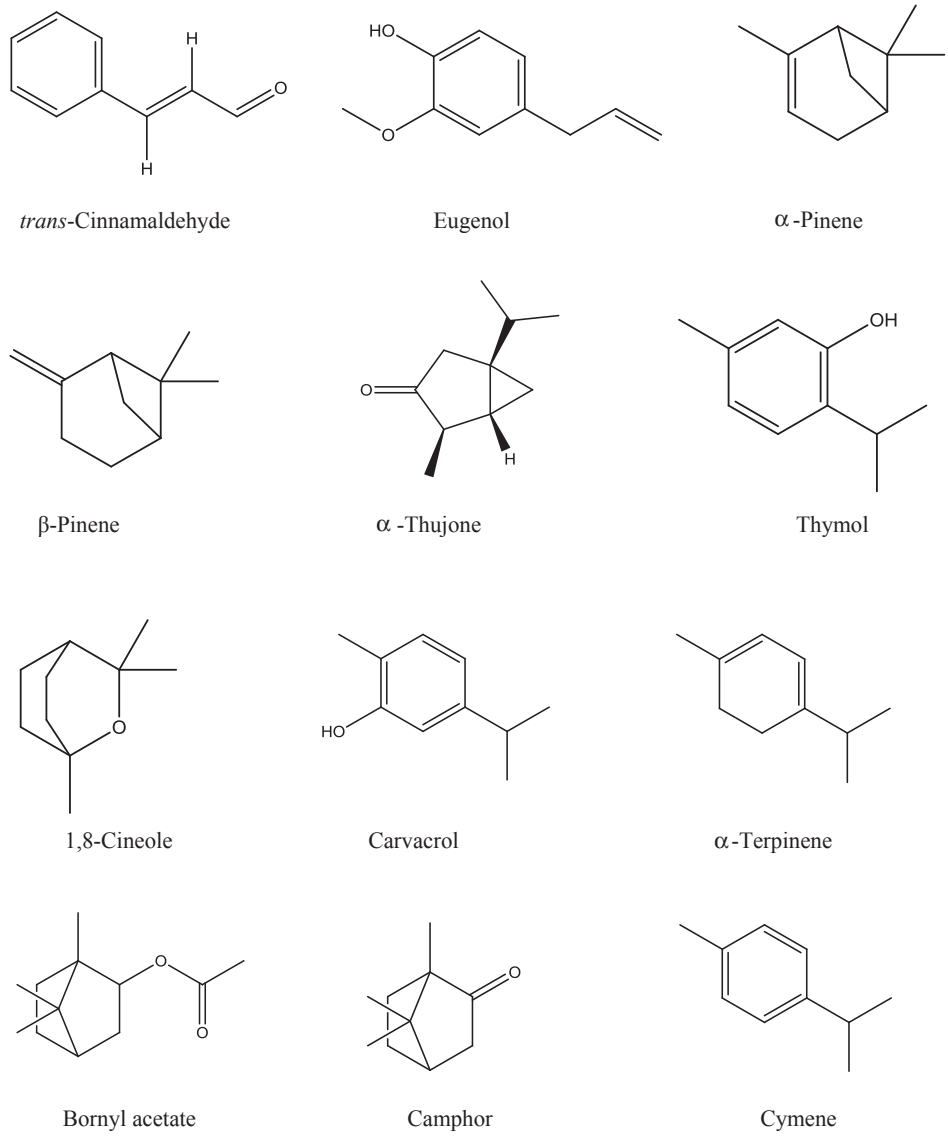
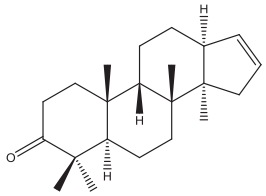


FIGURE 23.4 Common antimicrobial monoterpenes from essential oils.

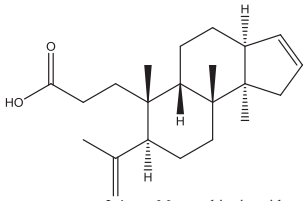
Gram-negative) and fungi were found in MICs in the range 25–100 μg/mL [66].

Uraria picta Desv. (Syn. *Doodia picta* Roxb; Fam. Papilionaceae), a suffruticose sparingly branched perennial herb, has been traditionally used as an antidote to the venom of a dangerous Indian snake, *Echis carinata* [67]. Rahman and coworkers [68] reported the isolation a number of isoflavanones including 5,7-dihydroxy-2′-methoxy-3′,4′-methylenedioxyisoflavanone and 4′,5-dihydroxy-2′,3′-dimethoxy-7-(5-hydroxyoxychromen-7yl)-isoflavanone (Figure 23.6) from this plant with antimicrobial activity against bacteria and fungi with MICs in the range of 12.5–100 μg/mL [68].

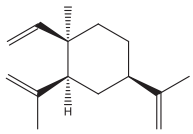
A simple salicylic acid derivative, 2-carboxy-3-(2-hydroxypropanyl)phenol,3-hydroxy-4-methoxycinnamaldehyde (Figure 23.7) and isoflavones including 5,7,4′-trihydroxy-8-(1,1-dimethyl-prop-2-enyl)-isoflavone, 5,7,2′,4′-tetrahydroxy-8-(1,1-dimethyl-prop-2-enyl)-isoflavone, and 5,2′,4′-trihydroxy-4″,4″,5″(ξ)-trimethyl-4″,5″-dihydrofurano-(7,6,2″,3″)-isoflavone (Figure 23.6) isolated from the stem bark of *Flemingia paniculata* [69] revealed significant activities against the test organisms with MIC values in the range of 1.57–200 μg/mL against bacteria (both Gram-positive and Gram-negative) and fungi. The highest potency (MIC = 1.57 μg/mL; 0.005 μmol) was exhibited by a new isoflavone, 5,7,4′-trihydroxy-8-(1,1-dimethyl-prop-2-enyl)-isoflavone (Figure 23.6), against *S. aureus* [70]. Its activity was counted as half of that of amoxycillin (MIC = 0.89 μg/mL; 0.0024 μmol). Polygosumic



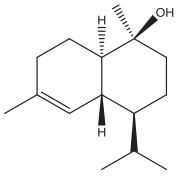
Mansumbinone



3,4-*seco*-Mansumbinoic acid

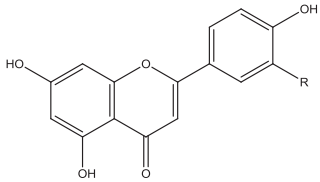


β-Elemene

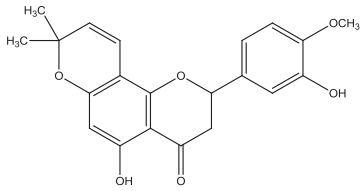


T-Cadinol

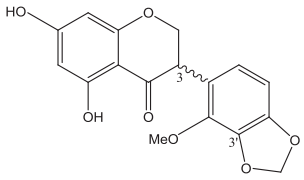
FIGURE 23.5 Antibacterial terpenes from *Commiphora molmol*.



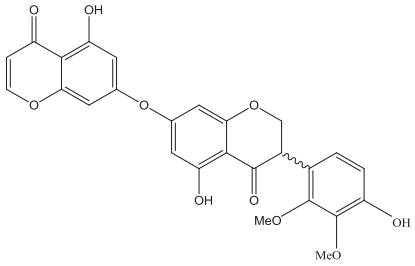
Apigenin, R= H; luteolin R= OH



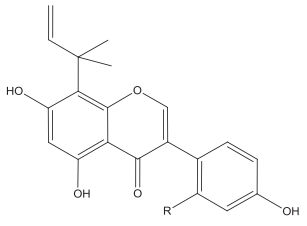
(-)-(2S)-5,3′-Dihydroxy-4′-methoxy-6″,6″-dimethylchromeno-(7,8,2″,3″)-flavanone



5,7-Dihydroxy-2′-methoxy-3′,4′-methylenedioxyisoflavanone

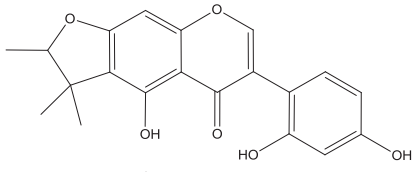


4′,5-Dihydroxy-2′,3′-dimethoxy-7-(5-hydroxyoxychromen-7yl)-isoflavanone



5,7,4′-Trihydroxy-8-(1,1-dimethylprop-2-enyl)-isoflavone (R= H)

5,7,2′,4′-Tetrahydroxy-8-(1,1-dimethylprop-2-enyl)-isoflavone(R= OH)



5,2′,4′-Trihydroxy-4″,4″,5″(ξ)-trimethyl-4″,5″-dihydrofurano-(7,6,2″,3″)-isoflavone

FIGURE 23.6 Some plant-derived antimicrobial flavonoids and isoflavonoids.

acid (Figure 23.7), a cadinane sesquiterpene, has been reported from *Polygonum viscosum*, which revealed good inhibition of the growth of drug-resistant *Escherichia coli* and MRSA [71]. Phenyl ethanoidglycosides (72; Figure 23.7) isolated from *Phlomis lanceolata* revealed antibacterial activity against MDR strains of *S. aureus* [72]. Alkaloids are another class of secondary metabolites with potential bioactivity. For example, vinca alkaloids (vincristine and vinblastine) isolated from *Catharanthus roseus* are now in use as anticancer drugs. There are overflowing reports of different types of alkaloids with antibacterial activities. Carbazole alkaloid, clausenal ([73]; Figure 23.8), isolated from *Clausena heptaphylla* (Fam. Rutaceae) was reported to show strong antibacterial activity with MICs of 3, 6, and 20 against *S. aureus*, *E. coli*, and *P. aeruginosa*, respectively.

Rahman and Gray [74] reported isolation of benzoisofuranone and several carbazole alkaloids including a dimericcarbazole, 3,3′-[oxybis(methylene)]bis(9-methoxy-9*H*-carbazole) (Figure 23.8), from *Murraya koengii* (Fam. Rutaceae). These compounds showed activity against four bacteria and two fungi with MICs of 3.13−100 μg/mL. Among these compounds, girinimbine (Figure 23.8), a carbazole alkaloid, exhibited the highest activity with MICs of 3.1 mLand 12.5 μg/mL against *S. aureus* and *Proteus vulgaris*, respectively [74]. In West and Central Africa, *Cryptolepis sanguinolenta* is

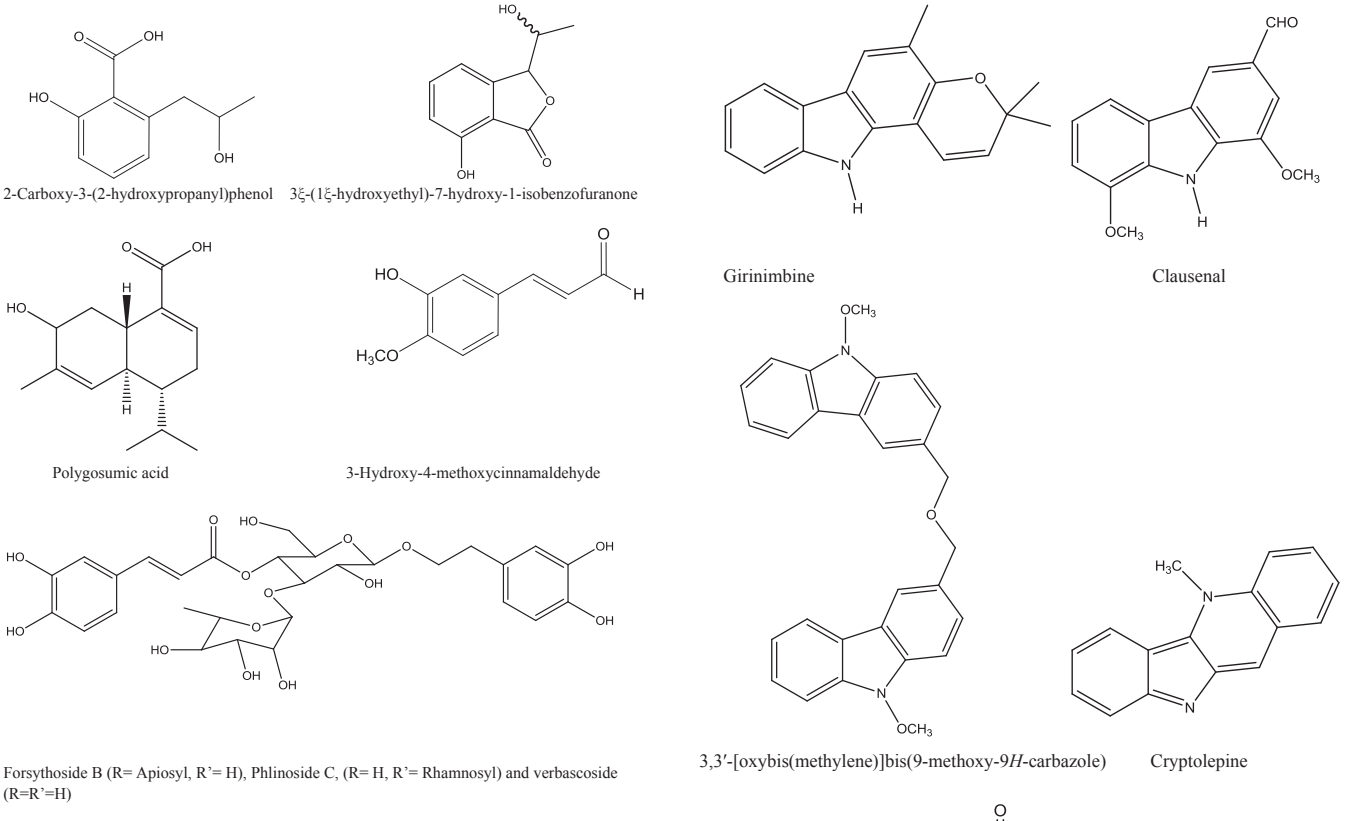


FIGURE 23.7 Some plant-derived antibacterial phenolic compounds.

widely used traditionally for the treatment of infectious diseases. Cryptolepine (Figure 23.8), an alkaloid, revealed antimicrobial activities against an extensive panel of Gram-positive, Gram-negative bacteria, and yeast [75]. Its MICs against *S. aureus* were found to be 7.8 μg/mL and 60 μg/mL, as free base and as hydrochloride salt, respectively. An unusual azaanthraquinone alkaloid, benz[g]isoquinoline-5,10-dione (Figure 23.8), isolated from *Mitracarpus scaber*, another member of the family Rutacae, revealed an MIC of 6.25 μg/mL against *S. aureus* [76]. Quaternary alkaloids have been isolated from the Southern prickly ash, *Zanthoxylum clava-herculis*, another plant belonging to the family Rutacae. Chelerythrine (Figure 23.8), a quaternary alkaloid, exhibited promising antibacterial activity against *S. aureus* (MIC = 4 μg/mL) [77]. Rahman and coworkers [78] reported the isolation of lignans and alkaloids from *Zanthoxylum budrunga* (Fam. Rutaceae) with antimicrobial activity. The MICs of the alkaloids against the test bacteria (*S. aureus*, *E. coli*, *P. vulgaris*, and *Klebsiella aerogenes*) were found to be 0.114−1.568 μmol. Canthine-6-one (Figure 23.8) revealed the highest activity among the alkaloids.

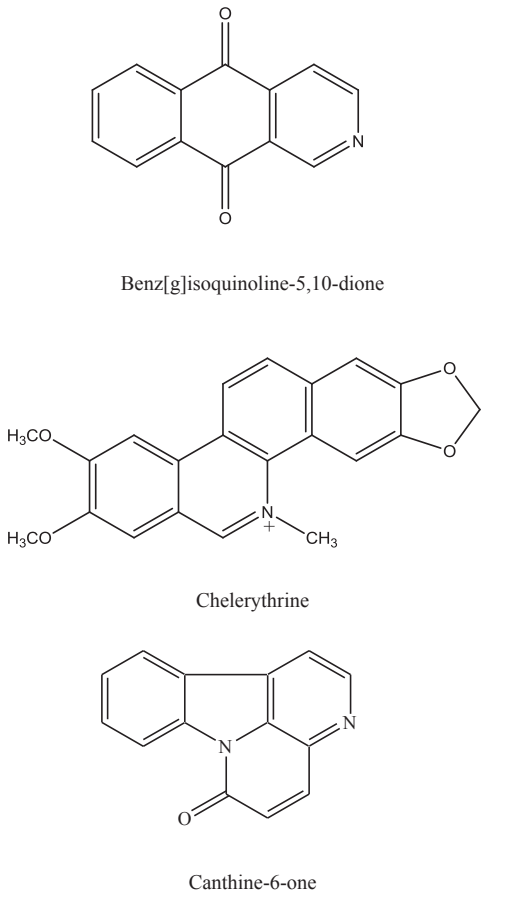


FIGURE 23.8 Some plant-derived antimicrobial alkaloids.

23.5.2 Antimicrobial Agents from Microbes

Microbes are considered as main natural sources for the development of new antibiotics since the accidental discovery of penicillin from the mold, *P. notatum*. Scientists worldwide are still focusing on various microbes, including bacteria and fungi, for the discovery of lead antibacterial agents. This section outlines some antimicrobial compounds from microbial sources. Nisin, a polycyclic antibacterial peptide composed of 34 amino acid residues, has been produced by *Lactococcus lactis* and used as a food preservative because of its bactericidal activities against Gram-positive and spore-forming food-borne bacteria including *S. aureus* and *Listeria monocytogenes* [79].

Reuterinis (Figure 23.9), β-hydroxypropionaldehyde, produced by *Lactobacillus reuteri*, is a broad spectrum antimicrobial agent with activity against food-borne pathogens and spoilage organisms. In combination with nisin, it demonstrates synergistic activity. It is reported to significantly reduce the growth of Gram-positive *S. aureus*, *Lactobacillus monocytogenes*, and Gram-negative *E. coli* and *S. Typhimorium* [80].

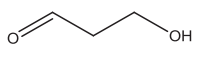
Three simple amides have been reported from an ethyl acetate extract of the cultural broth of *Streptomyces hygroscopicus* and exhibited antimicrobial activity against a wide range of bacteria and fungi [81].

Indoles and dithiolopyrrolones, isolated and identified from *Xenorhabdus bovienii* A2, showed a significant to strong activity against *Cryptococcus neoformans*, *Botrytis cinerea*, and *Phytophthora infestants* [82].

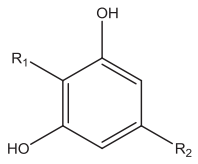
Dialkyl resorcinols (Figure 23.9), 2-butyl-5-propylresorcinol, 2-hexyl-5-methylresorcinol, 2-hexyl-5-propylresorcinol, and 2-hexyl-5-pentylresorcinol, isolated from the cultural broth of *Pseudomonas* sp. Ki19 have been reported to exhibit the inhibition of the growth of Gram-positive bacteria, such as *S. aureus*, at concentrations of ≤10 μg/mL and two fungi, namely, *Aspergillus fumigatus* and *Fusarium culmorum*, at a concentration of 50 μg/mL [83].

Khamthong and coworkers [84] reported the isolation of anthraquinone−citrinin derivatives (Penicillanthranin A; Figure 23.9) from the sea fan-derived fungus *Penicillium citrinum* PSU-F51, which displayed moderate antibacterial activity against both *Staphylococcus aureus* and MRSA with equal MICs of 16 μg/mL. Diketopiperazine (Figure 23.9), (3,1′-didehydro-3[2″(3‴, 3‴-dimethyl-prop-2-enyl)-3″-indolylmethylene]-6-methyl piperazine-2,5-dione), obtained from the culture medium of *Penicillium chrysogenum* [85], revealed antibacterial activity comparable to standard antibiotic, streptomycin.

Klaiklay and coworkers [86] reported the isolation of tetrahydroanthraquinone derivative (2*R*,3*S*)-7-ethyl-1,2,3,4-tetrahydro-2,3,8-trihydroxy-6-methoxy-3-methyl-9,10-anthracene-dione (Figure 23.9) from the mangrove-derived fungus *Phomopsis* species, which exhibited weak cytotoxicity against breast cancer (MCF-7) cell lines as well as weak antibacterial activity against *S. aureus* ATCC25923 including an MRSA strain SK1. A diphenyl ether, butenolide pestalolide (Figure 23.9), isolated from the mangrove-derived fungus *Pestalotiopsis* sp. PSU-MA69 displayed weak antifungal activity against *C. albicans* and *C. neoformans* [87].



Reuterinis

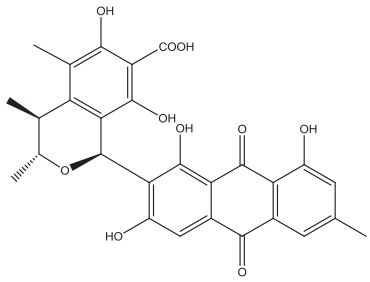


2-Butyl-5-propylresorcinol (R_1= butyl, R_2= propyl)

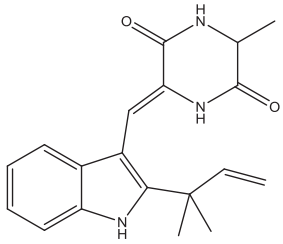
2-Hexyl-5-methylresorcinol (R_1= hexyl, R_2= methyl)

2-Hexyl-5-propylresorcinol (R_1= hexyl, R_2= propyl)

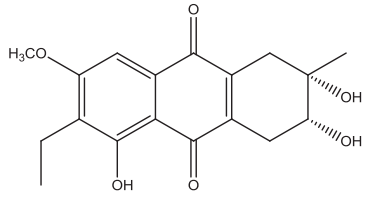
2-Hexyl-5-pentylresorcinol (R_1= hexyl, R_2= pentyl)



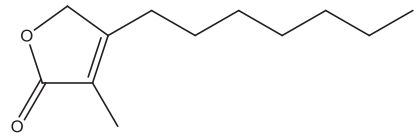
Penicillanthranin A



3,1′-Didehydro-3[2″(3‴,3‴-dimethyl-prop-2-enyl)-3″-indolylmethylene]-6-methyl pipera-zine-2,5-dione



(2*R*,3*S*)-7-ethyl-1,2,3,4-tetrahydro-2,3,8-trihydroxy-6-methoxy-3-methyl-9,10-anthracenedione



Butenolidepestalolide

FIGURE 23.9 Some microbial secondary metabolites with antibacterial activity.

23.5.3 Antimicrobial Agents from Marine Sources

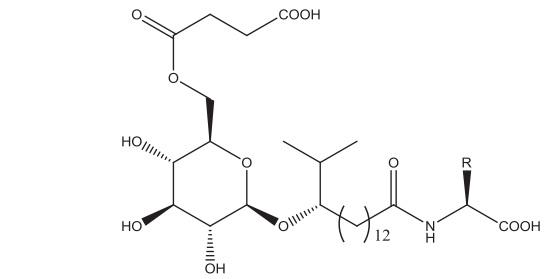
Recently, there have been enormous research efforts focusing on marine organisms and phytoplankton for the discovery of lead bioactive compounds. Naturally occurring diterpenes with unprecedented tetracyclic skeletons, ioniols I and II [88], tetracyclic brominated diterpenes [89,90], were isolated from the organic extract of *Sphaerococcus coronopifolius* collected from the rocky coasts of Corfu island in the Ionian Sea. These metabolites were evaluated for their antibacterial activity against a panel of MDR *S. aureus* and MRSA with MICs in the range 0.5–128 μg/mL. Kladi and coworkers [91] reported the isolation of some new C_{15} eight-membered cyclic ethers with a characteristic terminal *cis* ene–yne moiety from the organic extract of the red alga, *Laurencia glandulifera*, collected at the Crete island in South Greece and found MICs in the range of 8–256 μg/mL when tested against a panel of MDR *S. aureus* and MRSA. Ioannou and coworkers [92] reported the isolation of 17 diterpenes featuring the dolabellane skeleton 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 and methicillin-resistant variants.

Neomaclafungins A-I [93], 26-membered macrolides of the oligomycin subfamily, have been isolated from the fermentation broth of *Actinoalloteichus* sp. NPS702, marine sediment collected from USA Bay, Kochi Prefecture, Japan. Neomaclafungins A-I exhibited significant antifungal activity in vitro against *Trichophyton mentagrophytes* (ATCC 9533) with MICs of 1–3 μg/mL.

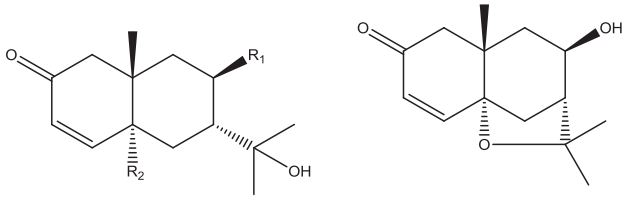
Tareq and coworkers [94] reported the isolation of glycolipopeptides (Figure 23.10), Ieodoglucomides A and B, from a Marine-derived bacterium *Bacillus licheniformis*, which revealed moderate in vitro antimicrobial activity while Ieodoglucomides B showed cytotoxic activity against lung cancer and stomach cancer cell lines. Lipopeptides, peptidolipins B-F [95], have been isolated from a marine *Nocardia* sp., which was obtained from the ascidian *Trididemnum orbiculatum*. Peptidolipins B and E demonstrated moderate antibacterial activity against MRSA and methicillin-sensitive *S. aureus*.

Eeudesmene-type sesquiterpenes, kandenols A–E (Figure 23.10), isolated from *Streptomyces* sp. HKI0595, showed weak to moderate antimicrobial activities against *Bacillus subtilis* ATCC 6633 and *Mycobacterium vaccae* [96].

Polycyclic secondary metabolites, citreamicin A, citreamicin B, citreaglycon A, and dehydrocitreaglycon A (Figure 23.10), isolated from marine-derived *Streptomyces caelestis* exhibited antibacterial activity against *Staphylococcus haemolyticus*, *S. aureus*, and *B. subtillis*. Citreamicin A, citreamicin B, and citreaglycon A revealed strong activities against MRSA ATCC 43300 with MIC values of 0.25, 0.25, and 8.0 μg/mL, respectively [97]. A dimeric diketopiperazine, brevianamide S (Figure 23.10), isolated from *Aspergillus versicolor* (collected from the Bohai Sea, China), exhibited selective antibacterial activity [98] against Bacille Calmette-Guérin, suggesting a new mechanism of action that could be considered as a new lead for antitubercular drugs. Isorhodoptilometrin -1-methyl ether, emodin, 1-methyl emodin, siderin, arugosin C, and variculanol obtained from the marine fungus *A. versicolor* were reported to exhibit antimicrobial activity, anticancer activity, and inhibition of Hepatitis C virus protease [99]. Yang and coworkers [100] reported the isolation of anthraquinone derivative, 3-acetoxy-4-deoxybostrycin (Figure 23.10), from a marine-derived fungus *Nigrospora*, which exhibited promising activity against *B. cereus* with an MIC value of 48.8 nM, which was stronger than that

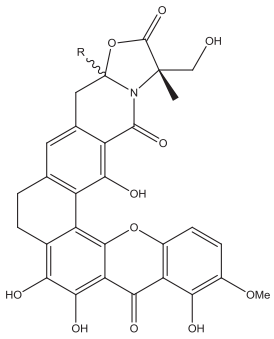


Eodoglucomide A (R= Me), Eodoglucomide B (R= H)



Kandenol A (R_1= OH, R_2= H); Kandenol B (R_1= R_2=OH) Kandenol E

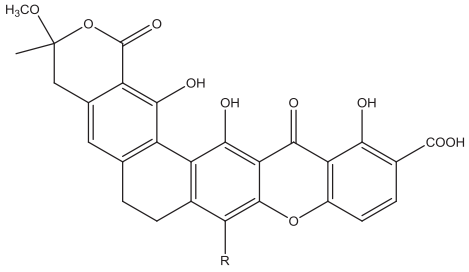
Kandenol C (R_1= OH, R_2=OOH);Kandenol D (R_1= H, R_2=OOH)



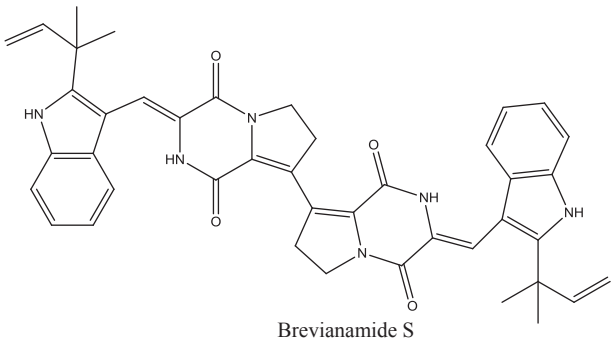
Citreamicin A, R= Me

Citreamicin B R= Me

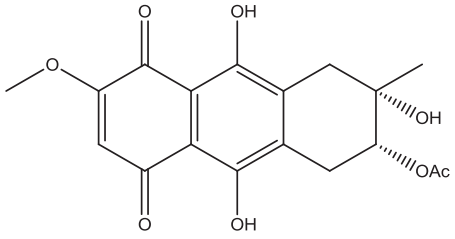
FIGURE 23.10 Some marine-derived secondary metabolites with antimicrobial activity.



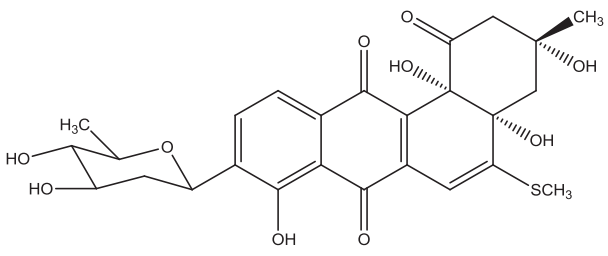
Citreaglycon A (R=OH), dehydrocitreaglycon A (R=H)



Brevianamide S



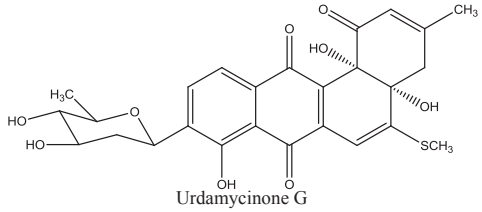
3-Acetoxy-4-deoxybostrycin



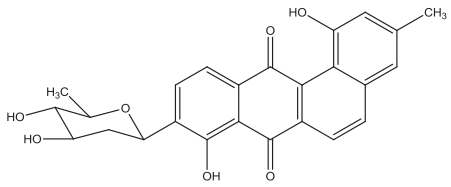
Urdamycinone E

FIGURE 23.10 cont'd.

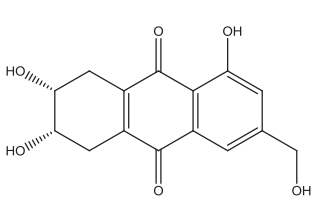
of the positive control ciprofloxacin (MIC = 1250 nM). *C*-glycosylated benz[α]anthraquinone derivatives [101], urdamycinone E, urdamycinone G, dehydroxyaquayamycin (Figure 23.10), isolated from the marine *Streptomycetes* sp. exhibited potent activity against *M. tuberculosis* MICs of 3.13–12.50 μg/mL. Trichodermaquinone and trichodermaxanthone (Figure 23.10) were isolated from the marine-derived fungus *Trichoderma aureoviride* PSU-F95 [102], which displayed strong antibacterial activity against MRSA with MIC values of 8 and 4 μg/mL, respectively.



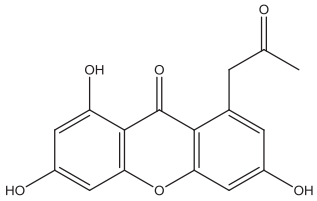
Urdamycinone G



Dehydroxyaquayamycin



Trichodermaquinone



Trichodermaxanthone

FIGURE 23.10 cont'd.

23.5.4 Antimicrobial Agents from Animal Sources

There are reports of isolation of antibacterial agents from animal sources as well. For example, European fire-bellied toad, *Bombina* sps., produces skin secretions with peptides, such as bombinins and bombinins H, with activity against Gram-positive and Gram-negative bacteria and also fungi [103].

Lactoferrin, an iron-binding glycoprotein present in the milk of human and mammals, is reported to have significant antimicrobial activity against a wide range of bacteria and viruses [104]. Chitosan, a polycationic biopolymer, produced from the exoskeletons of crustaceans and anthropods, has been reported to suppress fungal colony growth and inhibited fungal spore germination at a 0.01% (w/v) concentration [105].

23.6 CONCLUSION

A huge number of antibiotics are available for the treatment of infectious diseases. Most of them are either natural or modified from natural ones (semisynthetics). Although there are an enormous number of antibiotics available, infectious disease is still considered as the second leading cause of death worldwide amounting to

approximately 17 million people, particularly children and the elderly, per year. Microorganisms are slowly but surely developing resistance to antibiotics. MRSA and multiMDR Gram-negative bacteria are big concerns in recent years. The number of death certificates of MRSA victims issued in the United Kingdom has recently been decreased due to public carefulness about health hygiene. However, it is necessary to discover new antibiotics that are required to tackle such a fatal disease. So, research will continue to focus on the natural resources in order to find lead compounds for the development of safe but highly effective antibiotics.

References

[1] Pelczar MJ, Chan ECS, Krieg NR, editors. Microbiology. 5th ed. New York: McGraw-Hill Book Company; 1986. p. 510–40.

[2] Patrick G. An introduction to medicinal chemistry. 5th ed. Oxford: Oxford Publishing Press; 2013. 413–414.

[3] Petri Jr WA. Penicillins, cephalosporins, and other beta-lactam antibiotics. In: Brunton LL, Lazo JS, Parker KL, editors. Goodman & Gilman's the pharmacological basis of therapeutics. 10th ed. New York: McGraw-Hill; 2006. p. 1127–54.

[4] Schatz A, Bugie E, Waksman SA. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc Soc Exp Biol Med 1944;55:66–9.

[5] Ehrlich J, Bartz QR, Smith RM, Joslyn DA, Burkholder PR. Chloromycetin, a new antibiotic from a soil actinomycete. Science 1947;106:417.

[6] Duggar BM. Aureomycin: a product of the continuing search for new antibiotics. Ann NY Acad Sci 1948;51:177–81.

[7] Gerzon K, Flynn EH, Sigal MV, Wiley PF, Monahan R, Quarck UC. Erythromycin. VIII. Structure of dihydroerythronolide. J Am Chem Soc 1956;78:6396–408.

[8] McCormick MH, Stark WM, Pittenger GE, Pittenger RC, McGuire JM. Vancomycin, a new antibiotic. I. Chemical and biologic properties. Antibiot Annu 1955-56;121:606–11.

[9] Harris CM, Kopecka H, Harris TM. Vancomycin: structure and transformation to CDP-I. J Am Chem Soc 1983;105:6915–22.

[10] Martinelli E, Schulz K, Mansoori GA. Supercritical fluid extraction/retrograde condensation (SFE/RC) with applications in biotechnology. In: Bruno TS, Ely JF, editors. Supercritical fluid technology. Boca Raton: CRC Press; 1991. p. 451–78.

[11] Coll JC, Bowden BF. The application of vacuum liquid-chromatography to the separation of terpene mixtures. J Nat Prod 1986;4:934–6.

[12] Pelletier SW, Chokshi HP, Desai HK. Separation of diterpenoid alkaloid mixtures using vacuum liquid-chromatograph. J Nat Prod 1986;49:892–900.

[13] Stahl E. Thin layer chromatography. p. 855. 2nd ed. New York: Springer-Verlag, Berlin, Heidllberg; 1966.

[14] Touchtone JC, Dobbins MF. Practice of thin layer chromatography. p. 170. New York: John Willey & Sons Ltd; 1977.

[15] Sadler IH. The use of NMR spectroscopy in the structure determination of natural products—one dimensional methods. Nat Prod Rep 1988;5:101–27.

[16] Bendall MR, Doddrell DM, Pegg DT. Editing of ^{13}C NMR spectra—a pulse sequence for the generation of sub-spectra. J Am Chem Soc 1981;103:4603–5.

[17] Bendall MR, Pegg DT, Doddrell DM. Pulse sequences utilizing the correlated motion of coupled heteronuclei in the transverse plane of the doubly rotating frame. J Mag Resonance 1983;52: 81–117.

[18] Duddeck H, Dietrich W. Structure elucidation by modern NMR. Springer Verlag; 1989.

[19] Abraham RJ, Fisher J, Loftus P. Introduction to the NMR Spectroscopy. p.144. Chichester, New York: John Willey & Sons Ltd; 1993.

[20] Derome AE. The use of NMR spectroscopy in the structure determination of natural-products- two-dimensional methods. Nat Prod Rep 1989;6:111–41.

[21] Bax A, Freeman R. Investigation of complex networks of spin-spin coupling by two-dimensional NMR. J Mag Reson 1981;44: 542–61.

[22] Williams DH, Fleming I. Spectroscopic methods in organic chemistry. p.122. 5th ed. London: McGraw-Hill Book Company; 1995.

[23] Mirau PA, Bovey FA. In: Bovey FA, editor. Nuclear magnetic resonance spectroscopy. 2nd ed. San Diego: Academic Press, Inc.; 1988. p. 325.

[24] Bax A. Broad-band homonuclear decoupling in heteronuclear shift correlation NMR-spectroscopy. J Mag Reson 1983;53: 517–20.

[25] Bax A, Summers MF. H-1 and C-13 assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. J Am Chem Soc 1986;108:2093–4.

[26] Caceres A, Alvarez AV, Ovando AE, Samayoa BE. Plants used in Guatemala for the treatment of respiratory-diseases- screening of 68 plants against gram-positive bacteria. J Ethnopharmacol 1991; 31:193–208.

[27] Ghisalberti EL. Detection and isolation of bioactive natural products. In: Colegate SM, Molyneux RJ, editors. Bioactive natural products. Boca Raton, London, Tokyo: CRC Press; 1993. p. 19.

[28] Drummond AJ, Waigh RD. In: Pandalai SG, editor. Recent research developments in phytochemistry, vol. 4. India: Research Signpost; 2000. p. 143–52.

[29] Shiu WKP, Gibbons S. Anti-staphylococcal acylphloroglucinols from *Hypericum beanii*. Phytochemistry 2006;67:2568–72.

[30] Ananthan S, Faaleolea ER, Goldman RC, Hobrath JV, Kwong CD, Laughon BE, et al. High-throughput screening for inhibitors of *Mycobacterium tuberculosis* H37Rv. Tuberculosis 2009;89:334–53.

[31] Gruppo V, Johnson CM, Marietta KS, Scherman H, Zink EE, Crick DC, et al. Rapid microbiologic and pharmacologic evaluation of experimental compounds against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2006;50:1245–50.

[32] Collins L, Franzblau SG. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob Agents Chemother 1997;41:1004–9.

[33] Schmitt S, Lopez MM, Kuipers O, Panke S, Hel M. High-throughput nL-reactor screening for antimicrobial peptides. New Biotechnol 2014;31:S73.

[34] Cragg GM, Grothaus PG, Newman DJ. Impact of natural products on developing new anti-cancer agents. Chem Rev 2009; 109:3012–43.

[35] Butler A, Hensman T. Drugs for the fever. Educ Chem 2000;37: 151.

[36] Hollman A. Digoxin comes from Digitalis lanata. British Med J 1996;7035:912.

[37] Gautam R, Saklani A, Jachak SM. Indian medicinal plants as a source of antimycobacterial agents. J Ethnopharmacol 2007;110: 200–34.

[38] Wang Z, Yu P, Zhang G, Xu L, Wang D, Wang L, et al. Design, synthesis and antibacterial activity of novel andrographolide derivatives. Bioorg Med Chem 2010;18:4269–74.

[39] Oluwatuyi M, Kaatz GW, Gibbons S. Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochemistry 2004; 65:3249–54.
[40] O'Donnell G, Poeschl R, Zimhony O, Gunaratnam M, Moreira JB, Neidle S, et al. Bioactive pyridine-*N*-oxide sulphides from *Allium stipitatum*. J Nat Prod 2009;72:360–5.
[41] Schempp CM, Pelz K, Wittmer A, Schöpf E, Simon JC. Antibacterial activity of hyperforin from St John's wort against multiresistant *Staphylococcus aureus* and gram-positive bacteria. The Lancet 1999;353:2129.
[42] Gibbons S, Ohlendorf B, Johnsen I. The genus Hypericum—a valuable resource of anti-Staphylococcal leads. Fitoterapia 2002; 73:300–4.
[43] Shiu WKP, Rahman MM, Curry J, Stapleton P, Zloh M, Malkinson JP, et al. Antibacterial acylphloroglucinols from *Hypericum olympicum* L. cf. *Uniflorum*. J Nat Prod 2012;75:336–43.
[44] Osman K, Evangelopoulos D, Basavannacharya C, Gupta A, McHugh TD, Bhakta S, et al. An antibacterial from *Hypericum acmosepalum* inhibits ATP-dependent MurE ligase from *Mycobacterium tuberculosis*. Int JAntimicrob Agents 2012;39:124–9.
[45] Wang W, Zeng YH, Osman K, Shinde K, Rahman MM, Gibbons S, et al. Norlignans, acylphloroglucinols and a dimeric xanthone from *Hypericum chinense*. J Nat Prod 2010;73:1815–20.
[46] Burt S. Essential oils: their antibacterial properties and potential applications in food—a review. Int J Food Microbiol 2004;94: 223–53.
[47] Azzouz MA, Bullerman LB. Comparative antimycotic effects of selected herbs, spices, plant components and commercial antifungal agents. J Food Prot 1982;45:1298–301.
[48] Conner DE, Beuchat LR. Effect of essential oils plants on growth of food spoilage yeasts. J Food Sci 1984;49:429–34.
[49] Suksrikarm B. Herb and spice. Thailand: Amorn Printing; 1987.
[50] Soliman KM, Badeaa RI. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food Chem Toxicol 2002;40:1669–75.
[51] August KT. Cysteine–onion oil interaction: its biological importance and the saporation of interaction product by chromatography. Food Sci Tech Abstracts 1978;10:12.
[52] Bauer K, Garbe D, Surburg H. Common fragrance and flavor materials: preparation, properties and uses. Weinheim: Wiley-VCH; 2001. p. 293.
[53] Pinto E, Vale-Silva L, Cavaleiro C, Salgueiro L. Antifungal activity of the clove essential oil from *Syzygium aromaticum* on *Candida, Aspergillus* and *Dermatophyte* species. J Med Microbiol 2009;58:1454–62.
[54] Devi KP, Nisha SA, Sakthivel R, Pandian SK. Eugenol (an essential oil) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. J Ethnopharmacol 2010;130: 107–15.
[55] Farag DS, Daw ZY, Hewedi FM, El-Baroty GS. Antimicrobial activity of some Egyptian spice essential oils. J Food Prot 1989;52: 665–7.
[56] Hammer KA, Carson CF, Riley TV. Antimicrobial activity of essential oils and other plant extracts. J Appl Microbiol 1999; 86:985–90.
[57] Marino M, Bersani C, Comi G. Impedance measurements to study the antimicrobial activity of essential oils from *Lamiaceae* and *Compositae*. Int J Food Microbiol 2001;67:187–95.
[58] Lens-Lisbonne C, Cremieux A, Maillard C, Balansard G. Methods for evaluation of antibacterial activity of essential oils: application to essences of thyme and cinnamon. J Pharm Belg 1987;42:297–302.
[59] McGimpsey JA, Douglas MH, van Klink JW, Beauregard DA, Perry NB. Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgaris* L. in New Zealand. Flavour Fragrance J 1994;9:347–52.
[60] Vardar-Unlü G, Candan F, Sökmen A, Daferera D, Polissiou M, Sökmen M, et al. Antimicrobial and antioxidant activity of the essential oil and methanol extracts of *Thymus pectinatus* Fisch. et Mey. Var. *pectinatus* (Lamiaceae). J Agric Food Chem 2003;51:63–7.
[61] Rahman MM, Garvey M, Piddock LJ, Gibbons S. Antibacterial terpenes from the oleo-resin of *Commiphora molmol* (Engl.). Phytother Res 2008;22:1356–60.
[62] Dolara P, Corte B, Ghelardini C, Pugliese AM, Cerbai E, Menichetti S, et al. Local anaesthetic, antibacterial and antifungal properties of sesquiterpenes from myrrh. Planta Med 2000;66: 356–8.
[63] Ulubelen A, Oksüz S, Topcu G, Gören AC, Voelter W. Antibacterial diterpenes from the roots of *Salvia blepharochlaena*. J Nat Prod 2001;64:549–51.
[64] Pauletti PM, Araújo AR, Young MCM, Giesbrecht AM, Bolzani VDS. nor-Lignans from the leaves of *Styraxferrugineus* (Styracaceae) with antibacterial and antifungal activity. Phytochemistry 2000;55:597–601.
[65] Sato Y, Suzaki S, Nishikawa T, Kihara M, Shibata H, Higuti T. Phytochemical flavones isolated from *Scutellaria barbata* and antibacterial activity against methicillin-resistant *Staphylococcus aureus*. J Ethnopharmacol 2000;72:483–8.
[66] Rahman MM, Gray AI. Antimicrobial constituents from the stem bark of *Feronia limonia*. Phytochemistry 2002;59:73–7.
[67] Kirtikar KR, Basu BD, An ICS. Indian medicinal plants. India: Bishen Singh Mahendra Pal Singh; 1993.
[68] Rahman MM, Gibbons S, Gray AI. Isoflavanones from *Uraria picta* and their antimicrobial activity. Phytochemistry 2007;68: 1692–7.
[69] Rahman MM, Sarker SD, Byres M, Gray AI. New salicylic acid and isoflavone derivatives from *Flemingia paniculata*. J Nat Prod 2004;67:402–6.
[70] Rahman MM, Khondkar P, Gray AI, Sarker SD. Antibacterial and antifungal activity of the constituents of *Flemingia paniculata*. Pharm Biol 2008;46:356–9.
[71] Datta BK, Rahman MM, Gray AI, Nahar L, Hossein AA, Auzi AA, et al. Polygosumic acid, a new cadinane sesquiterpene, from *Polygonum viscosum* inhibits the growth of drug-resistant *Escherichia coli* and *Staphylococcus aureus (MRSA)* in vitro. J Nat Med 2007;61:391–6.
[72] Nazemiyeh H, Rahman MM, Gibbons S, Nahar L, Delazar A, Ghahramani MA, et al. Assessment of antibacterial activity of phenylethanoid glycosides from *Phlomislanceolata* against multiple-drug resistant (MDR) strains of *Staphylococcus aureus*. J Nat Med 2008;62:91–5.
[73] Chakraborty A, Saha C, Podder G, Chowdhury BK, Bhattacharyya P. Carbazole alkaloid with antimicrobial activity from *Clausena heptaphylla*. Phytochemistry 1995;38: 787–9.
[74] Rahman MM, Gray AI. A benzoisofuranone derivative and carbazole alkaloids from *Murrayakoenigii* and their antimicrobial activity. Phytochemistry 2005;66:1601–6.
[75] Cimanga K, De Bruyne T, Lasure A, Van Poel B, Pieters L, Claeys M, et al. In vitro biological activities of alkaloids from *Cryptolepis sanguinolenta*. Planta Med 1996;62:22–7.
[76] Okunade AL, Clark AM, Hufford CD, Oguntimein BO. Azaanthraquinone: an antimicrobial alkaloid from *Mitracarpus scaber*. Planta Med 1999;65:447–8.
[77] Gibbons S, Leimkugel J, Oluwatuyi M, Heinrich M. Activity of *Zanthoxylum clava-herculis* extracts against multi-drug resistant methicillin-resistant *Staphylococcus aureus* (mdr-MRSA). Phytother Res 2003;17:274–5.
[78] Rahman MM, Khondkar P, Islam MA, Gray AI. Antimicrobial activities of alkaloids and lignans from *Zanthoxylum budrunga*. Nat Prod Commun 2008;3:45–7.

[79] Arqués JL, Rodríguez E, Nuñez M, Medina M. Antimicrobial activity of nisin, reuterin, and the lactoperoxidase system on *Listeria monocytogenes* and *Staphylococcus aureus* in cuajada, a semisolid dairy product manufactured in Spain. J Dairy Sci 2008;91:70–5.
[80] Bian L, Molan A-L, Maddox I, Shu Q. Antimicrobial activity of *Lactobacillus reuteri* DPC16 supernatants against selected food borne pathogens. World J Microbiol Biotech 2011;27:991–8.
[81] Hossain MS, Hossain MA, Rahman MM, Gray AI, Bhuiyan MSA, Mondol MAM, et al. Amides from the fungus *Streptomyces hygroscopicus* and their antimicrobial activity. Phytochemistry 2004;65: 2169–73.
[82] Li J, Chen G, Webster JM, Czyzewska E. Antimicrobial metabolites from a bacterial symbiont. J Nat Prod 1995;58:1081–6.
[83] Pohanka A, Levenfors J, Broberg A. Antimicrobial dialkyl resorcinols from *Pseudomonas* sp. Ki19. J Nat Prod 2010;73: 825–30.
[84] Khamthong N, Rukachaisirikul V, Phongpaichit S, Preedanon S, Sakayaroj J. Bioactive polyketides from the sea fan-derived fungus *Penicillium citrinum* PSU-F51. Tetrahedron 2012;68: 8245–50.
[85] Devi P, Rodrigues C, Naik CG, D'Souza L. Isolation and characterization of antibacterial compound from a mangrove-endophytic fungus, *Penicillium chrysogenum* MTCC 5108. Indian J Microbiol 2012;52:617–23.
[86] Klaiklay S, Rukachaisirikul V, Phongpaichit S, Pakawatchai C, Saithong S, Buatong J, et al. Anthraquinone derivatives from the mangrove-derived fungus *Phomopsis* sp. PSU-MA214. Phytochemistry Lett 2012;5:738–42.
[87] Klaiklay S, Rukachaisirikul V, Tadpetch K, Sukpondma Y, Phongpaichit S, Buatong J, et al. Chlorinated chromone and diphenyl ether derivatives from the mangrove-derived fungus *Pestalotiopsis* sp. PSU-MA69. Tetrahedron 2012;68:2299–305.
[88] Smyrniotopoulos V, Vagias C, Rahman MM, Gibbons S, Roussis V. Ioniols I and II, tetracyclic diterpenes with antibacterial activity from *Sphaerococcus coronopifolius*. Chem Biodivers 2010;7:666–76.
[89] Smyrniotopoulos V, Vagias C, Rahman MM, Gibbons S, Roussis V. Structure and antibacterial activity of brominated diterpenes from the red alga *Sphaerococcus coronopifolius*. Chem Biodivers 2010;7:186–95.
[90] Smyrniotopoulos V, Vagias C, Rahman MM, Gibbons S, Roussis V. New brominated diterpenes with antistaphylococcal activity from the red alga *Sphaerococcus coronopifolius*. J Nat Prod 2008;71:1386–92.
[91] Kladi M, Vagias C, Stavri M, Rahman MM, Gibbons S, Roussis V. C_{15}Acetogeninswith antistaphylococcal activity from the red alga *Laurencia glandulifera*. Phytochemistry Lett 2008;1:31–6.
[92] Ioannou E, Quesada A, Rahman MM, Gibbons S, Vagias C, Roussis V. Dolabellnes with antibacterial activity from the brown alga *Dilophus spiralis*. J Nat Prod 2011;74:213–22.
[93] Sato S, Iwata F, Yamada S, Katayama M. NeomaclafunginsA-I: oligomycin-class macrolides from a marine-derived actinomycete. J Nat Prod 2012;75:1974–82.
[94] Tareq FS, Kim JH, Lee MA, Lee HS, Lee YJ, Lee JS, et al. Ieodoglucomides A and B from a marine-derived bacterium *Bacillus licheniformis*. Org Lett 2012;14:1464–7.
[95] Wyche TP, Hou Y, Vazquez-Rivera E, Braun D, Bugni TS. Peptidolipins B-F, antibacterial lipopeptides from an ascidian-derived *Nocardia* sp. J Nat Prod 2012;75:735–40.
[96] Ding L, Maier A, Fiebig HH, Lin WH, Peschel G, Hertweck C. Kandenols A-E, eudesmenes from an endophytic *Streptomyces* sp. of the mangrove tree *Kandelia candel*. J Nat Prod 2012;75: 2223–7.
[97] Liu LL, Xu Y, Han Z, Li YX, Lu L, Lai PY, et al. Four new antibacterial xanthones from the marine-derived actinomycetes *Streptomyces caelestis*. Mar Drugs 2012;10:2571–83.
[98] Song F, Liu X, Guo H, Ren B, Chen C, Piggott AM, et al. Brevianamides with antitubercular potential from a marine-derived isolate of *Aspergillus versicolor*. Org Lett 2012;14:4770–3.
[99] Hawas UW, El-Beih AA, El-Halawany AM. Bioactive anthraquinones from endophytic fungus *Aspergillus versicolor* isolated from red sea algae. Arch Pharm Res 2012;35:1749–56.
[100] Yang KL, Wei MY, Shao CL, Fu XM, Guo ZY, Xu RF, et al. Antibacterial anthraquinone derivatives from a sea anemone-derived fungus *Nigrospora* sp. J Nat Prod 2012;75:935–41.
[101] Supong K, Thawai C, Suwanborirux K, Choowong W, Supothina S, Pittayakhajonwut P. Antimalarial and antitubercular *C*-glycosylated benz[α]anthraquinones from the marine-derived *Streptomyces* sp. BCC45596. Phytochem Lett 2012;5:651–6.
[102] Khamthong N, Rukachaisirikul V, Tadpetch K, Kaewpet M, Phongpaichit S, Preedanon S, et al. Tetrahydroanthraquinone and xanthone derivatives from the marine-derived fungus *Trichoderma aureoviride* PSU-F95. Arch Pharm Res 2012;35: 461–8.
[103] Simmaco M, Kreil G, Barra D. Bombinins, antimicrobial peptides from *Bombina* species. Biochim Biophys Acta (BBA) – Biomembr 2009;788:1551–5.
[104] Lönnerdal B. Biological effects of novel bovine milk fractions. Nestle Nutr Workshop Ser Pediatr Program 2011;67:41–54.
[105] Tikhonov VE, Stepnova EA, Babak VG, Yamskov IA, Palma-Guerrero J, Jansson,H-B., et al. Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2(3)-(dodec-2-enyl)succinoyl/-derivatives. Carbohydr Polym 2006;64: 66–72.

LIST OF ABBREVIATIONS

AMR Antimicrobial resistance
APCI Atmospheric pressure chemical ionization
API Active pharmaceutical ingredient
APT Attached proton test
CC Column chromatography
CI Chemical ionization
COSY Correlation spectroscopy
COSY-lr Correlation spectroscopy long range
DEPT-135 Distortionless enhancement by polarization transfer-135
EI Electron impact
ESI Electrospray ionization
FAB Fast atom bombardment
FD/FI Field desorption/field ionization
FID Free induction decay
GC–MS Gas chromatography mass spectrometry
HMBC Heteronuclear multiple bond coherence
HMQC Heteronuclear multiple quantum correlation
HPLC High-performance liquid chromatography
HSQC Heteronuclear single quantum coherence
HREIMS High-resolution electron impact mass spectra
IR Infrared
LC–MS Liquid chromatography mass spectrometry
MALDI Matrix-assisted laser desorption ionization
MDR Multidrug resistant
MIC Minimum inhibitory concentration
MRSA Methicillin-resistant *Staphylococcus aureus*
MS Mass spectrometry
MTT Methyl thiazolyldiphenyl-tetrazolium bromide

NMR Nuclear magnetic resonance
NOESY Nuclear Overhauser enhancement spectroscopy
ODS Octadecyl silane
PDA Photodiode array
PDA Potato dextrose agar
PTLC Preparative thin layer chromatography
R_f Retardation factor
SFC Supercritical fluid extraction
SPE Solid phase extraction
TLC Thin layer chromatography
TOCSY Total correlation spectroscopy
VLC Vacuum liquid chromatography
UV Ultraviolet
UV−Vis Ultraviolet−visible

This page intentionally left blank

CHAPTER

24

Uses of Herbals in Cardiac Diseases: Priority of Evidence Over Belief

Subir K. Maulik[1], *Sanjay K. Banerjee*[2]

[1]Department of Pharmacology, All India Institute of Medical Sciences, New Delhi, India; [2]Drug Discovery Research Center, Translational Health Science and Technology Institute (THSTI), Faridabad, Haryana, India

OUTLINE

24.1 INTRODUCTION

Cardiovascular diseases (CVD) refer to any disease that affects the cardiovascular system, principally cardiac disease, vascular diseases of the brain and kidney, and peripheral arterial disease. Major CVD include coronary artery disease, hypertension, heart failure, cardiac arrhythmias, and cerebrovascular diseases such as stroke, peripheral arterial disease, congenital heart disease, and rheumatic heart disease. The causes of CVD are diverse, but atherosclerosis and/or hypertension are the most common. In addition, a number of physiological and morphological changes alter cardiovascular function with age and lead to increased risk of CVD.

CVD is the leading cause of deaths worldwide and is increasing at a fast rate in low- and middle-income countries [1]. Evidence suggests there are a number of major risk factors for heart diseases: age, gender, high blood pressure, hyperlipidemias, diabetes mellitus, obesity, smoking, excessive alcohol consumption, sugar consumption, family history, lack of physical activity, psychosocial factors, and environmental pollution [2]. While the individual contribution of each risk factor varies between different communities or ethnic groups, the consistency of the overall contribution of these risk factors to epidemiological studies is remarkably strong [3]. Some of these risk factors, such as age, gender, or family history, are immutable; however, many important cardiovascular risk factors can be modified by lifestyle change, social change, drug treatment, and prevention of Serrano's Cardiac Triad: hypertension, hyperlipidemia, and diabetes.

Evidence-Based Validation of Herbal Medicine
http://dx.doi.org/10.1016/B978-0-12-800874-4.00024-6

24.2 EPIDEMIOLOGY OF CVD

By 2030, it is projected that noncommunicable diseases will account for more than three-quarters of deaths worldwide. CVD alone will be responsible for more deaths in developing and low-income countries [4]. By 2005, the total number of deaths due to CVD (mainly coronary heart disease, stroke, and rheumatic heart disease) had increased globally to 17.5 million from 14.4 million in 1990. Of these, 7.6 million and 5.7 million were attributed to coronary heart disease and stroke, respectively. Among all deaths due to CVD, more than 80% of the deaths occurred in low- and middle-income countries [5]. The World Health Organization (WHO) estimates that cardiovascular deaths will account for 30% of all deaths worldwide [6]. Therefore, CVD is the largest single contributor to global mortality and will continue to dominate in future [5].

The Prevention of Occurrences of Myocardial Infarction and Stroke (PREMISE) study, led by WHO, included developing countries from the Middle East, Asia, and Latin America [7] and suggested several gaps for patients with CVD. The relative lack of implemented use of pharmacological interventions as shown in the PREMISE study is illustrative of the potential link between rising trends in risk factors, lack of availability of and access to medicines, and inadequate delivery of health care services in developing countries. To prevent the growing trend of CVD, herbal medicine, which has good efficacy with multiple effects, can offer a better therapeutic option.

24.3 HERBAL DRUG VERSUS MODERN MEDICINE

Although most of the cardiovascular disorders usually affect older adults, the process begins in early life. Hence, primary prevention should be initiated right from childhood. Most of the cardiovascular disorders, i.e., hypertension, heart failure, coronary artery disease and hyperlipidemias, are chronic progressive disorders and lead to cellular injury, inflammation, oxidative stress, endoplasmic reticulum (ER) stress, and alteration of cellular metabolism that ultimately result in organ dysfunction. An interaction and cross talk among all of these injurious parameters is very important, and this cross talk is highlighted by an aberration in the critical feedback loop. Thus, application of pharmacological agents with multiple roles against all kinds of pathological stress as stated above would be essential to return this feedback loop to healthy communication. The herb or its different combinations may inhibit the critical feedback loop to restore the functional integrity of different organs.

Modern medicines used for the treatment of different cardiovascular disorders mostly offer symptomatic relief or, at most, may slow down the progression of the disease. Even medical practitioners may not recommend the drugs as a preventive measure in the early stage of the disease due to the chances of adverse effects outweighing the benefits. In fact, in some cardiac diseases such as heart failure, frustrations arising out of therapeutic inadequacies have prompted scientists to look for some extreme alternatives like gene therapy and stem cell therapy. In the treatment of uncontrolled systemic hypertension, nonpharmacological therapies, like renal sympathetic denervation, are being tried with limited success.

Under such circumstances, considering the high risk of morbidities and mortality of CVD coupled with the limitations of modern medicines, there is an urgent need to explore herbs for the prevention and, if possible, “cure” of CVD. In the present chapter, the uses of herbal medicines against CVD in India and other countries is discussed. At the same time, we examine whether the current scientific data obtained from different scientific studies are enough to integrate the herbal medicine into evidence-based medical therapy.

24.4 VALIDATION OF INDIAN MEDICINAL PLANTS FOR CARDIOPROTECTION

Several Indian medicinal plants have been explored in animals and humans for their usefulness against different CVDs. Herbal products or plant extracts are tested in different animal models and looked at for their molecular mechanisms. Some of the products are also evaluated for finding the chemical constituents for bioactivity.

The mechanisms of action of most of the herbal medicines, whether single herbal or multiple herbal formulations, have not been elucidated due to the lack of knowledge about their active compounds and phytochemical constituents. The same problem is applicable to their pharmacokinetics and bioavailability studies. In the case of single-molecule pharmaceuticals, there is no uncertainty as to which chemical compound is to be used for pharmacokinetic and bioavailability studies, but that is not always the case with herbals. Herbal medicines are constrained by their unknown and/or unidentifiable active chemical constituents. Nevertheless, some investigators have attempted to conduct such studies.

24.5 EXPERIMENTAL DATA OBTAINED FROM INDIAN MEDICINAL PLANTS FOR CARDIOVASCULAR ACTIVITY

There are several Indian medicinal plants and their single components which have been shown to have beneficial effects against cardiovascular disorders (Table 24.1)[8–51]. Out of all Indian medicinal plants, *Terminalia arjuna* showed a great potential to be developed as an herbal medicine for cardiac disorders. *Terminalia arjuna* is commonly known as arjuna or arjun tree in the Indian subcontinent, which includes India, Pakistan, and Bangladesh. The stem bark of arjuna was introduced into Ayurveda as a treatment for heart disease (termed as *Hridroga*). The Arjuna bark has been extensively studied over the last century in different animal models to demonstrate its cardioprotective properties, like positive inotropic, hypolipidemic, coronary vasodilatory, antioxidant effects and the induction of stress protein in the heart. Stem bark of *T. arjuna* has been reported to attenuate myocardial fibrosis and oxidative stress induced by chronic beta-adrenoceptor stimulation. *Terminalia arjuna* treatment significantly attenuated cardiac dysfunction and myocardial injury in rats with chronic heart failure. This cardioprotective action of *T. arjuna* was comparable to fluvastatin, a synthetic drug [8]. A study suggested that the protection might be through maintaining endogenous antioxidant enzyme activities, inhibiting lipid peroxidation and cytokine levels. Chronic oral administration of *T. arjuna* in rabbits caused augmentation of myocardial antioxidants, i.e., superoxide dismutase, catalase, and glutathione along with induction of heat shock protein72 (HSP72) [9]. In vivo ischemic-reperfusion injury oxidative stress, tissue injury of the heart, and hemodynamic effects were prevented in the *T. arjuna*-treated rat and rabbit hearts [9,10]. These studies are significant in view of the fact that oxidative stress plays an important role in the pathogenesis of coronary artery disease and its associated effects on cardiac functions. *Terminalia arjuna* bark extract also attenuated myocardial fibrosis and oxidative stress induced by chronic beta-adrenoceptor stimulation [52]. A beneficial cardioprotective effect of *T. chebula* against isoproterenol-induced myocardial necrosis was reported through stabilization of the lysozomal membrane [23].

Several studies provide a scientific basis for the putative therapeutic effect of Arjuna in ischemic heart disease. *Terminalia arjuna* bark extract has significant prophylactic and therapeutic effects on protection of the heart against heart failure. The bark has been reported to contain several bioactive compounds that alone or in combination may have beneficial effects against cardiac disorder. *Terminalia chebula* is another Ayurvedic drug recommended for the treatment of heart diseases.

Garlic (*Allium sativum*) is another potential herb for use in cardiovascular disorder. Garlic has been shown to protect against myocardial infarction, doxorubicin-induced cardiotoxicity, cardiac arrhythmias, cardiac hypertrophy, and ischemia-reperfusion injury [11]. The induction of cardiac endogenous antioxidants and the reduction of lipid peroxidation by garlic have been reported previously. Other mechanisms, such as regulating ion channels, modulating Akt signaling pathways, histone deacetylase inhibition, and cytochrome P450 inhibition, may also be responsible for the cardioprotective effect of garlic [12].

Tinospora cordifolia has been investigated for its antiarrhythmic activity in rats. The PQRST waves were normalized, and atrial, as well as ventricular, fibrillation was controlled in rats treated with *T. cordifolia* [14]. This study indicated that *T. cordifolia* can be used in antiarrhythmic clinical settings and beneficial in atrial and ventricular fibrillation and flutter, and may be useful in ventricular tachyarrhythmias. Rao et al. [15] also reported the dose-dependent reduction in infarct size and lipid peroxide levels in infarct heart tissue with the prior treatment of *T. cordifolia*. However, properly designed clinical trials are absolute prerequisites for their use in patients.

Emblica officinalis, commonly known as amla in India, is an important medicinal plant reputed for its dietary and therapeutic uses in traditional systems of medicine. A study demonstrated the cardioprotective potential of *E. officinalis* by improvement in hemodynamic, contractile function, and tissue antioxidant status [53]. Rajak et al. [16] reported that amla has a property to adapt tissues against cardiac ischemic injury by enhancing endogenous antioxidants. The cardioprotective effects of *Glycyrrhiza glabra*, another important herb, were also reported against ischemia-reperfusion injury induced by ligation of the left anterior descending coronary artery in rats [17]. *Saussurea lappa* is commonly known as *kushta*. In Ayurveda, it is mentioned that the aqueous extract of the root *S. lappa* is used for treatment of angina pectoris [18]. The cardioprotective effect of *S. lappa* against isoproterenol-induced myocardial injury was also observed in rats.

Withania somnifera, commonly known as *ashwagandha*, is a potent medicinal plant and has been used for thousands of years as an important medicine in the Ayurvedic system. Chronic administration of *W. somnifera* in rats remarkably augmented endogenous antioxidants and significantly reduced myocardial injury after ischemia and reperfusion [20]. *Eclipta prostrata* has been used as a traditional medicinal plant to prevent dyslipidemia and atherosclerosis in some Asian countries. Experimental data in rats showed that *E. prostrata*

TABLE 24.1 List of Indian Medicinal Plants and Their Application against Cardiovascular Diseases in Animals

Name of plants	Pharmacological actions	Proposed mechanisms	References
Arjun (*Terminalia Arjuna*)	Cardioprotective, cardiac failure, cardiotoxicity	Increased cardiac contractibility, increased endogenous antioxidant, lipid-lowering effect.	[8–10]
Garlic (*Allium sativum*)	Myocardial infarction, cardiotoxicity, arrhythmia, cardiac hypertrophy, and ischemia-reperfusion injury	Regulating ion channels, modulating Akt signaling pathways, histone deacetylase inhibition, antioxidant	[11–13]
Giloy or guduchi (*Tinospora cordifolia*)	Arrhythmias, ischemia-reperfusion –induced myocardial necrosis.	Decreased calcium and sodium levels and increased potassium levels in blood, reduced lipid peroxide levels	[14,15]
Amla (*Emblica officinalis*)	Isoproterenol-induced cardiotoxicity	Reducing lipid peroxidation, antioxidant	[16]
Mulethi (*Glycyrrhiza glabra*)	Dyslipidemia and ischemic injury	Antioxidant effect	[17]
Kustha (*Saussurea lappa*)	Myocardial injury	Antioxidant effect	[18]
Jatamanshi (*Nordostachys jatamanshi*)	Lipidemia	Hypolipidemic effect	[19]
Ashwagandha (*Withania somnifera*)	Cardioprotection and attenuation of ischemic cardiac injury	Antioxidant	[20]
Bach (*Acorus calamus*)	Cardiomyopathy	Attenuation of calcineurin activity, antioxidant	[21]
Badranj boya (*Nepeta hindostana*)	Myocardial infarction	Antioxidant	[22]
Haritaki (*Terminalia chebula*)	Myocardial infarction	Antioxidant	[23]
Bhringaraja (*Eclipta alba* Syn. *E. prostrata*)	Atherosclerosis	Lipid-lowering action	[24]
Shatavari (*Asparagus racemosus*)	Cardioprotection	Lipid-lowering action	[25]
Punarnava (*Boerhaavia diffusa*)	Cardiac injury	Improves mitochondrial function	[26]
Guggulu (*Balsamodendron mukul* Syn. *Commiphora wightii*)	Antihypertensive	Lipid-lowering action and lowering blood pressure	[27]
Mandukaparni (*Centella asiatica*)	Cardioprotection	Antioxidant	[28–30]
Shankhapushpi (*Convolvulus pluricaulis*)	Cardioprotection	Antioxidant	[31]

TABLE 24.1 List of Indian Medicinal Plants and Their Application against Cardiovascular Diseases in Animals—cont'd

Name of plants	Pharmacological actions	Proposed mechanisms	References
Vishnu priya (*Ocimum sanctum* Syn. *O. tenuiflorum*)	Cardioprotection	Anti-apoptotic	[32–34]
Jatamansi (*Nardostachys jatamansi*)	Antihyperlipidemia and cardioprotection	Antioxidant and lipid-lowering	[19]
Pippali (*Piper longum*)	Cardioprotection	Antioxidant	[35]
Sunthi (*Zingiber officinale*)	Cardioprotection	Antioxidant	[36]
Musta (*Cyperus rotundus*)	Cardioprotection	Antioxidant	[37]
Vacha (*Acorus calamus*)	Cardioprotection	Attenuating calcineurin activity and antioxidant	[21]
Vidanga (*Embelia ribes*)	Cardioprotection	Antioxidant	[38]
Lavanga (*Syzygium aromaticum*)	Cardioprotection	Antioxidant	[39]
Jyotishmati (*Celastrus paniculatus*)	Antihyperlipidemic	Lipid-lowering action	[40]
Chandana (*Santalum album*)	Antihyperlipidemic	Lipid-lowering action	[41]
Ela (*Elettaria cardamomum*)	Antihypertensive and antihyperlipidemic	Lowers blood pressure and lipid levels	[42]
Shatapushpa (*Foeniculum vulgare*)	Antithrombotic	Vasorelaxant and clot dissolution	[43]
Satapatrika (*Rosa damascena* Syn. *R. centifolia*)	Cardiac failure	Inotropic effect	[44]
Tvak patra (*Cinnamomum cassia*)	Ischemic heart injury	Increased NO, anti-inflammatory and antioxidant	[45]
Kumkuma (*Crocus sativus*)	Cardioprotective	Antioxidant	[46]
Terminalia bellirica	Cardiotonic	Cardiotonic	[47]
Pomegranate (*Punica granatum*)	Antihypertensive	Inhibit angiotensin converting enzymes (ACE)	[48]
Curcumin (*Curcuma longa*)	Antihyperlipidemic, cardioprotective	Antioxidant, PTP1B inhibition, SIRT-1 activation,	[49–51]

significantly increased high-density lipoprotein-cholesterol levels. Atherogenic indices were decreased by 10–30% in animals fed diets supplemented with *E. prostrata*. The protective effect of *E. prostrata* is due to its beneficial effect on serum lipid and oxidative metabolism [24]. The health-promoting effects of *E. prostrata* may have implications for atherosclerosis and hypercholesterolemia in humans too.

Centella asiatica has beneficial effects on the tissue antioxidant defense system and has a beneficial effect against adriamycin-induced cardiac damage in rats. Experimental data also strongly suggests the cardioprotective activity of *Hydrocotyle asiatica* in limiting ischemia-reperfusion–induced myocardial injury. Similarly, the cardioprotective effect of *Curcuma longa* has also been reported in the ischemia-reperfusion injury

of rat hearts. *Curcuma longa* attenuated cell death due to apoptosis and prevented the impairment of cardiac performance [32].

The cardioprotective effect of the fruit of *Embelia ribes* was reported in acute myocardial infarction, induced by isoproterenol in rats [38]. *Acorus calamus* has been used as a traditional remedy since ancient days. Recently, experimental data showed that *A. calamus* was effective in attenuating isoproterenol-induced cardiomyopathy by virtue of its calcineurin-attenuating activity and antioxidant effects [21].

Nardostachys jatamansi is commonly used as a folk medicine in India for its hypolipidemic activity, which has a direct relationship with cardioprotection. Experimental data with rats showed that *N. jatamansi* exhibits a fair amount of antioxidant and hypolipidemic activities [19]. *Crocus sativus* L. (saffron) attenuated isoproterenol-induced myocardial injury by strengthening the antioxidant defense system and, thus, preserved cardiac functions [46]. The protection was also attributable to an anti-inflammatory effect as well as increased NO level. *Cinnamomum cassia* is a well-known traditional herb that is widely used for the treatment of ischemic heart disease. It is one of several species of *Cinnamomum* that are used primarily for their aromatic bark, which is used as a spice in India and China. The effects of cinnamic aldehyde and cinnamic acid isolated from *C. cassia* also offered protection against isoproterenol-induced myocardial ischemia in rats [45].

Besides medicinal purposes, *Foeniculum vulgare* (fennel) seed is also used by different cultures in India, Pakistan, Afghanistan, Iran, and the Middle East in their cookery. Essential oil from *F. vulgare* provided significant protection against thrombosis. Data demonstrated that anethole, a component of *F. vulgare*, showed antithrombotic activity that appeared to be due to the broad-spectrum antiplatelet activity, clot destabilizing effect, and vasorelaxant action [43].

24.6 MEDICINAL PLANTS USED FOR CARDIOVASCULAR DISEASES OTHER THAN INDIAN MEDICINAL PLANTS

In the last few decades, many scientific efforts have been made into investigating local plants from different countries for their potential beneficial effects in cardiovascular disorders. Some of these have been validated in properly designed experimental studies. Table 24.2 [54–84] shows a list of plants that are used in China, Africa, and other countries that have so far been scientifically validated and reported to have a protective effect against cardiovascular disorders.

Among several medicinal plants, danshen (*Salvia miltiorrhiza*), jiao gu lan *(Gynostemma pentaphyllum)* and *Monascus purpureus* have shown a lipid-lowering effect. Besides lipid-lowering and antiatherosclerotic effects, danshen is also effective in different other cardiovascular disorders, such as blood clotting abnormalities, ischemic heart disease, angina pectoris, LDL oxidation, myocardial infarction, and congestive heart failure. Dong quai (*Angelicae sinensis*), Chinese hawthorn (*Crataegus pinnatifida*), and danshen (*S. miltiorrhiza*) have shown an antithrombic effect through inhibition of platelet aggregation.

The number of medicinal plants have been explored and have shown good activity against hypertension. These include *Camellia sinensis*, *G. pentaphyllum* (jiao gu lan), *Helianthus annuus*, *Trachycarpus fortunei* (hook.), *Viscum album* (mistletoe), *Capparis cartilaginea* (lasaf), *Hibiscus sabdariffa* (roselle), *Panax ginseng* (ginseng), *Coleus forskohlii* (forskolin), *Cardiospermum halicacabum*, *Aristolochia manshuriensis* (guan mu tong), *Desmodium styracifolium* (osbeck), *Lepidium latifolium* (rompepiedra or stone breaker), *Bassla axillaris*, and *Musanga cecropiodes* (umbrella tree or cork wood).

Cardiotonic activity was also reported from *C. forskohlii* (forskolin), *Cocculus hirsutus*, and *Nerium oleander*. However, the mechanism(s) behind this effect need to be explored properly. *Polygonum cuspidatum* and *Rhodiola rosea (*hong jing tian) have shown to have a cardioprotective effect that needs to be translated into humans. Promising beneficial effects of *S. miltiorrhiza (*danshen) and *P. ginseng* also need to be investigated in more detail to prove their reported effects regarding congestive heart failure.

24.7 DATA OBTAINED FROM HUMAN STUDIES WITH MEDICINAL PLANTS FOR CARDIOVASCULAR EFFECTS

Several medicinal plants that showed promising data in experimental studies were also explored in various clinical trials for their efficacy and safety in cardiovascular disorders (Table 24.3)[85–106]. Most of the animal studies with *T. arjuna* have reported antioxidant, anti-ischemic, antihypertensive, and antihypertrophic effects, which have relevance to its therapeutic potential in CVDs in humans. The stem bark of *T. arjuna* is used in India by the Ayurvedic physicians for the treatment of various CVDs. Several clinical studies have reported its efficacy, mostly in patients with ischemic heart disease, hypertension, and heart failure, and have been reviewed elsewhere [107]. A number of clinical studies have also reported its beneficial effects in patients of chronic stable angina, endothelial dysfunction, heart failure, and even ischemic mitral regurgitation [108]. There was a 50% reduction in anginal episodes in stable angina patients after the treatment of *T. arjuna*. It has

TABLE 24.2 List of Medicinal Plants from Countries Other than India and Their Application against Cardiovascular Diseases in Animals

Name of plants	Pharmacological actions	Proposed mechanisms	References
Danshen (*Salvia miltiorrhiza*)	Cardiovascular disorders such as atherosclerosis or blood clotting abnormalities; ischemic heart disease, angina pectoris, myocardial infarction and congestive heart failure.	Relax coronary arteries, elicit an antioxidant salvage effect upon the myocardium, reduce intimal thickness in air-injured carotid arteries, inhibit platelet aggregation, and prevent low-density lipoprotein (LDL) oxidation	[54]
Dong quai (*Angelicae sinensis*)	Antiarrhythmic, antithrombotic	Ferulic acid may cause platelet dysfunction by inhibiting production of thromboxane A2,improve the blood circulation of the injured nerve.	[55]
Ginseng (*Panax ginseng*)	For angina pectoris, myocardial infarction, congestive heart failure, and hypertension	Improved diastolic relaxation,hypotensive effects through enhanced synthesis of nitric oxide	[55]
Monascus purpureus	Lower cholesterol levels, improved blood circulation	Blocking the action of HMG-CoA reductase	[56]
Polygonum cuspidatum	Cardioprotective activity	Due to presence of resveratrol	[57]
Hong jing tian (*Rhodiola rosea*)	Cardioprotective	_	[58]
Tetrandrine (*Stephania tetrandra*)	Its cardiovascular effects may particularly be related to its blockage on L-type calcium channels.	Block multiple ion channels, such as L-type, T-type calcium channels, and Ca^{2+} release-activated Ca^{2+} channels	[59]
Jiao gu lan (*Gynostemma pentaphyllum*)	Antihypertension,antihyperlipidemic	Stimulates the release of nitric oxide, causing blood vessels to relax.Lowers serum cholesterol,triglycerides and LDL while raising HDL levels	[60]
Monodora myristica	Antihypertensive	Presence of high level of unsaturated fatty acid, reduces coronary heart disease	[61]
Bassla axillaris	Reduced systolic and diastolic blood pressure	_	[62]
Starchytarpheta jamaicensis	Cardiovascular effects	The acute hypotensive effect may be partly due to the negative chronotropic effect or to a direct effect on vascular smooth muscle.	[62]
Camellia sinensis	Hypertension,hematemesis	Activation of vascular endothelial layer to release vasorelaxant	[63]
Helianthus annuus	Hypertensive	_	[63]
Trachycarpus fortunei (Hook.)	Hypertensive	_	[63]
Forskolin (*Coleus forskohlii*)	Hypotensive, cardiotonic	Positive inotropic action on cardiac tissue via increased cAMP levels	[64]
Cardiospermum halicacabum	Hypotensive	_	[47]
Cocculus hirsutus	Cardiotonic	_	[47]
Nerium oleander	Cardiotonic	An increase in heart rate, cardiac flow, and force of contraction	[47]

Continued

TABLE 24.2 List of Medicinal Plants from Countries Other than India and Their Application against Cardiovascular Diseases in Animals—cont'd

Name of plants	Pharmacological actions	Proposed mechanisms	References
Custard apple (*Annona muricata*)	Antihypertensive	Decreasing the peripheral vascular resistance	[65]
Celery (*Apium graveolens*)	Reduce systolic and diastolic blood pressure	Might block voltage-dependent and receptor-operated Ca^{2+} channels	[66]
Guan mu tong (*Aristolochia manshuriensis*)	Hypotensive	Inhibited lipid accumulation in blood vessels	[67]
Breadfruit (*Artocarpus altilis*)	Decreased the tension of phenylephrine-stimulated isolated guinea pig aorta rings by 15–35%	Exerted weak negative chronotropic effect to reduce left ventricular pulse pressure and negative inotropic effect on right ventricular myocardial strips	[65]
Lasaf (*Capparis cartilaginea*)	Produces a dose-dependent decrease in blood pressure and slight bradycardia in anesthetized rats	Caused inhibition of norepinephrine or K^{+}-induced contractions	[66]
Virginia day flower (*Commelina virginica*)	Decrease the tension of phenylephrine-stimulated isolated guinea pig aorta rings	_	[65]
Chinese hawthorn (*Crataegus pinnatifida*)	Lowers blood pressure, inhibits platelet aggregation and thrombosis, prevents strokes and reduces the risk of heart attack by lowering blood pressure, increasing circulation, and inhibiting both the formation of plaque on arterial walls and formation of blood clots in the brain, heart, and arteries	Vasorelaxation resulting from nitric oxide stimulation,significant antioxidant activity, and a tonic action on cardiac myocytes.	[67–69]
River lily, swamp lily (*Crinum glaucum*) Western Nigeria	Decrease in systolic and diastolic pressures	Decrease the frequency of cardiac contractions, cause bradycardia or atrioventricular conduction disturbance	[70]
Osbeck (*Desmodium styracifolium*)	Hypotensive	Produces two successive hypotensive actions: the first one mediated through cholinergic receptor stimulation, whereas the second was potentiated by blockades of autonomic ganglion and alpha-adrenoceptor	[57]
Hardy fuchsia, chiko, tilco (*Fuchsia magellanica*) Southern Argentina and Chile	Lowers blood pressure, strong reduction in the mean arterial pressure	Diuretic effect may be responsible for reducing blood pressure.	[71]
Roselle (*Hibiscus sabdariffa*) West African,Nigeria	Antihypertensive	Reduces free radical due to presence of vitamin C and anthocyanins	[72]
French lavender (*Lavandula stoechas*)	Produces a fall in blood pressure and heart rate	_	[73]
Rompepiedra or stone breaker (*Lepidium latifolium*)	Hypotensive effect	Hypotensive effect due to its diuretic action in rats	[74]
Murungai (*Moringa oleifera*)	Reduces systolic, diastolic, and mean blood pressure, acts as a hypolipidimic	_	[75]
Umbrella tree, cork wood (*Musanga cecropiodes*) West Africa	Vasorelaxant, hypotensive agent	_	[76]

TABLE 24.2 List of Medicinal Plants from Countries Other than India and Their Application against Cardiovascular Diseases in Animals—cont'd

Name of plants	Pharmacological actions	Proposed mechanisms	References
Basil (*Ocimum basilicum*	Reduces systolic, diastolic, and mean blood pressure	Eugenol, one of the components of the plant, exerts its effect by blocking the calcium channels	[77]
Nela nelli (*Phyllanthus amarus*)	Produces cardiotonic and a fall in mean diastolic, systolic, and mean arterial pressure	The cardiotonic activity was exhibited due to positive ionotropic and chronotropic effect.	[78]
Kudzu (*Pueraria lobata*) China	Decrease in blood catecholamine levels, blood pressure, and heart rate.	Improve vascular function due to their lipid-lowering, antioxidant and nitric oxide production, as well as their phytoestrogenic properties	[79]
Radish (*Raphanus sativus*)	Reduces blood pressure and heart rate mediated through an atropine-sensitive pathway	Effects are mediated through activation of muscarinic receptors	[80]
Coriaceum oliver	Reduces blood pressure	Reduce blood pressure through calcium channel blockade activity	[81]
Sesame (*Sesamum indicum*)	Antihypertensive	Presence of acetylcholine-like substance in the seeds might be responsible.	[82]
Cat's claw herb (*Uncaria rhynchophylla*)	Reduces blood pressure and relieves various neurological symptoms	Inhibits Ca^{2+} influx mainly through a voltage-dependent Ca^{2+}channel.	[83]
Mistletoe (*Viscum album*)	Antihypertensive	Possible inhibition of sympathetic stimulation	[84]

been reported that *T. arjuna* reduced left ventricular mass (cardiac hypertrophy) along with improvement in LV ejection fraction in patients of angina. Similarly, a hydroalcoholic extract of *T. arjuna* demonstrated some beneficial effects in 12 patients of severe refractory heart failure (NYHA class IV). Patients treated with *T. arjuna* have shown a significant decrease in total cholesterol and LDL cholesterol. *Terminalia arjuna* reversed smoking-related endothelial dysfunction very much similar to the reported reversal of endothelial dysfunction in smokers with the use of vitamins C and E [108]. However, major shortcomings in all these human studies are the absence of phytochemical standardization of the extracts, preclinical toxicity studies along with pharmacological interactions with other drugs, and large multicenter randomized clinical trials.

Similar to *T. Arjuna*, garlic (*A. sativum*) has been widely recognized for prevention and treatment of CVDs like atherosclerosis, hyperlipidemia, thrombosis, hypertension, myocardial necrosis, and cardiomyopathy, which has been reviewed [11]. Effectiveness of garlic in CVDs was more encouraging in different animal studies, which prompted many clinical trials, many of which showed its positive effect on almost all cardiovascular conditions like hyperlipidemias, thrombosis, platelet aggregation, and hypertension [11]. However, several issues regarding the proper use of garlic, i.e., use of different preparations available, dose, duration, and interaction with generic drugs, should be optimized before its recommended use along with modern medicines.

Apium graveolens showed effectiveness in two different human studies against hypertension [66,91]. *Achllea Wilhelmsi* showed antihyperlipidemic and antihypertensive effects in a clinical study. A significant decrease in triglycerides and total cholesterol and LDL-C were evidenced after four months of intervention, whereas HDL-C levels were increased after six months of treatment. Appreciable decreases in diastolic and systolic blood pressures were also observed after two and six months of *A. wilhelmsii* treatment [92]. *Zingiber officinale* (ginger) administration in humans for 45 days reduced triglycerides and LDL-C and increased HDL-C levels [96]. Two studies in India and the United Kingdom with artichoke (*Cynara cardunculus var. scolymus*) showed the reduction of total cholesterol and LDL-C in the artichoke-treated group [97,98].

TABLE 24.3 List of Medicinal Plants and Their Application aagainst Cardiovascular Diseases in Humans

Name of plant/ herbal product	Country of study	Diseases indication	Efficacy	No of patients	References
Terminalia arjuna	India	Post myocardial infarctionIschemic cardiomyopathy	↓ Symptoms of angina, left ventricular ejection fraction, and left ventricular mass *Terminalia* group, severity of cardiomyopathy also improved from NYHA class III to Class I in two patients during the study	N = 10 (myocardial infarction) N = 2 (ischemic cardiomyopathy)	[85]
Terminalia arjuna	India	Congestive heart failure with severe refractory heart failure (NYHA class IV).	Improvement of edema, fatigue and dyspnea along with walking tolerance, stroke volume, left ventricular ejection fraction, with decreases in end-diastolic and end-systolic left ventricular volume	N = 12	[86]
Terminalia arjuna	India	Patients with coronary artery disease (CAD)	↓ Total cholesterol, ↓ LDL cholesterol, ↓ lipid peroxide levels after 30-day follow-up.	N = 105	[87]
Allium sativam	Australia	Progression of carotid atherosclerosis	Anti-atherosclerotic effect on carotid atherosclerosis	N = 196	[88]
Allium sativam	India	Hypertension, Oxidative stress	↓ 8-hydroxy-2-deoxyguanosine, ↓ nitric oxide level, ↓ lipid peroxidation, ↑ vitamins C, ↑ vitamin E	N = 20	[89]
Allium sativam	India	Effect of garlic powder (Kwai) on plasma lipids and lipoproteins in mild hypercholesterolemia	No change in blood lipids and lipoproteins levels	N = 28	[90]
Apium graveolens	China	Hypertension	↓ Blood pressure[a] ↓ Blood pressure[b]	N = 14[a] N = 16[b]	[66,91]
Achllea Wilhelmsi	Iran	Antihyperlipidemic and antihypertensive effects	↓ Triglyceride after 2 months, ↓ total cholesterol and LDL-C after 4 months ↑ HDL-C levels after 6 months of treatment, ↓ diastolic and systolic blood pressure after 2 and 6 months, respectively	N = 120	[92]
Fenugreek *Trigonella foenum*	India	Coronary artery disease (CAD) patients and patients with type 2 diabetes with or without CAD	↓ Total cholesterol ↓ triglycerides	N = 30	[93]
Curcumin	India	Lipid level in patients with acute coronary syndrome	Moderate-dose curcumin showed the minimal effect of increase, followed by the low-dose curcumin and finally, the high-dose curcumin that showed the highest effect of increase.	N = 75	[94]
Curcumin	India	Overweight hyperlipidemia	Reduction in lipid profiles such as serum total cholesterol, triglyceride and	N = 53	[95]

TABLE 24.3 List of Medicinal Plants and Their Application aagainst Cardiovascular Diseases in Humans—cont'd

Name of plant/ herbal product	Country of study	Diseases indication	Efficacy	No of patients	References
(Ginger) *Zingiber officinale*	Saudi	Lipid levels in patients with hyperlipidemia after 45 days.	LDL-cholesterol and VLDL-cholesterol ↓Triglyceride, ↓ LDL-C, ↑ HDL-C	N = 45	[96]
Artichoke (*Cynara cardunculus var. scolymus*)	India	Hyperlipoproteinemia	↓ Total cholesterol, ↓ LDL-C	N = 143	[97]
Artichoke (*Cynara cardunculus var. scolymus*)	UK	Hypercholesterolemia	↓ Total cholesterol	N = 75	[98]
Rhubarb (*Rheum rhapontiam*)	USA	Cholesterol-lowering effect in hypercholesterolemic men	↓ Total cholesterol (8%) and LDL-C (9%), while HDL-C concentrations remained unchanged. The depressed total and LDL-C levels returned to baseline after the fiber supplementation was withdrawn for 1 month.	N = 10	[99]
Hibiscus sabdariffa L. tea	Bosten	Pre- and mildly hypertensive adults	↓ Blood pressure in pre- and mildly hypertensive adults	N = 65	[100]
Combination of *Commiphora mukul* and *Inula racemosa* (1:1 ratio)	India	Ischemic heart disease	↓ Total cholesterol ↓ Triglycerides ↓ Total blood lipids An improvement in the ECG was observed in 59% of the patients at the end of six-month study period.	N = 200	[101]
Inula racemosa	India	Ischemic heart disease	All nine subjects showed improvement in ST-segment depression on ECG.	N = 9	[102]
Crataegus	China	Cardiac insufficiency stage NYHA II heart failure	Reduced performance in the exercise tolerance test, fatigue, palpitation, and exercise dyspnea.	N = 1011	[103]
C. mukul Guggul	USA	Hypercholesteromia	No improvement of cholesterol level	N = 103	[104]
C. mukul (Guggul) in combination with *Inula racemosa*	India	Ischemic heart disease	Improved electrocardiogram readings and decreased episodes of dyspnea and chest pain	N = 200	[105]
Crataegus monogyna (Hawthorn)	USA	Heart failure	Provides no symptomatic or functional benefit when given with standard medical therapy to patients with heart failure	N = 120	[106]

While most of the human studies were smaller, Tauchert et al. carried out a relatively bigger study after recruiting 1011 patients with cardiac insufficiency of stage NYHA II, treated with standardized hawthorn (crataegus) extract WS 1442 (Crataegutt novo® 450, one tablet b.i.d.) over a period of 24 weeks. A significant improvement in clinical symptoms (reduced performance in the exercise tolerance test, fatigue, palpitation,

and exercise dyspnea, ankle edema, and nocturia) was observed at the end of the study. An improved ejection fraction was also observed. Almost two-thirds of the patients felt better or much better following the 24 weeks of treatment, while more than three-fourths of the participating physicians recorded a good or a very good efficacy, and 98.7% noted a good or a very good tolerance. This study concluded that high-dose hawthorn therapy is an efficient, well-tolerated, and easily regulated therapeutic alternative for patients suffering from cardiac insufficiency stage NYHAII [103].This study showed a good example for exploring plant or herbal products in humans. Basic scientists and clinicians should look experimental data carefully and translate that knowledge in humans.

24.8 INDIAN INITIATIVES

Safety is a major concern for the use of herbals in humans. In many countries like India, traditional systems of medicines have been using these plants for hundreds of years and enjoy both governmental and cultural patronage. Therefore, they are widely believed to be "safe." However, that is not always true. Various factors, like major changes in ecosystems, geographical variations, faulty botanical identifications, etc., can render toxicities to plants. So it is recommended that screening for any potential toxic effects should be carried out. In this respect, Indian Council of Medical Research (ICMR), an apex governmental organization has come out with some guidelines which are available at http://icmr.nic.in/ethical_guidelines.pdf.

The process of toxicity studies is lengthy and expensive. In the absence of any major industrial participation in the field of development of herbals, it is rather difficult, if not impossible, to carry out the battery of toxicity studies, as required for new chemical entities (NCE), which could have been ideal. Therefore, ICMR recommends limited toxicity studies for those plants which are mentioned in Indian traditional medicine (Ayurveda and Unani) and for those plants for which a different part of the plant or a different extract other than that mentioned in the traditional text is used.

The standardization of phytoconstituents of the medicinal plants used in Indian traditional medicine has been recently described in detail in Ayurvedic Pharmacopoeia of India and quality standard monographs of ICMR. The best way of instilling evidence into the claims of medicinal values of herbs is a properly designed clinical trial, like a randomized clinical trial. In India, AYUSH, a governmental organization looking after traditional systems of medicine, has taken a big step forward by promoting clinical trials of herbal medicines. We expect a paradigm shift in our knowledge about their efficacy and safety in coming years that will help in the integration of the traditional system with the mainstream.

24.9 CONCLUSION

There are several ways by which we can integrate herbal and traditional medicine with modern medicine: (1) incorporating it as an integral part of a country's health care system, (2) practicing it along with modern medicine by individual health care practitioners, (3) incorporating elements of both traditional and modern practices to form a new branch to solve complex health problems, (4) modern medicine practitioners imparting training in traditional medicine, and (5) documenting successful integration between traditional and modern medicine in clinical practice. During the last two to three decades, developed countries, such as the United States, Canada, Australia, and members of the European Union, have judiciously promoted the use of herbal medicine in the form of complementary and alternative medicine. However, efficacy or safety of the majority of herbal medicines in CVDs has not been fully established through an evidence-based approach. Further, other issues, such as scientific, cultural, educational, economical, and legal, need to be addressed.

References

[1] Finegold JA, Asaria P, Francis DP. Mortality from ischaemic heart disease by country, region, and age: statistics from World Health Organisation and United Nations. Int J cardiol 2012;168(2): 934–45.
[2] Howard BV, Wylie-Rosett J. Sugar and cardiovascular disease: a statement for healthcare professionals from the Committee on Nutrition of the Council on Nutrition, Physical Activity, and Metabolism of the American Heart Association. Circulation 2002;106(4):523–7.
[3] Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, et al. INTERHEART Study Investigators. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet 2004;364(9438):937–52.
[4] Eaglehole R, Bonita R. Global public health: a scorecard. Lancet 2008;372(9654):1988–96.
[5] WHO. World health statistics. Geneva: World Health Organization; 2009. 2009e.
[6] WHO Department of Child and Adolescent Health and Development. The current evidence for the burden of group a streptococcal diseases. Discussion papers on child health. Geneva: World Health Organization; 2005.
[7] Mendis S, Abegunde D, Yusuf S, Ebrahim S, Shaper G, Ghannem H, et al. WHO study on prevention of recurrences of myocardial infarction and stroke (WHO-PREMISE). Bull World Health Organ 2005;83(11):820–8.
[8] Parveen A, Babbar R, Agarwal S, Kotwani A, Fahim M. Mechanistic clues in the cardioprotective effect of Terminalia arjuna

bark extract in isoproterenol-induced chronic heart failure in rats. Cardiovasc Toxicol 2011;11(1):48–57.
[9] Gauthaman K, Banerjee SK, Dinda AK, Ghosh CC, Maulik SK. *Terminalia arjuna* (Roxb.) protects rabbit heart against ischemic-reperfusion injury: role of antioxidant enzymes and heat shock protein. J Ethnopharmacol 2005;96(3):403–9.
[10] Gauthaman K, Maulik M, Kumari R, Manchanda SC, Dinda AK, Maulik SK. Effect of chronic treatment with bark of *Terminalia arjuna*: a study on the isolated ischemic-reperfused rat heart. J Ethnopharmacol 2001;75(2–3):197–201.
[11] Banerjee SK, Dinda AK, Manchanda SC, Maulik SK. Chronic garlic administration protects rat heart against oxidative stress induced by ischemic reperfusion injury. BMC Pharmacol 2002; 2:16.
[12] Khatua TN, Adela R, Banerjee SK. Garlic and cardioprotection: insights into the molecular mechanisms. Can J Physiol Pharmacol 2013;91(6):448–58.
[13] Banerjee SK, Maulik M, Mancahanda SC, Dinda AK, Gupta SK, Maulik SK. Dose dependent induction of endogenous antioxidants in rat heart by chronic administration of garlic. Life Sci 2002;70(13):1509–18.
[14] Sharma AK, Kishore K, Sharma D, Srinivasan BP, Agarwal SS, Sharma A, et al. Cardioprotective activity of alcoholic extract of *Tinospora cordifolia* (Willd.) Miers in calcium chloride-induced cardiac arrhythmia in rats. J Biomed Res 2011;25(4): 280–6.
[15] Rao PR, Kumar VK, Viswanath RK, Subbaraju GV. Cardioprotective activity of alcoholic extract of *Tinospora cordifolia* in ischemia-reperfusion induced myocardial infarction in rats. Biol Pharm Bull 2005;28(12):2319–22.
[16] Rajak S, Banerjee SK, Sood S, Dinda AK, Gupta YK, Gupta SK, et al. *Emblica officinalis* causes myocardial adaptation and protects against oxidative stress in ischemic-reperfusion injury in rats. Phytother Res 2004;18(1):54–60.
[17] Ojha S, Golechha M, Kumari S, Bhatia J, Arya DS. *Glycyrrhiza glabra* protects from myocardial ischemia-reperfusion injury by improving hemodynamic, biochemical, histopathological and ventricular function. Exp Toxicol Pathol 2013;65(1–2):219–27.
[18] Saleem TS, Lokanath N, Prasanthi A, Madhavi M, Mallika G, Vishnu MN. Aqueous extract of *Saussurea lappa* root ameliorate oxidative myocardial injury induced by isoproterenol in rats. J Adv Pharm Technol Res 2013;4(2):94–100.
[19] Krishnamoorthy G, Shabi MM, Ravindhran D, Uthrapathy S, Rajamanickam VG, Dubey GP. *Nardostachys jatamansi*: cardioprotective and hypolipidemic herb. J Pharm Res 2009;2:574–8.
[20] Gupta SK, Mohanty I, Talwar KK, Dinda A, Joshi S, Bansal P, et al. Cardioprotection from ischemia and reperfusion injury by *Withania somnifera*: a hemodynamic, biochemical and histopathological assessment. Mol Cell Biochem 2004;260(1–2): 39–47.
[21] Singh BK, Pillai KK, Kohli K, Haque SE. Isoproterenol-induced cardiomyopathy in rats: influence of Acorus calamus Linn.: *a. calamus* attenuates cardiomyopathy. Cardiovasc Toxicol 2011; 11(3):263–71.
[22] Ahmad N, Maheshwari TV, Zaidi S, Nasiruddin M. Cardioprotective potential of hydro-alcoholic extract of *Nepeta hindostana* (roth) on isoproternol induced myocardial infarction in rats. Ira J Pharm Res 2004;141(3):50–5.
[23] Suchalatha S, Devi CS. Protective effect of *Terminalia chebula* against lysosomal enzyme alterations in isoproterenol-induced cardiac damage in rats. Exp Clin Cardiol 2005;10(2):91–5.
[24] Kim D, Lee S, Choi J, Lillehoj HS, Yu M, Lee G. The butanol fraction of *Eclipta prostrata* (Linn) effectively reduces serum lipid levels and improves antioxidant activities in CD rats. Nutrition Res 2008;28(8):550–4.
[25] Khanna AK, Chander R, Kapoor NK. Hypolipidaemic activity of Abana in rats. Fitoterapia 1991;62:271–5.
[26] Prathapan A, Vineetha VP, Raghu KG. Protective effect of *Boerhaavia diffusa* L. against mitochondrial dysfunction in angiotensin II induced hypertrophy in h9c2 cardiomyoblast cells. PLoS One 2014;9(4):e96220.
[27] Panneerselvam J, Sambandam G, Nalini N. Single- or double-blind treatment with *Balsamodendron mukul* and nifedipine in hypertensive patients. J Clin Hypertens 2005;7(6):340–5.
[28] Jamil S, Nizami Q, Salam M. *Centella asiatica* (Linn.) urban a review. Nat Prod Rad 2007;6(2):158–70.
[29] Gnanapragasam A, Ebenezar KK, Sathish V, Govindaraju P, Devaki T. Protective effect of *Centella asiatica* on antioxidant tissue defense system against adriamycin induced cardiomyopathy in rats. Life Sci 2004;76(5):585–97.
[30] Pragada RR, Veeravalli KK, Chowdary KPR, Routhu KV. Cardioprotective activity of Hydrocotyle asiatica L. in ischemia-reperfusion induced myocardial infarction in rats. J Ethnopharmacol 2004;93(1):105–8.
[31] Agarwa CP, Sharma B, Fatima A, Jain SK. An update on ayurvedic herb Convolvulus pluricaulis. Asian Pac J Trop Biomed 2014; 4(3):245–52.
[32] Mohanty I, Arya DS, Gupta SK. Effect of *Curcuma longa* and *Ocimum sanctum* on myocardial apoptosis in experimentally induced myocardial ischemic-reperfusion injury. BMC Complement Altern Med 2006;6:3.
[33] Sood S, Narang D, Dinda AK, Maulik SK. Chronic oral administration of *Ocimum sanctum* Linn. augments cardiac endogenous antioxidants and prevents isoproterenol-induced myocardial necrosis in rats. J Pharm Pharmacol 2005;57(1):127–33.
[34] Sood S, Narang D, Thomas MK, Gupta YK, Maulik SK. Effect of *Ocimum sanctum* Linn. on cardiac changes in rats subjected to chronic restraint stress. J Ethnopharmacol 2006;108(3):423–7.
[35] Wakade AS, Shah AS, Kulkarni MP, Juvekar AR. Protective effect of *Piper longum* L. on oxidative stress induced injury and cellular abnormality in adriamycin induced cardiotoxicity in rats. Ind J Exp Biol 2008;46(7):528–33.
[36] Ansari MN, Bhandari U, Pillai KK. Ethanolic *Zingiber officinale* R. extract pretreatment alleviates isoproterenol-induced oxidative myocardial necrosis in rats. Ind J Exp Biol 2006;44(11):892–7.
[37] Jahan N, Rahman KU, Ali S. Cardioprotective and antilipidemic potential of *Cyperus rotundus* in chemically induced cardiotoxicity. Int J Agr Biol 2012;14:989–92.
[38] Bhandari U, Ansari MN, Islam F. Cardioprotective effect of aqueous extract of *Embelia ribes* Burm fruits against isoproterenol-induced myocardial infarction in albino rats. Indian J Exp Biol 2008;46(1):35–40.
[39] Atale N, Chakraborty M, Mohanty S, Bhattacharya S, Nigam D, Harma M, et al. Cardioprotective role of *Syzygium cumini* against glucose-induced oxidative stress in H9C2 cardiac myocytes. Cardiovas Toxicol 2013;13(3):278–89.
[40] Patil RH, Prakash K, Maheshwari VL. Hypolipidemic effect of *Celastrus paniculatus* in experimentally induced hypercholesterolemic wistar rats. Ind J Clin Biochem 2010;25(4):405–10.
[41] Kulkarni CR, Joglekar MM, Patil SB, Arvindekar AU. Antihyperglycemic and antihyperlipidemic effect of *Santalum album* in streptozotocin induced diabetic rats. Pharm Biol 2012;50(3): 360–5.
[42] Verma SK, Jain V, Katewa SS. Blood pressure lowering, fibrinolysis enhancing and antioxidant activities of cardamom (*Elettaria cardamomum*). Ind J Biochem Biophys 2009;46(6):503–6.
[43] Tognolini M, Ballabeni V, Bertoni S, Bruni R, Impicciatore M, Barocelli E. Protective effect of *Foeniculum vulgare* essential oil and anethole in an experimental model of thrombosis. Pharmacol Res 2007;56(3):254–60.

[44] Boskabady MH, Vatanprast A, Parsaee H, Boskabady M. Possible mechanism of inotropic and chronotropic effects of *Rosa damascena* on isolated guinea pig heart. DARU 2013;21:38.
[45] Song F, Li H, Sun J, Wang S. Protective effects of cinnamic acid and cinnamic aldehyde on isoproterenol-induced acute myocardial ischemia in rats. J Ethnopharmacol 2013;150(1): 125–30.
[46] Sachdeva J, Tanwar V, Golechha M, Siddiqui KM, Nag TC, Ray R, et al. *Crocus sativus* L. (saffron) attenuates isoproterenol-induced myocardial injury via preserving cardiac functions and strengthening antioxidant defense system. Exp Toxicol Pathol 2012;64(6): 557–64.
[47] Joy PP, Thomas J, Mathew S, Skaria BP. Medicinal plants. In: Tropical horticulture, vol. 2; 2001. p. 449–632.
[48] Aviram M, Dornfeld L. Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. Atherosclerosis 2001;158:195–8.
[49] Chen TH, Yang YC, Wang JC, Wang JJ. Curcumin treatment protects against renal ischemia and reperfusion injury-induced cardiac dysfunction and myocardial injury. Transplant Proc 2013; 45(10):3546–9.
[50] Yang Y, Duan W, Lin Y, Yi W, Liang Z, Yan J, et al. SIRT1 activation by curcumin pretreatment attenuates mitochondrial oxidative damage induced by myocardial ischemia reperfusion injury. Free Radic Biol Med 2013;65:667–79.
[51] Li JM, Li YC, Kong LD, Hu QH. Curcumin inhibits hepatic protein-tyrosine phosphatase 1B and prevents hypertriglyceridemia and hepatic steatosis in fructose-fed rats. Hepatology 2010; 51(5):1555–66.
[52] Kumar S, Enjamoori R, Jaiswal A, Ray R, Seth S, Maulik SK. Catecholamine-induced myocardial fibrosis and oxidative stress is attenuated by *Terminalia arjuna* (Roxb.). J Pharm Pharmacol 2009;61(11):1529–36.
[53] Ojha S, Golechha M, Kumari S, Arya DS. Protective effect of *Emblica officinalis* (amla) on isoproterenol-induced cardiotoxicity in rats. Toxicol Ind Health 2012;28(5):399–411.
[54] Wang B. *Salvia miltiorrhiza*: chemical and pharmacological review of a medicinal plant. J Med Plants Res 2010;4(25):2813–20.
[55] Valli G, Giardina EG. Benefits, adverse effects and drug interactions of herbal therapies with cardiovascular effects. J Am Coll Cardiol 2002;39(7):1083–95.
[56] Liu J, Zhang J, Shi Y, Grimsgaard S, Alraek T. Fønnebø V. Chinese red yeast rice (*Monascus purpureus*) for primary hyperlipidemia: a meta-analysis of randomized controlled trials. Chin Med 2006; 1:4.
[57] Stef G, Csiszar A, Lerea K, Ungvari Z, Veress G. Resveratrol inhibits aggregation of platelets from high-risk cardiac patients with aspirin resistance. J Cardiovasc Pharmacol 2006;48(2):1–5.
[58] Ho CS, Wong YH, Chiu KW. The hypotensive action of *Desmodium styracifolium* and *Clematis chinensis*. Am J Chin Med 1989; 17:189–202.
[59] Yao WX, Jiang MX. Effects of tetrandrine on cardiovascular electrophysiologic properties. Acta Pharmacol Sin 2002;23(12): 1069–74.
[60] Mishra RN, Joshi D, Lan JG. (*Gynostemma pentaphyllum*): the chinese rasayan- current research scenario. Int J Res Pharm Biomedical Sci 2011;2(4):1483–1502.
[61] Idu M, Omoruyi O. Effect of different pretreatments on germination and seedling development of *Monodora myristica* (Gaertn.) dunal Seeds. Forests Trees Livelihoods 2002;12:221–8.
[62] Idu M, Omonhinmin AC, Ogboghodo IA. Germination ecology of two Savanna tree species. *Tamarindus indica* and *Prosopis africana*. Seed Tech 2002;24(1):103–7.
[63] Au DT, Jialin WU, Jiang Z, Chen H, Lu G, Zhao Z. Ethnobotanical study of medicinal plants used by Hakka in Guangdong, China. J Ethnopharmacol 2008;117:41–50.
[64] Paul M, Radha A, Kumar DS. On the high value medicinal plant, *Coleus forskohlii* Briq. Hygeia – J Drug Med 2013;5(1):69–78.
[65] Hasrat JA, Pieters L, Vlietinck AJ. Medicinal plants in Suriname. J Pharm Pharmacol 2004;56:381–7.
[66] Gharooni M, Sarkarati AR. Application of *Apium graveolens* in treatment of hypertension. Tehran Univ Med J 2000;58:67–9.
[67] Mashour NH, Lin GI, Frishman WH. Herbal medicine for the treatment of cardiovascular disease: clinical considerations. Arch Intern Med 1998;158:2225–34.
[68] Bensky D, Gamble A. USA: chinese herbal medicine. Materia Medica. Rev ed. Seattle: Eastland Press; 1993.
[69] Brixius K, Willms S, Napp A, Tossios P, Ladage D, Bloch W, et al. *Crataegus* special extract WS 1442 induces an endothelium-dependent, NO-mediated vasorelaxation via eNOS-phosphorylation at serine 1177. Cardiovasc Drugs Ther 2006;20: 177–84.
[70] Ajayi GO, Adegunloye BJ, Oroye O. Effects of *Crinum glaucum* on cardio-respiratory function in anaesthetized cat. Nig J Nat Prod Med 1997;1:15–6.
[71] Schmeda-Hirschmann G, Loyola JI, Sierra J, Ratama, Retamal R, Rodrigue J. Hypotensive effect and enzyme inhibition activity of mapuche. Med Plant Extract Phytother Res 1992;6:184–8.
[72] Mojiminiyi FB, Dikko M, Muhammad BY, Ojobor PD, Ajagbonna OP, Okolo RU, et al. Antihypertensive effect of an aqueous extract of the calyx of *Hibiscus sabdariffa*. Fitoterapia 2007;78:292–7.
[73] Gilani AH, Aziz N, Khan MA, Shaheen F, Jabeen Q, Siddiqui BS, et al. Ethnopharmacological evaluation of the anticonvulsant, sedative and antispasmodic activities of *Lavandula stoechas* L. J Ethnopharmacol 2000;71:161–7.
[74] Navarro E, Alonso J, Rodriguez R, Trujillo J, Boada J. Diuretic action of an aqueous extract of *Lepidium latifolium* L. J Ethnopharmacol 1994;41:65–9.
[75] Faizi S, Siddiqui BS, Saleem R, Aftab K, Shaheen F, Gilani AH. Hypotensive constituents from the pods of *Moringa oleifera*. Planta Med 1998;64:225–8.
[76] Kamanyi A, Bopelet M, Aloamaka CP, Obiefuna PC, Ebeigbe AB. Endothelium-dependent rat aortic relaxation to the aqueous leaf extract of *Musanga cecropioides*. J Ethnopharmacol 1991;34:283–6.
[77] Azhar I, Aftab K, Usmanghani K. Naturally occurring calcium channel blockers. Hamdard Medicus 1995;38:5–16.
[78] Amaechina FC, Omogbai EK. Hypotensive effect of aqueous extract of the leaves of *Phyllanthus amarus* Schum and Thonn (Euphorbiaceae). Acta Pol Pharm 2007;64:547–52.
[79] Fan LL, Zeng GY, Zhou YP, Zhang LY, Cheng YS. Pharmacologic studies on *Radix puerariae*: effects of puerariae flavones on coronary circulation, cardiac hemodynamics and myocardial metabolism in dogs. Chin Med J 1982;95:145–50.
[80] Ghayur MN, Gilani AH. Radish seed extract mediates its cardiovascular inhibitory effects via muscarinic receptor activation. Fund Clin Pharmacol 2006;20:57–63.
[81] Ajagbonna OP, Oneyeyili PA. Effects of ethanol extract of *Rhaptopetalum coriaceum* Oliv. Stem bark on mean arterial pressure and heart rate in rats. Nig J Exp Clin Anal 2002;2:30–3.
[82] Nakano D, Itoh C, Takaoka M, Kiso Y, Tanaka T, Matsumura Y. Antihypertensive effect of sesamin IV inhibition of vascular superoxide production by sesamin. Biol Pharm Bull 2002;25: 1247–9.
[83] Horie S, Yano S, Aimi N, Sakai S, Watanabe K. Effects of hirsutine, an antihypertensive indole alkaloid from *Uncaria rhynchophylla*, on intracellular calcium in rat thoracic aorta. Life Sci 1992;50:491–8.
[84] Ben EE, Eno AE, Ofem OE, Aidem U, Itam EH. Increased plasma total cholesterol and high density lipoprotein levels produced by the crude extract from the leaves of *Viscum album* (mistletoe). Niger J Physiol Sci 2006;21:55–60.

[85] Dwivedi S, Jauhari R. Beneficial effects of *Terminalia arjuna* in coronary artery disease. Ind Heart J 1997;49:507–10.
[86] Bharani A, Ganguly A, Bhargave KD. Salutary effect of *Terminalia arjuna* in patients with severe refractory heart failure. Int J Cardiol 1995;49:191–9.
[87] Gupta R, Singhal S, Goyle A, Sharma VN. Antioxidant and hypocholesterolaemic effects of *Terminalia arjuna* tree-bark powder: a randomised placebo-controlled trial. J Assoc Phys India 2001; 49:231–5.
[88] Orekhov AN, Sobenin IA, Korneev NV, Kirichenko TV, Myasoedova VA, Melnichenko AA, et al. Anti-Atherosclerotic therapy based on botanicals. Recent Patents Cardiovasc Drug Discov 2010;8(1):56–66.
[89] Dhawan V, Jain S. Garlic supplementation prevents oxidative DNA damage in essential hypertension. Mol Cell Biochem 2005;275:85–94.
[90] Simons LA, Balasubramaniam S, von Konigsmark M, Parfitt A, Simons J, Peters W. On the effect of garlic on plasma lipids and lipoproteins in mild hypercholesterolaemia. Atherosclerosis 1995;113(2):219–25.
[91] Somanadhan B, Varughese G, Palpu P, Sreedharan R, Gudiksen L, Smitt UW, et al. An ethnopharmacological survey for potential angiotensin converting enzyme inhibitors from Indian medicinal plants. J Ethnopharmacol 1999;65:103–12.
[92] Asgary S, Naderi GH, Sarrafzadegan N, Mohammadifard N, Mostafavi S, Vakili R. Antihypertensive and antihyperlipidemic effects of Achillea wilhelmsii. Drugs Exp Clin Res 2000;26:89–93.
[93] Bordia A, Verma SK, Srivastava KC. Effect of ginger (*Zingiber officinale Rosc.*) and fenugreek (*Trigonella foenum graecum* L.) on blood lipids, blood sugar and platelet aggregation in patients with coronary artery disease. Prostaglandins Leukot Essent Fatty Acids 1997;56:379–84.
[94] Alwi I, Santoso T, Suyono S, Sutrisna B, Suyatna FD, Kresno SB, et al. The effect of curcumin on lipid level in patients with acute coronary syndrome. Acta Med. Indones 2008;40:201–10.
[95] Pashine L, Singh JV, Vaish AK, Ojha SK, Mahdi AA. Effect of turmeric (*Curcuma longa*) on overweight hyperlipidemic subjects: double blind study. IJCH 2012;24(2):113–7.
[96] Alizadeh-Navaei R, Roozbeh F, Saravi M, Pouramir M, Jalali F, Moghadamnia AA. Investigation of the effect of ginger on the lipid levels. A double blind controlled clinical trial. Saudi Med J 2008;29:1280–4.
[97] Englisch W, Beckers C, Unkauf M, Ruepp M, Zinserling V. Efficacy of artichoke dry extract in patients with hyperlipoproteinemia. Arzneimittelforschung 2000;50:260–5.
[98] Bundy R, Walker AF, Middleton RW, Wallis C, Simpson HC. Artichoke leaf extract (*Cyanara scolymus*) reduces plasma cholesterol in otherwise healthy hypercholesterolemic adults: a randomized, double-blind placebo controlled trial. Phytomedicine 2008;15:668–75.
[99] Goel V, Ooraikul B, Basu TK. Cholesterol lowering effects of rhubarb stalk fiber in hypercholesterolemic men. J Am Coll Nutr 1997;16:600–4.
[100] McKay DL, Chen CY, Saltzman E, Blumberg JB. *Hibiscus sabdariffa* L. tea (tisane) lowers blood pressure in prehypertensive and mildly hypertensive adults. J Nutr 2010;140:298–303.
[101] Singh RB, Niaz MA, Ghosh S. Hypolipidemic and antioxidant effects of Commiphora mukul as an adjunct to dietary therapy in patients with hypercholesterolemia. Cardiovasc Drugs Ther 1994;8(4):659–64.
[102] Tripathi SN, Upadhyaya BN, Gupka VK. Beneficial effect of *Inula racemosa* (Pushkarmoola) in angina pectoris: a preliminary report. Ind J Physio Pharmac 1984;28:73–5.
[103] Tauchert M, Gildor A, Lipinski J. High-dose Crataegus extract in the treatment of NYHA stage II heart failure. Herz 1999;24(6): 465–74.
[104] Szapary PO, Jwolfe M, Bloedon LT, Cucchira AJ, Dermarderosian AH, Cirigliano MD, et al. Guggu lipid for the treatment of hypercholestremia randomised control trail. JAMA 2003;290(6):765–72.
[105] Singh RP, Singh R, Ram P, Batliwala PG. Use of *Pushka Guggul*, an indigenous antiischemic combination, in the management of ischemic heart disease. Int J Pharmacog 1993;31:147–60.
[106] Zick SM, Vautaw BM, Gillespie B, Aaronson KD. Hawthorn extract randomized blinded chronic heart failure (HERB CHF) trial. Eur J Heart Fail 2009;11:990–9.
[107] Maulik SK, Talwar KK. Therapeutic potential of *Terminalia arjuna* in cardiovascular disorders. Am J Cardiovasc Drugs 2012;12(3): 157–63.
[108] Maulik SK, Katiyar CK. *Terminalia arjuna* in cardiovascular diseases: making the transition from traditional to modern medicine in India. Curr Pharm Biotechnol 2010;11(8):855–60.

LIST OF ABBREVIATIONS

ACE Angiotensin converting enzymes
CAD Coronary artery disease
cAMP Cyclic adenosine monophosphate
CVD Cardiovascular diseases
ECG Electrocardiogram
ER Endoplasmic reticulum
HDL High density lipoprotein
HDL-C High density lipoprotein-cholesterol
HMG-CoA reductase 3-Hydroxy-3-methyl-glutaryl-CoA reductase
HSP72 Heat shock protein 72
LDL Low density lipoprotein
LDL-C Low density lipoprotein-cholesterol
LV Left ventricle
NCE New chemical entities
NO Nitric oxide
NYHA New York Heart Association
PTP1B Protein-tyrosine phosphatase 1B
VLDL Very low density lipoprotein
WHO World Health Organization

This page intentionally left blank

Index

Note: Page numbers followed by "f" and "t" indicate figures and tables respectively.

This page intentionally left blank