Pharmacognosy

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Fundamentals, Applications and Strategy

Edited by

Simone Badal
University of the West Indies, Kingston, Jamaica

Rupika Delgoda
University of the West Indies, Kingston, Jamaica

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List of Contributors

F. Abas, University Putra Malaysia, Serdang, Selangor, Malaysia

N.A. Al-Dhabi, King Saud University, Riyadh, Saudi Arabia

I.I. Amarakoon, University of the West Indies, Mona, Kingston 7, Jamaica

S. Amos, Cedarville University, Cedarville, OH, United States

K. Andrae-Marobela, University of Botswana, Gaborone, Botswana

S. Badal, University of the West Indies, Kingston, Jamaica

S. Bahadur, Jadavpur University, Kolkata, West Bengal, India

T. Baj, Medical University of Lublin, Lublin, Poland

M.A.B. Baker, University of New South Wales, Kensington, NSW, Australia

G.F. Barclay, The University of the West Indies, St. Augustine, Trinidad and Tobago

M. Bartnik, Medical University of Lublin, Lublin, Poland

E.N. Barton, University of the West Indies, Kingston, Jamaica

T.J. Booth, Technical University of Denmark, Kongens Lyngby, Denmark

C.S. Bowen-Forbes, The University of the West Indies, Kingston, Jamaica

K.J. Brown, University of the West Indies, Kingston, Jamaica

M.C. Brown, University of Technology, Kingston, Jamaica

G. Byfield, North Carolina A&T State University, Greensboro, NC, United States

J.E. Campbell, The University of the West Indies, Mona, Jamaica

C.-T. Che, University of Illinois at Chicago, Chicago, IL, United States

A. Clayton, University of the West Indies, Kingston, Jamaica

D. Cohall, The University of the West Indies, Cave Hill, Barbados

W.C. Cole, University of Calgary, Calgary, AB, Canada

D.K. Daley, Biotech R&D Institute, Kingston, Jamaica; University of Technology, Jamaica, Kingston, Jamaica

R. Delgoda, University of the West Indies, Mona campus, Jamaica, West Indies

L.L. Dilworth, University of the West Indies, Kingston, Jamaica

V. Duraipandiyan, King Saud University, Riyadh, Saudi Arabia

T.O. Elufioye, University of Ibadan, Ibadan, Oyo State, Nigeria

P.C. Facey, University of the West Indies, Kingston, Jamaica

S. Francis, University of the West Indies, Kingston, Jamaica

W. Gallimore, University of the West Indies Mona, Kingston, Jamaica

V. George, Amity Institute of Phytochemistry and Phytomedicine, Thiruvananthapuram, Kerala, India

M.I. Georgiev, The Stephan Angeloff Institute of Microbiology, Plovdiv, Bulgaria

A. Goldson-Barnaby, The University of the West Indies, Kingston, Jamaica

B.J. Gurley, University of Arkansas for Medical Sciences, Little Rock, AR, United States

C.-L. Hamilton, University of the West Indies, Mona, Kingston 7, Jamaica

S-A. Hartley, Biotech R&D Institute, Kingston, Jamaica

R.K. Harwansh, Jadavpur University, Kolkata, West Bengal, India

T.P. Ijinu, Amity Institute for Herbal and Biotech Products Development, Thiruvananthapuram, Kerala, India

I.S. Ismail, University Putra Malaysia, Serdang, Selangor, Malaysia

K.-D. James, Florida Atlantic University, Davie, FL, United States

W.A. Kukula-Koch, Medical University of Lublin, Lublin, Poland

N. Lajis, University Putra Malaysia, Serdang, Selangor, Malaysia

N. Laurieri, University of Oxford, Oxford, United Kingdom

Y. Lawrence, Comfort Medical, Coral Springs, FL, United States

A.C. Liwa, University of Calgary, Calgary, AB, Canada; Catholic University of Health and Allied Sciences, Mwanza, Tanzania

F.B. Lopez, The University of the West Indies, Cave Hill, Barbados

A. Ludwiczuk, Medical University of Lublin, Lublin, Poland

M. Maulidiani, University Putra Malaysia, Serdang, Selangor, Malaysia

S.A. Mitchell, University of the West Indies, Mona, Kingston 7, Jamaica

P.K. Mukherjee, Jadavpur University, Kolkata, West Bengal, India

J.E. Murray, University of the West Indies, Mona campus, Jamaica, West Indies

C.R. Nwokocha, University of the West Indies, Kingston, Jamaica

D. Picking, University of the West Indies, Mona campus, Jamaica, West Indies

P. Pushpangadan, Amity Institute for Herbal and Biotech Products Development, Thiruvananthapuram, Kerala, India

W.F. Reynolds, University of Toronto, Toronto, ON, Canada

C.K. Riley, Scientific Research Council, Kingston, Jamaica

J. Roach, University of the West Indies, Cave Hill Campus, St. Michael, Barbados

M.E. Roye, University of the West Indies, Mona, Kingston 7, Jamaica

M. Shields, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, United States

E. Sieniawska, Medical University of Lublin, Lublin, Poland

D. Simpson, Cedarville University, Cedarville, OH, United States

K. Skalicka-Woźniak, Medical University of Lublin, Lublin, Poland

K.N. Smith, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States

L.A. Stanley, Consultant in Investigative Toxicology, Linlithgow, United Kingdom

D.K. Stennett, University of the West Indies, Kingston, Jamaica

P.F. Tennant, University of the West Indies, Mona, Kingston 7, Jamaica

W.F. Tinto, University of the West Indies, Cave Hill Campus, St. Michael, Barbados

S.C. Turfus, University of the West Indies, Mona campus, Jamaica, West Indies

I. Vandebroek, The New York Botanical Garden, Bronx, NY, United States

J. Widelski, Medical University of Lublin, Lublin, Poland

R. Young, University of the West Indies, Kingston, Jamaica

Foreword

Pharmacognosy is the pharmaceutical science that is hidden in plain sight. In part that is because it is a very broad-based science with applications in pharmacy, agriculture, nutraceuticals, traditional medicine and phytotherapy, cosmetics, perfumery and essential oils, aromatherapy, and the food industry. At almost any time during the day, there is an application of pharmacognosy observable; it is amazingly pervasive in everyday life! Consequently, a single volume discussing all of the aspects of pharmacognosy in society is impossible to imagine.

Although older descriptions restricted the definition to studies related to medicinal plants, originally focusing on their identification and then on some of their chemical constituents, contemporary attempts at a definition of pharmacognosy seek a broader perspective. Thus, for me, pharmacognosy may be defined as "the study of biologically active natural resources." The resources involved in those studies may be any living organism, including plants, microorganisms, marine organisms, animals, insects, etc. The nature of the studies are similarly broad, and are frequently focused on examining traditional medicines for issues related to safety and effectiveness, searching for new, single agent molecules for drug discovery and development, for preparations which may serve as natural herbicides, insecticides, as well as developing new and established resources for nutraceuticals, cosmeceuticals, and veterinary agents, and assessing foods for their safety in the marketplace. Underpinning these studies is the fundamental aspect of sustainability; that the resource, whatever it is, can be maintained over time without damaging the environment or threatening the habitats of those resources. The introduction of this factor is a component of "ecopharmacognosy."

More recently, studies at the genome level in microorganisms have begun to characterize the major metabolic pathways in detail at the molecular level, and determine how they may be controlled to optimize metabolite formation, as well as unearthing new sources of established metabolites. In order to achieve and accumulate scientific evidence and make these dramatic advances, some of the most advanced technologies in the areas of chromatography, spectroscopy, biotechnology, and biological evaluation are fundamentally important and routinely used.

This volume represents a comprehensive compilation of the philosophical, scientific, and technological aspects of contemporary pharmacognosy. It examines the impact of the advanced techniques of pharmacognosy on improving the quality, safety, and effectiveness of traditional medicines, and how pharmacokinetics and pharmacodynamics have a crucial role to play in discerning the relationships of active metabolites to bioavailability and function at the active sites, as well as the metabolism of plant constituents. As a precursor, the importance of traditional medicines in global health care is discussed, and other aspects which contribute to pharmacognosy, including legal and regulatory issues, such as the intellectual property rights which relate to traditional knowledge, access to the biome, and the global issues concerning the registration of finished products.

Most organisms, plants, animals, marine systems, and microorganisms are essentially factories for the formation of a structurally diverse collection of natural products. Individual chapters are devoted to the most important of these metabolites, such as the various types of glycosides, the numerous alkaloid classes, the tannins, the various kinds of terpenoids, and a range of other plant metabolites. Marine organisms have been an impressive source of completely new metabolic systems in the past 40 years, and, as this area has succumbed to improved structure elucidation techniques, chapters are also devoted to the investigations and importance of the metabolites of marine animals, and to the broad range of microbial metabolites that have dramatically changed chemotherapy in the past 50 years. Also embraced within pharmacognosy and discussed in this volume are important commercial products used in many diverse industries such as the carbohydrates, various important oils, resins, balsams, fats, and waxes. Proteins from various natural sources are discussed and their importance in determining biological function emphasized. The fundamental importance of vitamins is also presented.

To explain some of the biological effects of natural products, the form and function of animal cells is presented, together with some of the applications of natural products as chemotherapeutic agents, as well as their specific use and mechanism of action for the treatment of cardiovascular disease, and selected other malignant states. Some of the metabolites that cross the blood–brain barrier are well-established psychoactive drugs, and they may also be of use as therapeutic agents in the future for pain and for addiction. Further progress in the investigation of natural products for new

drugs will depend on the development of new models and new targets for drug discovery, recognizing that the philosophical and practical frontier now lies in the burgeoning area of network pharmacology. High throughput screening has been in vogue for a while now for synthetic libraries and microfluidics plays an essential role in such studies.

Also presented in this volume are four areas of science which have assumed lead roles in the contemporary development of pharmacognosy, and why it is often viewed as being the most high tech of the pharmaceutical sciences. These are the diverse impacts of biotechnology, the applications of nanoscale technology, the tremendous advances in spectroscopic techniques, particularly nuclear magnetic resonance spectroscopy, and the significant impact that metabolomic approaches have had in the analysis of plants with respect to the quality control of phytotherapeuticals and traditional medicines, and to the clinical metabolic processes following chemotherapy. A final chapter discusses the significance that bioscience companies can have in small developing economies.

This very diverse compilation of chapters related to the broad perspective of pharmacognosy reminds scientists in many areas of research that pharmacognosy is far from dead. It is, indeed, very, very much full of life, is thriving as new technologies are integrated into common practice, and provides societally important, scientifically-based contributions to human health and well-being in numerous areas of our lives.

Geoffrey A. Cordell, Ph.D.

College of Pharmacy, University of Florida, Gainesville, FL, United States; Natural Products Inc., Evanston, IL, United States

Preface

Drugs of natural origin, which have roots in many medical traditions, are of inordinate significance due to the substantial growth in usage around the world. In addition, nature-based medicines are the topic of increased inquiry in the quest for novel pharmacophores that hold the prospect of enhanced therapy. The award of the 2015 Nobel Prize in Physiology or Medicine to nature-based drugs Avermectin and Artemisinin, used in the treatment of infections caused by roundworm parasites and malaria, respectively, underscores such trends, and highlights, in particular, the potential value of naturally derived medicines in targeting neglected tropical diseases. This development follows the World Health Organization's 2008 ratification of The Beijing Declaration, which promotes the safe and effective use of traditional and alternative medicines and calls for greater assimilation of these into national health care systems. Issues of quality, safety, efficacy measurements, commercial production, regulation, and ethics of natural drugs are now, more than ever, of paramount importance.

Pharmacognosy has evolved from a descriptive botanical subject to a multidisciplinary field inclusive of continuous advances in cell and molecular biology, ethnobotany, phytotherapy, analytical chemistry, and phytochemistry. It has embraced innovations for functional analysis of molecular targets that aid the development of targeted therapies. This book therefore aims to provide the student of pharmacognosy, and the related fields of pharmacy, medicine, medical herbalism, nursing, medicine, and pharmacology, a fundamental comprehension of naturally derived drugs within the historical context of their development, in addition to providing an update on recent developments in the field.

The text comprises eight sections. The first section includes an overview of the fabric of pharmacognosy based on plant metabolites, their origins, their diverse chemistry, and their impact on human diseases. A subsequent section, unique to this text (as far as we are aware), highlights secondary metabolites and drugs derived from animal sources. The section on animal anatomy and physiology will assist the student to comprehend the functional application of these natural drugs. Several later sections of the book focus on a variety of topics covering latest opinions on industrial, technological, regulatory, ethical, and sustainability developments, which are of potential relevance to undergraduate and graduate students, and researchers in the field, as well as policy makers. As humans exploit nature's unique gifts for alleviating disease, this should be achieved with safety, sustainability, and equitable benefit-sharing considerations in mind. These were some of the ideas that inspired the content of this book, although it is in no way an attempt to be fully comprehensive on all aspects of pharmacognosy.

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Spain), Colin Henderson (University of Dundee), Cheryl Patten (University of Brunskwick), Hanokh Czosnek (Hebrew University of Jerusalem), Gordon Roberts (University of Leicester), Rushika Perera (Colorado State University), Edith Sim (University of Oxford), Anthony Andrady (North Carolina State University), Rainer Bussmann (Missouri Botanical Gardens), Nancy Turner (University of Victoria) and David Picking (University of the West Indies, Mona). In addition, special appreciation to Geoffrey Cordell who not only reviewed multiple chapters but also wrote the "Foreword" for this book.

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Chapter 1

Background to Pharmacognosy

T.O. Elufioye[1] **and S. Badal**[2]

[1]*University of Ibadan, Ibadan, Oyo State, Nigeria,* [2]*University of the West Indies, Kingston, Jamaica*

Chapter Outline

General introduction into Pharmacognosy as a field of science
Its scope
Emerging areas
Relevance to the society.

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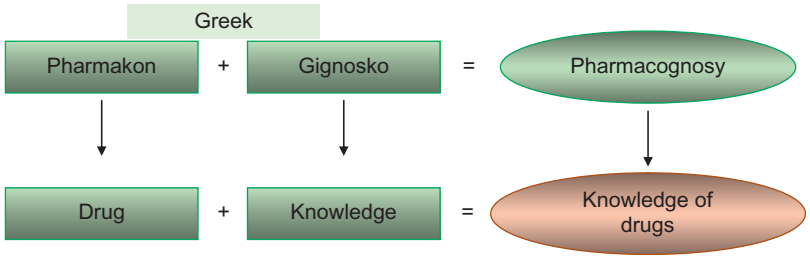
1.1 DEFINITIONS



The discipline of Pharmacognosy has evolved in definition over the years. While the fundamental connectedness among all definitions includes an understanding of drug entities, the combined descriptive variations from subtle to distinctive need highlighting. In the 1800s, Flückiger (1828–94) defined Pharmacognosy as "the simultaneous application of various scientific disciplines with the object of acquiring knowledge of drugs from every point of view" [1]. Approximately one hundred years later, the application of science toward the understanding of drugs was still the focus. However, this time, the origin of drugs was specifically toward a natural source as Pharmacognosy was defined by Tyler [2] as "an applied science that deals with the biologic, biochemical, and economic features of natural drugs and their constituents." Within this same era, the definition of Pharmacognosy evolved from an application-based understanding of natural drugs to an overall systematic knowledge of not just natural drugs, but more specifically, crude drugs from animal and vegetable origin as described by Greenish [3] who suggested that "Pharmacognosy is that science which aims at a complete and systematic knowledge of crude drugs of animal and vegetable origin" [1].

Almost a decade later, the Pharmacognosy definition transitioned to an understanding of plant-based products only, from the previous animal- and plant-based definitions, as outlined by Kraemer [4], who described it as "the study of medicinal plants and their crude products commonly designated as drugs." Still, a century later, the meaning of Pharmacognosy was once again redefined to incorporate the scientific study of crude drugs from animal origin in addition to the plant-based products; however, the definition also included the study of crude drugs from other natural sources, metals, and minerals [5].

Other definitions were more specific to the type of drug understanding as the discipline has been defined as "a molecular science that explores naturally occurring structure–activity relationships with a drug potential" [6], while others focus more on the structural, physical, chemical, and sensory characters of crude drugs of vegetable, animal, or mineral origin [7]. A more succinct, yet broad definition of Pharmacognosy is "The study of biologically active natural products" [8].

Pharmacognosy is connected to many other areas of science and a complete understanding of this field has to also taken into account, but not be limited to, botany, chemistry, enzymology, genetics, pharmacology, toxicology, horticulture, quality control, and biotechnology as described in Chapter 3, Areas of Science Embraced by Pharmacognosy, as well as ancillary fields, such as pharmaceutics, pharmcoeconomics, pharmacovigilance, regulatory law, and conservation.

1.2 HISTORY OF PHARMACOGNOSY

1.2.1 General History

The word "Pharmacognosy" was first used in a work entitled "Analecta Pharmacognostical" by an Austrian physician Schmidt in 1811, and then by Anotheus Seydler in 1815. During these early days, the term "Pharmacognosy" was used to refer to a branch of medical science that was associated with drugs in their crude state. It was involved in the investigation of "medicinal substances from the plant, animal, and mineral kingdoms in their natural, crude or unprepared state or in the form of such primary derivatives as oil, waxes, gums, and resins" [9]. The descriptive and microscopic applications of Pharmacognosy were developed during the 19th and 20th centuries [10], and formed the regulatory basis for the use of plants in health systems based on pharmacopoeial definitions. However, in the 1960s and 1970s, there began a transition of Pharmacognosy from a descriptive botanical research discipline to a more integrated chemical and biologically focused one [10].

Many great scientists have contributed to the development of Pharmacognosy as a discipline, and as a speciality branch of academic pharmacy. These include Arthur E. Schwarting, Egil Ramstad, Varro E. Tyler, Jack L. Beal, and Norman R. Farnsworth, all from the United States of America. Others of note are James W. Fairbairn, Edward J.

Shellard, and Norman Bissett from the United Kingdom. Also included from Europe and East Asia are René R. Paris, Egon Stahl, Ludwig Hörhammer, Hildebert Wagner, Otto Sticher, Shoji Shibata, and Tsunematsu Takemoto [10].

As late as the 1960s, Pharmacognosy, as a discipline of pharmacy education, was primarily associated with botany. At this stage, it was mostly concerned with the macro- and microscopic identification, description, and authentication of drugs. This area of Pharmacognosy is referred to as classical Pharmacognosy, and is still of fundamental importance in the field as a whole, especially for the purpose of preliminary standardization and quality control processes which are helpful in the development of official pharmacopoeial standards. Specific attention was paid to the microscopic identification of plant materials and to the determination of quantitative data such as foreign matter, ash values, extractive values, and moisture content. Subsequently, chromatographic fingerprints, especially TLC, were used for identification and standardization purposes. This classical Pharmacognosy, which focused initially on the pharmacognostic evaluation of plants, has been further extended to include other natural forms, such as various types of microbes and marine organisms.

Over time, a shift occurred from the classical Pharmacognosy to a more chemically and biologically focused discipline, one which involves the isolation and characterization of bioactive principles from natural sources, as well as the evaluation of structure−activity relationships of the isolates for the purpose of optimizing their development into medicinal agents for clinical use. DNA identification of natural products as a regulatory standard is also a current focus in Pharmacognosy.

Today, Pharmacognosy, as an academic department, is well-established in some schools of pharmacy all over the world, although the name might have been replaced by terms such as pharmaceutical biology, phytochemistry, and natural product research in certain countries. The area of research pharmacognosists is involved in, includes analytical chemistry, bioactivity assessment methods development, biocatalysis, biosynthesis, biotechnology, cell biology, chemotaxonomy, clinical studies, cultivation of medicinal plants, ethnobotany, genetics, marine chemistry, microbial biotransformations, molecular biology, synthetic modification of natural products, pharmacology, phytochemistry, phytotherapy, standardization of traditional medicines, taxonomy, tissue culture, and zoopharmacognosy. [10].

Despite the promise of natural product research over the years, this field experienced a decline in attention relative to other areas of research. Nonetheless, there is now a renewed interest in medicines, herbicides, and insecticides from natural sources, and consequently Pharmacognosy and natural product research have experienced elevated attention.

The use of plants in the management of ailments dates as far back as the origin of man itself. Several cultures of the world used herbs for healing rituals and certain carnivorous animals, e.g., Jaguar are also known to eat plants when ill. Thus, the use of plants for medicinal purposes started long before any form of record. Notwithstanding, the first written documentation of drugs obtained from natural products dates as far back as to the Sumerians and Akkadians in the third millennium BC, as well as the Egyptian Ebers Papyrus (about 1550 BC) [10]. Galen, the first pharmacist, used many pain killers from natural origin, including opium. Between 460 and 377 BC, Hippocrates, "the father of medicines," contributed to general medical development by proposing that causes of disease are not necessarily spiritual [11]. Thus, he acknowledged treatment with plants and other natural products. By AD 40−80, Dioscorides had written "De Materia Medica" which contained about 100 medicinal plants [11].

After this came the Islamic era between AD 770 and 1197 during which time natural products use gained further attention in the treatment of diseases and infections. Abu Bakr Mohammad Ibn Zakariya Razi, also known as Rhazes (AD 865−925), born in Iran, is known for extending the analytical approach of his predecessors, Hippocrates and Galen, in some medical areas. His primary focus was urology, and he was the first to develop treatment for the kidney calculi [12]. Avicenna, a Muslim scientist of the 10th and 11th centuries contributed significantly to the medical community then, and continues to do so through his work, Al Canon from which many publications and teaching programs have arisen [13]. It is stated that Paracelsus (1493−1541), who played an important role in formulating drugs from minerals, burned Avicenna's Canon, the Bible of learned medicine, and justified his actions by saying "remove all the old books of learning and practice medicine as I have learnt in the real world" [14].

John Gerarde (1545−1612) a botanist and herbalist first published in 1597 the most widely circulated botany book in English of the 17th century "*The Generall Historie of Plantes*," which comprised of 1480 pages [15]. By the 18th century, researchers such as Johann Adam Schmidt contributed to the development of Pharmacognosy by being the first to ascribe the term "pharmacognosis," the precursor to Pharmacognosy. This in his handwritten manuscript described the necessary skillset involved or needed for the development of medicine [1]. At the end of this century, crude drugs were still being used as powders, simple extracts, or tinctures. By 1805, there began a new era in the history of medicine/pharmacognosy which was the era of compound isolation [1]. Pure secondary metabolites were isolated from several medicinal plants in the years that followed. These included morphine, strychnine, quinine, caffeine, nicotine, atropine, colchicine, and cocaine. In the 20th century, the discovery of many drugs from natural sources became a major

focus and this search was by no means limited to the plant kingdom. Drugs, particularly hormones and vitamins, were discovered from the animal kingdom. Microorganisms and marine flora also became important sources of drugs, particularly for antibiotics following Fleming's discovery of penicillin.

1.2.2 Regional History of Pharmacognosy

It is also important to introduce the global history of Pharmacognosy, an area discussed in more detail in Chapter 2, Traditional Medicine. Such an introduction allows the reader to appreciate the differential impact of this paradigm on our communities today, and the different contributions made to the medicinal plant systems in various communities over time.

1.2.2.1 The Egyptian Records

The ancient Egyptian records date back to 3000 BC (The first recorded prescriptions were found in Egyptian tombs and these include the Hieratic Papyri, Ebers Papyrus, and the Gynecologic Papyrus) [11]. Ebers Papyrus (1500 BC) contains 811 prescriptions and 700 drugs [16]. Egyptians were expert in using crude drugs for the treatment and cure of diseases. This was usually done by priest doctors who diagnosed, prescribed, and prepared the needed medicines. The crude drugs used included onions, coriander, melon, myrrh, aloes, gum, poppy, castor, anise, etc.

1.2.2.2 The Babylonian Records

The Babylonians used drugs of plant origin in their practice of medicine as far back as 1770 BC [17], and many drugs from plant and mineral sources were listed in Babylonian medical recipes [18]. The earliest record had 250 vegetable drugs including opium, ricinus, myrrh, menthe thymus, and 120 minerals [19].

1.2.2.3 The Indian Records

The Riveda and Ayurveda medicines were practiced around 2000 BC and continue today. This involved the use of sacred medicinal plants, the collection of which was done only by "innocent" religious persons. Important crude drugs used at this time include sandalwood, aloes, sesame oil, castor oil, ginger, benzoin, cannabis, caraway, clove, cardamom, and pepper, etc.

1.2.2.4 The Chinese Records

Chinese traditional medicine is well acknowledged for acupuncture and has experienced many changes over the years. It richly comprises plant-based products, as documented in the volume "Pen Ts'ao Kang Moa" (3000 BC) which contained records of medicinal plants and drugs of animal origin as well. These medicinal agents included anise, ginseng, rhubarb, ephedra, and pomegranate [11]. The Traditional Chinese medicine (TCM) system, believed to be more than 5000 years old, is based on two separate theories about the natural laws that govern good health and longevity, namely yin and yang, and the five elements (wuxing). The legendary emperor Shen Nung discussed medicinal herbs in his work, which was probably written about 2700 BC instead of their traditional date of 3700 BC [20]. However, TCM, as a series of practices, was systematized and written between 100 and 200 BC. A complete reference to Chinese medicine prescriptions is the *Modern Day Encyclopedia of Chinese Materia Medica* published in 1977. It lists nearly 6000 drugs of which 4800 are of plant origin [21]. However, the Chinese Pharmacopoeia of 2010 gives an updated record of medicinal plants in use in TCM.

1.2.2.5 Greek and Roman Records

Pythagoras (560 BC), a Greek philosopher and mathematician, used mustard and squill preparations to treat particular diseases, and the use of natural products toward the treatment of various ailments was also shared by Hippocrates, a Greek physician (466 BC). Dioscorides, also a Greek physician, in 77 AD was one of the first to describe drugs in his work, he is referred to as "the father of Pharmacogonosy" [22]. In his work "De Materia Medica," he documented the use of 944 drugs of which 657 were of plant origin [11,22]. Galen (CE 130−200), a Roman physician and Pharmacist mentioned earlier, was known to use "Galenical preparations" to manage several diseases, such as *Uvaeursi folium* used as an uroantiseptic and a mild diuretic continuing today. He also compiled the first list of drugs with supposedly similar or identical actions and thus interchangeable, "De succedanus," which are today not used given the pharmacological disparities.

1.2.2.6 The Arab and Persian Records

The physicians of Arabia added many new plants and medicaments to those already recorded by the Greeks and Romans. In the days of the Arab contribution, pharmacy as a subject, attained elevated attention and recognition allowing it to become an independent branch of medicine [23]. The medieval Persian traditional medicine was pioneered by Rhazes (865−925 AD) and Avicenna (AD 980−1037), who are regarded as founders of the golden days of the Persian medical sciences [24]. According to the literature, Freidoon used knife, fire, and many plant materials to treat injured soldiers. He was known as the first Iranian surgeon and the pioneer of the "Saenamargha" school of medicine [25].

1.3 DEFINITION OF TERMS

The following are some essential terms belonging to the discipline of Pharmacognosy which are widely used. A brief description is offered of some of these terms in anticipation that it will afford a more comprehensive understanding of the relevant field in subsequent chapters.

Chromatography: This is a laboratory method used for separating mixtures of chemical compounds based on the principle of adsorption. Different bonding properties of the molecules to be separated are very important in achieving good separation which is usually achieved when the relative amounts of each solute are distributed between a moving stream known as the mobile phase and a contiguous stationary phase. The mobile phase can be a liquid or a gas, while the stationary phase is either a solid or a liquid. Chromatography is used in quantitative and qualitative analysis of biological and chemical substances and is particularly useful in the separation of isomers and natural materials. Although chromatography is mainly associated with purification, it also has wider applications for the identification of compounds based on their chromatographic behavior.

Crude drugs: This refers to those natural products, such as plants or plant parts, and extracts and exudates that are not pure compounds, which have known pharmacological actions as discussed in more detail in Chapter 11, Terpenoids. In addition, crude drugs can be the harvested and usually dried plants, animals, or minerals of pharmaceutical or medicinal importance generally before processing or modification. Crude drugs can also be defined as "any products that have not been advanced in value or improved in condition by grinding, chipping, crushing, distilling, evaporating, extracting, artificial mixing with other process or treatment beyond what is essential to its proper packing and the prevention of decay or deterioration pending manufacture" [26].

Crude drugs can be classified into two groups: organized and unorganized. Examples are:

Organized

- Entire plants or animals: *Mentha* spp., *Lobelia* spp.
- Entire organ of plants or animals; Senna, Clove, Fennel, *Cinchona*, Liquorice.
- Minerals: Talc, Kaolin, Chalk.
- Marine sources: Sponges, Red algae, Agar.

Unorganized

- Mixed preparations derived from plants or animals, such as opium, aloes, tragacanth, balsams, resins, musk, gelatine, and bees wax.

Ethnobotany: This is the study of the relationships between plants and the people in the field. It includes the use of plants for furniture, shelter, transportation, as well studying the use of plants in medicine, alternative methods of healing, wild food, agricultural crops, and in religious ceremonies.

Ethnopharmacology: This is the scientific study correlating ethnic groups with their health and how it relates to their physical habitats, and their methodologies in creating and using plant-based medicines. According to the *Journal of Ethnopharmacology,* ethnopharmacological research involves "multidisciplinary effort in the documentation of indigenous medical knowledge, scientific study of indigenous medicines in order to contribute in the long-run to improved health care in the region of study as well as search for pharmacologically unique principles from existing indigenous remedies" [27].

Extraction: This is a way of separating the desired substance(s) from a mixture using a suitable solvent in which the desired substance is soluble. In other words, extraction techniques are used to separate compounds based upon their different solubility in two immiscible solvents. This is a laboratory procedure commonly used when isolating or purifying natural products. Phytochemistry uses solid−liquid, liquid−liquid, and acid−base extraction methods.

Herbs: This term is more appropriately applied when referring to culinary plants and refers to crude materials, which may be obtained from lichens, algae, fungi, or higher plants such as leaves, flowers, fruits, seeds, stem, back, roots, rhizomes, or other parts which may be entire, fragmented, or powdered [28]. It is a term that is probably misused when applied to medicinal plants for therapeutic use, unless there is a dual use, e.g., ginger, turmeric, or garlic.

Medicinal plants: These are either wild or cultivated plants used for the management and treatment of ailments, e.g., *Menthapiperita*.

Metabolomics: This is the systematic study of chemical processes that involve metabolites that represent unique fingerprints left behind producing somewhat of a metabolite profile [29]. The collection of these metabolites left is referred to as the metabolome [30]. The metabolome can be therefore through mRNA gene expression and proteomic analyses unlock hidden answers on cellular processes while revealing the physiology of that cell and so creates room for cellular manipulation toward disease amelioration.

Natural products: A generic term which can be an entire organism (plant, animal, microorganism, etc.), part of an organism (leaf, flower, isolated glands, etc.), an extract, an exudate, a partially fractionated preparation, or isolated pure compounds.

Phytotherapy: It is a part of Pharmacognosy that is concerned with the clinical use of crude drug extracts or partially purified mixtures from plants. It can also be referred to as the area dealing with the scientific studies of the bioactivities of plant-based medicines, as well as their clinical uses.

Spectroscopy: This is the study of the interaction of atoms and molecules with light also referred to as electromagnetic radiation. Such information provides insight into the measurement of the radiation intensity as a function of wavelength.

1.4 SCOPE OF PHARMACOGNOSY

The scope of Pharmacognosy has broadened in recent years to include the identification or authentication of crude drugs (using macroscopic, microscopic, or chemical methods), and their biopharmacological and clinical evaluations [31]. Research studies in Pharmacognosy currently include studies in the areas of phytochemistry, microbial chemistry, biosynthesis, biotransformation, bioinformatics, and chemotaxonomy. Pharmacognosy has also become an important link between pharmacology and medicinal chemistry [32]. It covers areas such as isolation and/or analysis of phytochemicals, structure−activity relationships, natural products as isolated or in silico models for the synthesis of new drugs, natural drugs of direct therapeutic use, the investigation of biosynthetic pathways, the cultivation and collection of medicinal plants, the preparation and qualitative and quantitative analysis of specific formulations, the development of plant tissue cultures, as well as the application of several spectroscopic and molecular techniques for natural product identification. Today such molecular biological techniques include DNA fingerprints (RAPD, RFLP, AFLP) which are used to identify and authenticate various herbs. As a result of such phytochemistry and pharmacological advances, testing methods are far more efficient [33].

1.5 EMERGING AREAS IN PHARMACOGNOSY

1.5.1 Forensic Pharmacognosy

There are many reported cases of the misuse of plant and plant-derived compounds. In most of these cases, the prosecution of offenders and criminals is often a very difficult task as sufficient evidence must be tendered in the court of law. Today, several on-the-spot methods, including remote sensing and hand-held devices, and laboratory techniques are employed for the identification of these substances, and this is the main thrust of Forensic Pharmacognosy. The word "forensic" comes from the Latin word *forēnsis*, which means "of or before the forum." It relates to, or denotes the application of, scientific methods and techniques for the investigation of a crime or suspicious incident. It is the use of science and technology to investigate and establish facts in criminal or civil courts of law. Hence, forensic science is the application of the full range of scientific techniques to answer questions of interest in a legal system, in relation to either criminal or civil action [34].

Forensic Pharmacognosy is the application of the methods and techniques in Pharmacognosy for the investigation of crimes arising from the misuse of plants and crude drugs obtained from plants, animals, and mineral origin. It utilizes the methods and techniques of both conventional and modern Pharmacognosy, such as macroscopic, microscopic, quantitative microscopy, and phytochemical methods to unravel crimes arising from the misuse of plants. Such misuse

includes for homicidal purposes, natural drug abuse, and the use of plants in sports to gain undue advantage; all are within the scope of forensic Pharmacognosy.

Most plant secondary metabolites leave small traces in the human body after ingestion which makes their extraction for analysis purposes a challenge [35]. Such challenges were and continue to be overcome with technological advances, and so the development of analytical methods, such as high pressure liquid chromatography (HPLC) in the late 20th century, allowed for the more effective detection of certain secondary metabolites like morphine, caffeine, and atropine given the heightened sensitivity of these detection methods. A chemist, Jean Servais Stas, isolated nicotine from body tissues thus becoming the first person to develop a method to extract plant alkaloids from organic material of the human body [36]. Other toxicologists then developed qualitative tests to determine the presence of a variety of alkaloids and other natural products, including various steroid formulations.

1.5.1.1 Analytical Methods Applicable in Forensic Pharmacognosy

1. Physical evaluation

   Physical evaluation of crude drugs is achieved by the determination of various physical parameters using physicochemical techniques. Such parameters include: the determination of solubility; specific gravity; optical rotation; viscosity; refractive index; water content; degree of fiber elasticity; ash values, extractive values; and foreign organic matter.
2. Biological evaluation

   This refers to the evaluation of therapeutic/pharmacological, enzymatic, gene modulating, and toxicological activity of the crude drug and/or its active principle using several models. A recent model of noted interest is network pharmacology where multitarget drugs may prove more efficacious than traditional ones [37]. Ultimately, biological evaluation determines therapeutic activity of the drug or active principle, potency, as well as toxicity, based on the chemical constituents present and their content.
3. Morphological evaluation

   This technique uses sensory organs (skin, eye, tongue, and nose) to obtain a qualitative evaluation of the plant. Such evaluations involve macroscopic observations such as color, odor, taste, size, shape, and other special features [38].
4. Microscopic examination

   This involves a detailed examination of the drug and is mostly used for the qualitative evaluation of established crude drugs in entire and powdered forms [39]. A microscope is used to detect various cellular tissues, such as trichomes, stomata, starch granules, calcium oxalate crystals, and aleurone grains. Crude drugs can also be identified microscopically by cutting the thin TS (transverse) or LS (longitudinal) sections of wood, and by staining them with staining reagents. Some of these agents include: iodine which stains blue with starch and hemicelluloses; phloroglucinol and HCl which stain pink with lignified tissue; and ruthenium red which stains pink with mucilage. Microscopic evaluation also includes the study of the constituents in a complex, powdered drug mixture. Quantitative aspects of microscopy comprise the stomata number and index, palisade ratio, vein-islet number, size of starch grains, and length of fibers [38,40].
5. Chemical analysis

   Chemical analysis is employed to either identify, quantitate, and/or evaluate the purity of drugs, secondary metabolites, and extracts of crude drugs. Firstly, preliminary phytochemical screening may be important for the chemical evaluation [38], such as the determination of acid saponification values. Methods that provide more definitive answers, such as identification of active constituents and/or the quantitation of these, include: photometric analysis; spectroscopic analysis (UV, IR, MS, and NMR); thin layer chromatography (TLC); high-performance liquid chromatography (HPLC); and gas chromatography (GC).

1.5.2 Molecular Pharmacognosy

Using the methods and technologies of molecular cloning, genetic engineering, tissue culture, and molecular markers, Pharmacognosy has developed rapidly in recent years, and now represents a highly interdisciplinary, cutting-edge science. Molecular Pharmacognosy involves the classification, identification, cultivation, and conservation of medicinal materials and the production of their components at a molecular level, as well as the modulation of secondary metabolites [41]. It investigates medicinal materials at the level of nucleic acids and proteins.

1.5.2.1 Concept of Molecular Pharmacognosy

a. *Molecular Identification of Medicinal Raw Materials*
- Molecular identification has the advantage of not being impacted by the environment.
- The identity of the medicinal raw materials is determined with the use of molecular markers.
- It allows the morphological variation of medicinal raw materials to be described more precisely.

b. *Phylogenetic Trees and the Evolution of Medicinal Plants and Animals*
- The phylogenetic trees of medicinal plant and animals are determined based on their gene sequence analysis of chloroplast and nuclear genome, and may be used for the identification of new potential drugs among species which are closely related to known medicinal species.

c. *Evaluation and Preservation of Germplasm Resources*
- Germplasm resources are used in the selection and propagation of new medicinal species, and in the sustainable use and development of available resources.

d. *Biosynthesis and Regulation of Active Components in Medicinal Plants and Microorganisms*

Molecular Pharmacognosy can also be involved in the biosynthesis and regulation of active plant metabolites. Methods used in the regulation of secondary metabolites are biotransformation and recombinant DNA. Transgenic techniques aimed at increasing the percentage of active components present in plants are also available.

e. *Mechanism of Endangerment and Protection of Endangered Medicinal Plants and Animals*

Conservation of genetic biodiversity is an essential objective in the protection of endangered species. Molecular markers, based on DNA polymorphisms and gene sequences, facilitate the evaluation of DNA variation, thereby identifying which plants and animals should be actively protected.

1.5.2.2 Techniques

Techniques in molecular Pharmacognosy include molecular markers, gene chips, recombinant DNA, and protein analysis.

1.5.2.3 Applications of Molecular Pharmacognosy

Molecular Pharmacognosy has diverse applications which include quality control and standardization of plant-based medicinal agents, identification and validation of new drugs, accumulation of secondary metabolites, DNA expression, and genetic diversity.

1.5.2.4 Prospects of Molecular Pharmacognosy

- Molecular identification of medicinal plants.
- Functional genome research related to plant secondary metabolites.
- New ideas and methods for the core identification and collection of genes.

1.5.3 Ecopharmacognosy

The emerging term of ecopharmacognosy is defined as "the study of sustainable, biologically active natural resources." As a philosophical approach, it provides a consensual framework for developing new strategies and new scientific perspectives which may improve future global product accessibility and assured beneficial outcome [42].

A wide range of ecological factors affects secondary metabolites. These include drought [43], salinity [44], temperature [45], climate change [46], light [47], and nutrient stress [48]. Therefore, if we continue to exploit our natural resources the way we have, then the question is asked, "what will be the source of natural medicines decades from now?"

1.6 PHARMACOGNOSISTS, WHAT THEY DO?

Pharmacognosy as a multiplex of overlapping and integrated sciences is at the forefront of numerous technological advances, both in the laboratory and the field. Thus, Pharmacognosists are involved in a number of selected activities and these include:

- Identification of natural drug sources.
- Determination of morphological characters.

- Planning for the cultivation of medicinal plants.
- Protocol development and/or implementation of processes involved in the collection, drying, and preservation of crude drug material.
- Evaluation of crude natural drugs microscopically, macroscopically, genetically, chemically, and biologically, for quality control purposes.
- Evaluation of the Pharmacology of crude extracts and active constituents.
- Isolation and characterization of active secondary metabolites from natural sources.
- Involved in interdisciplinary relationships with ethnobotany, ethnopharmacology, botany, chemistry, enzymology, genetics, pharmacology, horticulture, quality control, and biotechnology among others.
- Legal and regulatory issues.

1.6.1 The Function of Pharmacognosy in Society

Several gaps exist in the worldwide health care system and some of these are:

1. Per capita expenditures by countries on health care [49].
2. The number of trained physicians per thousand population in various countries [50].
3. The overall global access to medicinal agents including those for rare diseases [51–53].
4. The depletion of natural resources [40].

There is currently a major disparity in monetary allocations for medical research as approximately 90% of a US $110 billion expenditure is used to treat 10% of global health problems [54]. Such worldwide expenditure by the medical community targets treatments primarily for the wealthy, and stifles innovation in the drug discovery arena, as 75% of approved drugs in the United States, France, and Canada showed no added therapeutic benefit, and only 5% were "breakthrough" drugs. Secondly, to date, despite the critical role of natural product sciences in global health care, there is still a deficiency in the number of professionals in this arena, and the disparity of trained physicians per thousands of the population is too grave in certain parts of the world. Thirdly, accessing certain drugs in some parts of the world is a challenge, given the high cost of drug importation which makes traditional medicines the only rational alternative approach. Finally, if natural resources are exploited as in the past, what happens decades from now for our children and their children?

The question is then asked, how does Pharmacognosy fill these gaps? Encouraging collaborative efforts in natural products research between developing and developed countries is certainly one way to proceed and this can be initiated through an international context like WHO (World Health Organization) or through independent bodies like the recently developed Society for Scientific Advancement (SoSA). In 2006, the WHO Commission on Intellectual Property, Innovation, and Public Health (CIPIH) encouraged initiatives that boost patent protection within developing countries toward improving the declining quality and quantity of drug innovation. Such initiatives are also geared toward filling the gap of drug discoveries of the developing world. In addition, several funding agencies like TWAS, OWSD, and the Gates Foundation are aggressive in their efforts to pump funds into developing countries toward filling these gaps. Bodies like SoSA [55], facilitate intellectual discourse and collaborative ties with scientists from a first world context with scientists from the developing world.

The number of trained experts in natural product research and medicine needs attention and these individuals also need a forum of influence. Such efforts are evident in Japan, as the Japanese Liaison of Oriental Medicine (www.jlom.umin.jp) provides an environment where major scientific societies involved in traditional medicine and the WHO Collaborating Centers for Traditional Medicine based in Japan can come together. Such initiatives encourage an increase in professionals in these fields and act as a source of advice for the government, steering the way for future directional natural products research and drug development from these sources. In addition to these benefits, other benefits to be had are an open discourse of the need for more feasible global access to relevant medicaments.

This takes us to the point of the prudent and urgent need to conduct natural products research in a sustainable manner. Can Pharmacognosy fix this? Ecopharmacognosy, briefly mentioned previously, addresses this. It provides a critical area for us to consider and implement sustainable approaches to medicinal plant preparation and utilization, thereby safeguarding our natural resources and economic standing for the future. Every day, ecopharmacognosy has applications in plant sourcing, drug discovery, DNA barcoding, and conservation. Such applications have paved the way for safer recyclable solvents, enhanced efficient energy reactions, reduced use of chromatographic solvents, renewable feed-stocks, environmentally friendly by-products, and exploring common plants as chemical reagents [40].

1.7 CONCLUSIONS

The paradigms of Pharmacognosy have evolved over the years, from the application of various scientific disciplines used in the understanding of drugs to the study of sustainable biologically active natural resources. Pharmacognosy is connected to numerous disciplines that together aim to contribute to the management of several diseases. As new advances are developed and refined, such as network pharmacology, and forged with other regimes, old and new, Pharmacognosy promises to make its mark on the advancement of medicine in a substantial way.

1.8 PRACTICE QUESTIONS

1. Differentiate between classical and modern Pharmacognosy.
2. Several people contributed to the development of Pharmacognosy as a field of science, discuss.
3. Describe the relevance of Pharmacognosy to crime detection.
4. Discuss the history of Pharmacognosy as documented in certain areas of the world.
5. What is ecopharmacognosy?
6. Discuss the concepts, applications, and prospects of molecular Pharmacognosy.
7. What are crude drugs?

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Chapter 2

Traditional Medicine

C.-T. Che[1], V. George[2], T.P. Ijinu[3], P. Pushpangadan[3] and K. Andrae-Marobela[4]

[1]*University of Illinois at Chicago, Chicago, IL, United States,* [2]*Amity Institute of Phytochemistry and Phytomedicine, Thiruvananthapuram, Kerala, India,* [3]*Amity Institute for Herbal and Biotech Products Development, Thiruvananthapuram, Kerala, India,* [4]*University of Botswana, Gaborone, Botswana*

Chapter outline

Traditional medicine, as defined by the World Health Organization, is 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. Some traditional medicine systems are supported by huge volumes of literature and records of the theoretical concepts and practical skills; others pass down from generation to generation through verbal teaching. To date, in some parts of the world, the majority of the population continue to rely on their own traditional medicine to meet their primary health care needs. When adopted outside of its traditional culture, traditional medicine is often referred as "complementary and alternative medicine." Among others, the most widely used traditional medicine systems today include those of China, India, and Africa.

In this chapter, the Chinese, Indian, and African systems of traditional medicine are described.

2.1 TRADITIONAL CHINESE MEDICINE

Traditional Chinese medicine (TCM) is rooted in ancient Chinese philosophy and dates back over 3000 years. An integral part of Chinese cultural heritage, it has evolved over time and continues to serve a vast population of people in many places today by providing reliable, effective, and affordable health care options for patients. Other traditional systems of medicine in the Orient, such as the Kampo medicine in Japan and the Hanja medicine in Korea, are greatly influenced by TCM in many ways, including their fundamental principles, diagnostic approaches, and therapeutic modalities. In the West, knowledge of TCM reached the Middle East and Europe in the early days along the Silk Route. It further spread to the Western world after European trade was established with China during the 17th century. However, TCM has often been viewed by the mainstream medical profession with skepticism and it has never been widely applied in the modern Western context. Nevertheless, the last few decades have witnessed the movement toward alternative and complementary approaches to conventional medicine and it has notably changed the landscape of health care. There is an increasing interest in understanding and using TCM along with mainstream medicine. The resurgence of interest and desire for knowledge about the application, safety, and efficacy of TCM is now seen around the world and the impact cannot be ignored.

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2.1.1 Fundamental Principles of TCM

Like many other traditional medicine systems, TCM was developed under the influence of the philosophical framework that tried to explain the observations of events in the universe and the intertwining relationship among them. As a result, many medical principles were lent from the philosophical thinking of the early time. Thus, the concept of TCM is underpinned by the ideology of holism. The human body is viewed as a "small universe" (versus the surrounding environment—the greater universe) which is composed of organs, tissues, and vital substances, each having its own unique functions but yet they are correlated in a mutually interdependent manner with one another. Different parts of the body are perceived to be interconnected by a complex, yet invisible, channel system known as the meridians, and the entire body is nourished by the presence of vital substances, such as Qi (which has no counterpart in modern medicine but is often perceived as a form of vital energy necessary to keep the body alive), blood, and body fluids. Analogous to an idealistic world in which all components are interacting with one another in a dynamic and harmonious manner, good health is seen as a result of internal balance among various organs, tissues, vital substances, and emotions, as well as a harmony between men and the environment. Only in a balanced (homeostatic or equilibrium) state can the body function normally to maintain a healthy condition; and any disturbance of the harmony will lead to illness.

Most of the knowledge about TCM has been recorded in ancient texts and many of them pass on through the ages. The most outstanding piece of medical works available today that outlines the fundamental doctrines of TCM is the book of *Huang-di Nei-jing* ("Yellow Emperor's Canon of Internal Medicine"), which is a medical compilation completed sometime between 300 and 200 BC. The book describes the basic ideas of medical concepts and provides surprisingly detailed medical information such as body structure, physiological, and metabolic processes, causes and development of diseases, clinical presentations, and therapeutic approaches.

Another important piece of classic works of TCM is the *Shen-nong-ben-cao-jing* ("Herbal of the Divine Plowman" AD 100−200). It is considered the first Chinese pharmacopoeial text (*Materia Medica*), and perhaps the earliest herbal book in the world, in which 365 kinds of medicinal herbs are recorded together with morphological sketches. Interestingly, these herbs are categorized into three classes according to their therapeutic potentials and toxicity. In this regard, modern pharmacological concepts about therapeutic application and drug toxicity seem to echo the ancient Chinese view.

Chinese medicine is deeply influenced by two fundamental principles of the universe: the Yin−Yang and Five-Element theories. These principles give rise to the theories and practical guidelines of TCM and they are briefly described as follows.

2.1.1.1 Yin and Yang

In ancient China the world was seen as the interplay of a pair of opposite qualities, Yin and Yang. Thus, the daily cycle of the sun (representing brightness and warmness, ascribed to Yang) giving way to the moon (representing darkness and coldness, ascribed to Yin), and vice versa, provided the basis from which the principle of Yin and Yang was developed. The interrelation between Yin and Yang is complicated. On the one hand, they are opposite in nature and are antagonistic to each other. But on the other hand, these two elements within the same entity interact with each other and are complementary to each other. They are also interdependent to form a unity. More importantly, Yin and Yang are often seen to exist in a dynamic state in which they maintain an equilibrium condition. A simple expression of the Yin−Yang relationship is presented in Fig. 2.1.

When the Yin−Yang relationship is applied to the human body and health, they are used to classify the external and internal body structures, to explain physiological functions, to analyze clinical manifestations, to determine the etiology of diseases, to guide diagnosis, and to direct treatment protocols. Thus, an imbalance (either excess or deficiency) of

- Yin (black) and Yang (white) are apparently opposite and contrary elements.
- They are complementary to each other and bound together to form a mutual whole (the circle).
- They are interconnected.
- They are interdependent.
- They interact dynamically.
- They give rise to each other as they interrelate.

FIGURE 2.1 A simple expression of the Yin−Yang relationship.

Yin and Yang elements in an internal organ will lead to a disease state. Illnesses are therefore grossly classified into two major types: the Yang syndrome and the Yin syndrome. The Yang syndrome is caused by an excess of the Yang element to result in the manifestation of heat (hot) symptoms such as fever, pathogenic assault, inflammatory reaction, and stress. On the other hand, the Yin syndrome, arising from an overwhelming activity of the Yin element, will cause cold condition of the body such as hypothermia and general weakness. The same Yin−Yang principle also applies to therapies. To treat Yang (hot) syndromes, herbs possessing "cold" property are used to counteract the hot condition of the body (to "cool down" the body); by the same token, to treat Yin (cold) syndrome, herbs with "hot" property must be employed to dispel the excessive coldness in order to reestablish a balanced condition.

2.1.1.2 The Five Elements

In the ancient world, a person's livelihood always depended on the availability of five indispensable substances (elements), viz. wood, fire, earth, metal, and water. Each of these substances is seen not only to possess its own unique characteristic properties, but also to have an active and dynamic relationship with one another. Thus, they constantly interact with each other in either a promoting (generative) or restraining (inhibitory) manner (Fig. 2.2). The doctrine of Five Element thus describes two cycles, a promoting cycle and a restraining cycle, of interactions between the elements. Within Chinese medicine the effects of these two main relations are elaborated to explain how different parts of the body work and how diseases are formed. The concept is further extended to guide the selection of treatment strategy. For example, the five major visceral organs (i.e., heart, liver, spleen, lung, and kidney) are mapped onto the five elements with the notion that the former interplay with each other in the same manners as the latter. Each of the five visceral organs is seen to possess properties similar to one of the five elements, and therefore, correlations between the functions of the five visceral organs and attributes of a particular element can be established. Thus, the kidney corresponds to the element of water because it regulates water metabolism; liver is associated with the element of wood because of its ability to promote the flow of Qi just like plants flourish during the spring season; and the heart belongs to the element of fire because of its role to circulate the blood in order to keep the body warm, like fire producing heat. In clinical terms, the function of the kidney (water) can enhance the function of the liver (wood) just like water can help the growth of trees, and therefore, in order to treat diseases caused by the weakened liver, herbs may be given to nourish and strengthen the kidney functions. On the other hand, in the case where the liver (wood) is exceptionally strong and overacts to restrain the normal functions of the spleen (earth), treatment would focus on either calming (reducing) the liver activity and/or strengthening the spleen function in order to counteract the subduing action of the liver. To this end, it is well known that, apart from the control of clinical symptoms, Chinese medicine regime often aims at the modification of the functions of internal organs that may not be directly involved in pathological changes.

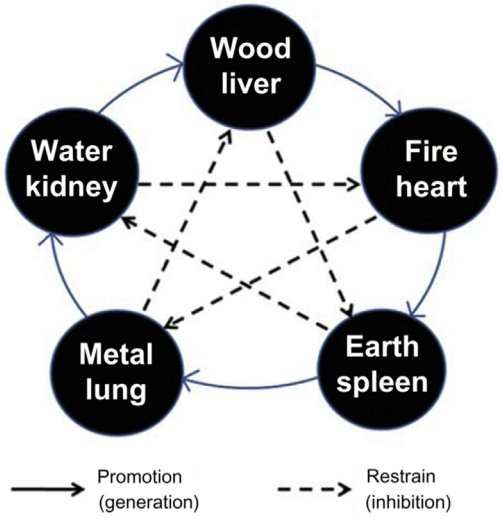


FIGURE 2.2 Dynamic relationships among the five elements and the major visceral organs.

2.1.2 Practice in Traditional Chinese Medicine

TCM embraces a holistic approach to prevent and treat diseases. It seeks to restore Qi (energy) and to strike for a balance among different components within the body through the use of a variety of modalities and therapies. While the best-known approaches are the use of medicinal herbs (herbology) and needles (acupuncture), others include moxibustion, medical massage and manipulation, cupping, exercise therapy, dietary therapy, and bone-setting techniques. Regardless of the therapeutic type used, they are all guided by the same fundamental set of theories.

In terms of therapeutic applications, the use of herbs is the major and most important approach in TCM. The Chinese herbal prescriptions normally compose of several herbal ingredients to make up the so-called polyprescription or multi-item medicinal formula. There are altogether over 10,000 standard prescriptions, which the herbal doctors may modify to suit individual needs of the patients. Each prescription is made up by one or more primary herbs which are responsible for the main therapeutic effect. Other herbs are added for secondary purposes such as enhancing the effect of the primary herbs, harmonizing the properties of the ingredients, reducing side effects, or improving the palatability of the prescription. Most often, the mixture is boiled in water to make a decoction (concoction) and taken orally. Other forms are available for topical or suppository applications. Modern pharmaceutical products are also available nowadays as pills, powders, extracts, soluble granules, tablets, capsules, syrups, and oral liquids.

Apart from using herbs as therapeutic drugs, some of them can be used in making medicinal diets, often referred to as dietary therapy. It is believed that a combination of food and herbs in the diet would have particular benefits, produces beneficial physiological responses, and restores internal balance. Dietary therapy involves careful selection of foods and herbs to treat mild visceral disorders, or address mild conditions of excess and deficiency. In addition, certain foods and herbs are applied during different seasons to provide counteracting effects in order to resist any undesirable impacts caused by environmental and weather changes. Good dietary practice is considered essential to assure a healthy life and longevity.

The last decades have witnessed a rapid growth of the health-food industry all over the world, in which the so-called functional foods, dietary supplements, or nutraceuticals are dominating the markets. Chinese medicinal herbs serve as important source materials for the production of many herbal health-foods. Indeed, many claims of health-food products are made on the basis of TCM theories, such as the energy-enhancing effect of ginseng root, blood-nourishing function of angelica root, and general health-promoting action of astragalus root.

2.1.3 Chinese Medicinal Herbs

The Chinese herbs are classified according to their ability to affect any of the functional status of the organs (such as reinforcing the Yang of the kidney or replenishing Qi of the lung) or to counteract the perceived pathological factors—the "evils" (such as dispelling excessive coldness from the stomach or calming the fire in the heart). Each herbal drug is characterized by specific properties that are correlated with their ultimate clinical effects. In general, drug properties are described in terms of their taste (viz. bitter, sweet, acrid, salty, and sour) and nature (viz. hot, cold, warm, or cool). They are then correlated to the therapeutic effects.

The principle of "opposite" applies in the selection of herbs. *Huang-di Nei-jing* states, "If the disease is due to excessive coldness, warm it; if the disease is due to excessive hotness, cool it." And the Herbal of the Divine Plowman echoes this, saying "Cure cold diseases with warm medications, and cure hot diseases with cold medications." On the other hand, experiential evidence has shown that each of the five tastes of the herbal drugs indicates a generalized therapeutic effect. For example, sweet drugs are tonics (substances taken to gain vigor or well-being of the body) and they can be used to treat various symptoms of deficiencies concerning Qi, blood, Yin or Yang. Thus, the Chinese/Korean ginseng (*Panax ginseng*) root, which possesses a sweet and slightly bitter taste and has a warm nature, is useful to nourish Yang function, to dispel coldness and to warm the body. On the other hand, the American ginseng (*Panax quinquefolium*) root is sweet and slightly bitter but it has a cool nature; it is therefore suitable for use to nourish Yin function and to remove excessive heat from the body. Some examples of commonly used Chinese medicinal herbs are included in Table 2.1.

The plant kingdom is a rich source of natural chemical substances, many of which have been found to be important natural pharmaceutical agents (such as caffeine, digitoxin, morphine, and paclitaxel). It is therefore not surprising that Chinese medicinal herbs constitute a vast pool of pharmacologically active chemical compounds. Indeed, through Pharmacognosy research, a number of active principles have been identified from Chinese medicinal herbs. These biologically active natural products not only provide evidence, at least partially, for rational use of the medicinal herbs, but also serve as drugs or templates for chemical modification into useful drugs. For example, ephedrine from *Ephedra*

TABLE 2.1 Examples of Commonly Used Chinese Medicinal Herbs

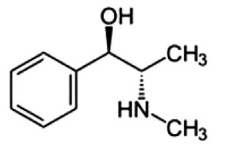
Botanical Name	Medicinal Part	Nature	Taste	Major Effect
Angelica sinensis	Root	Warm	Acrid and sweet	Blood tonic
Artemisia capillaris	Above-ground parts	Cool	Acrid and bitter	Diuretic and clear heat
Astragalus membranaceus	Root	Warm	Sweet	Tonify Qi
Atractylodes macrocephala	Root	Warm	Sweet and bitter	Tonic; diuretic
Bupleurum chinense	Root	Cool	Bitter	Clear heat
Chrysanthemum morifolium	Flower head	Cool	Sweet and bitter	Clear heat
Cinnamomum cassia	Young stem	Warm	Acrid and sweet	Diaphoretic
Citrus reticulata	Fruit rind	Warm	Acrid and bitter	Regulate Qi
Codonopsis pilosula	Root	Neutral	Sweet	Tonify Qi
Coptis sinensis	Rhizome	Cold	Bitter	Clear heat
Crataegus spinnatifida	Fruit	Warm	Sweet and Sour	Digestive; stomachic
Ephedra sinica	Stem	Warm	Acrid	Diaphoretic
Fritillaria cirrhosa	Corm	Cool	Bitter and sweet	Antitussive; expectorant
Glycyrrhiza uralensis	Root	Neutral	Sweet	Tonify Qi
Lonicera japonica	Flower	Cold	Sweet	Clear heat
Panax ginseng	Root	Warm	Sweet; slightly bitter	Tonify Qi
Poria cocos	Fungal body	Neutral	Sweet	Diuretic
Prunella vulgaris	Inflorescence	Cold	Acrid and bitter	Clear heat
Prunus armeniaca	Kernel	Warm	Bitter	Antitussive
Rehmannia glutinosa	Root (raw)	Cold	Sweet	Clear heat; cool blood
Rehmannia glutinosa	Root (steamed)	Warm	Sweet	Tonify Yin; blood tonic
Rheum palmatum	Rhizome	Cold	Bitter	Purgative; Laxative
Salvia miltiorrhiza	Root	Cool	Bitter	Promote circulation; tonify blood
Zingiber officinale	Rhizome	Warm	Acrid	Dispel cold
Ziziphus jujuba	Seed	Warm	Sweet and Sour	Tonify Qi; sedative

sinica is a sympathomimetic agent used as a central nervous system stimulant, artemisinin is an antimalarial drug obtained from the *Artemisia annua* herb, and camptothecin is an antitumor agent discovered from the tree *Camptotheca acuminata* (Fig. 2.3).

2.1.4 The Way Forward

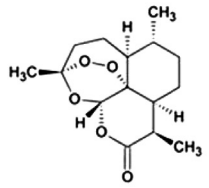
Over the last few decades, the world has witnessed the rapid and continuing growth of interest in TCM. The recognition that conventional medicine fails to offer satisfactory cure to many diseases (such as allergies, autoimmune diseases, chronic pains, and cancers) and that many drugs even have marked side effects have turned many attentions to search for alternative therapies and herb-based supplements. The movement is expedited by the increasing awareness of health issues, consciousness in disease prevention, and preference in improvement of the quality of life.

While Chinese medicine is a viable approach that holds great promise to improve people's health, there remains a clear and urgent need for a stronger evidence base to ensure and support the continuing development of this traditional medicine in modern societies. Some important issues are highlighted below.



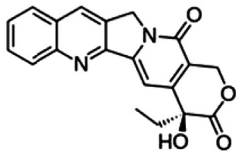
Ephedrine

- Found in *Ephedra sinica* (Ma-Huang)
- Acts as a sympathomimetic agent
- Used as a central nervous system stimulant
- A stereoisomer, pseudoephedrine, is a nasal decongestant



Artemisinin

- Found in *Artemisia annua* (Qing-Hao)
- Acts against the malaria parasite, *Plasmodium falciparum*
- Used as an antimalarial drug
- Analogs such as artemether and artesunate are also used in malarial therapy



Camptothecin

- Found in *Camptotheca acuminata* (Happy Tree)
- Acts as an inhibitor of topoisomerase I
- Useful as an anticancer drug
- Two analogs, topotecan and irinotecan, are used in cancer chemotherapy

FIGURE 2.3 Examples of active compounds found in Chinese medicinal herbs.

2.1.4.1 Clinical Evidence

In this era of evidence-base medicine, the biggest challenge to Chinese medicine is perhaps how to provide clinical evidence that is properly assessed. There is no doubt that a vast body of literature is available on the clinical outcome of Chinese medicine, but it is less clear what level of evidence it represents and how it can be properly assessed. It is therefore important to develop reliable and practical protocols to ensure the quality of clinical research on Chinese medicine so that the efficacy can be convincingly demonstrated.

2.1.4.2 Scientific Research

Developed largely on experiential basis, Chinese medicine is facing fierce challenges to demonstrate its safety, the mechanisms of action, and quality of the herbal products. In this respect, vigorous research is needed to confirm the evidence-based efficacy, to elucidate the mechanisms of action, to define pharmacological and toxicological profiles, to evaluate the safety, and to ensure the quality of the medicinal products.

2.1.4.3 Regulatory System

In the market, the quality of Chinese herbal products may vary. Problems include inconsistent composition, batch-to-batch variation, misleading labels, contamination, adulteration, and inclusion of undisclosed pharmaceutical ingredients. The situation has resulted in part from the lack of adequate regulations and policies. It is anticipated that many of these problems can be corrected under a well-planned regulatory framework.

2.1.4.4 Education

The strong consumer demand on alternative therapies including Chinese medicine requires better education and dissemination of information to the general public. For example, consumers' awareness of potential adverse reactions and herb–drug interactions associated with certain herbal products has to be raised. On the other hand, the mainstream medical professionals also need to receive adequate education about this kind of alternative therapy and to learn how to assess critically the validity of its claims.

2.2 THE INDIAN SYSTEMS OF MEDICINE

The health-promotive, preventive, and curative properties of herbs were recognized by the ancient sages and physicians of India to form the theoretical and conceptual foundations of the Indian Systems of Medicine. Deeply rooted in the traditions, culture, civilization, and religion of the people, these medicine systems can be broadly classified into two categories, the classical and the traditional systems. Among the "classical" Indian systems are Ayurveda, Siddha, Amchi, Unani, Yoga, and Naturopathy. They are universal in character and are neither location- nor language-specific. All these systems are supported by well-codified written treatises. The practitioners of these systems were trained under mentors in the old days and now in the schools of medicine. On the other hand, the "traditional" system of Indian medicine is oral in character, location- and community-specific, often only practiced by certain ethnic groups, families, or individuals. They are represented by the tribal, folkloristic, local health, and household remedies, as well as bone setters, practitioners in treatment of poisonous bites, and birth attendants.

2.2.1 Ayurveda

Ayurveda deals with the physical, mental, and spiritual world of mankind. It identifies man as an integral part of nature and stresses the necessity of maintaining harmony with all living and nonliving components of the surroundings (such as air, soil, and water). Ayurveda is a prevention-oriented holistic science of natural healing developed by the great masters of India. The term Ayur refers to "life" and Veda means "science," and thus translates into the "science of life." Ayurveda accomplishes its goal by treating diseases as well as coordinating the body, mind, and soul nexus with the help of vegetarian diet, medicinal herbs, exercise, and meditation. The origins of this science, though difficult to pinpoint, have been placed by Indian scholars somewhere around 6000 BC. According to the different interpretations made by the various scholars of Ayurveda, a number of treatises were derived.

The treatises of Charaka Samhita describe fundamental physiology, anatomy, etiology, and pathogenesis of diseases, as well as diagnostic criteria, treatment protocols, and prognosis. In addition, it describes the principles of prevention and social behavior conducive to emotional and physical health. There are detailed descriptions of treatments through the use of medicinal plants in the elimination (by the administration of carminatives, digestives, etc.) and rejuvenation therapies.

Sushruta is considered the father of surgery (particularly of plastic and reconstructive surgery). The Sushruta Samhita thus contains detailed descriptions of over 1000 surgical instruments including scalpels, scissors, forceps, and specula, and it includes procedures for the treatment of fractures, wounds, plastic reconstructions, abscesses, cesarian (legends relating to the birth of Buddha appear to indicate that he was born at a cesarian section), and bowel surgery. Anatomic details and their pathological alterations, embryology, toxicology, and therapeutics are impressively narrated.

2.2.1.1 Basic principles of Ayurveda

The following concepts in Ayurveda guide the preventive, health-promotive, and curative aspects of the practice.

2.2.1.1.1 The Three Principles of Nature

Satwa, Rajas, and Tamas are said to be the essences of nature in which all physical and physiochemical energies are included. Thus, energy existing in all matters is due to Rajas, resistance and stability of matters is due to Tamas, and all conscious manifestation of matters is due to Satwa. In nature, these three principles always exist in an interdependent manner.

2.2.1.1.2 Five Gross Elements

The five gross elements are earth, water, fire, air, and space. Creation of all forms of life is credited to these elements. The qualities of the five elements and their properties are given in Table 2.2.

Diseases are said to occur in the human body due to the imbalance of the five gross elements caused by a variety of reasons. Therefore, the main objective of the treatment is to restore the balanced state. To detect this imbalance due to the five gross elements, Ayurveda established the three humors, vital organs, and excretory products theories.

2.2.1.1.3 Three Humors

The three constituent humors are Vata, Pitta, and Kapha. These three concepts are broadly comparable to the modern concepts of motion, energy, and inertia. The concept of the Kapha Dosha (inertia) helps in synthesizing the building

TABLE 2.2 Five Gross Elements and Their Qualities

Five Gross Elements	Properties
Earth	Hardness, grossness, inertia, compactness, smell
Water	Coldness, fluidity, viscosity, softness, unctuousness, taste
Fire	Heat, color, form, lustier, digestive power, mental faculties, anger, velour, vision
Air	Movement, lightness, impulse, all functional activities in the organism, all vibrations, sense of touch
Space	Porosity, vacuum, sound, power of differentiation, hearing

blocks of cells and thus deals with cellular and intracellular structures of the human body. It is responsible for support to, and stability of, the body. Pitta Dosha (energy) refers to the energy state of the body and is concerned with the metabolic and biochemical processes which generate heat and energy. The function of the Vata Dosha (motion) is to regulate the proper use of energy by the different cellular structures. Ayurveda describes the Vata Dosha as the controller of the other two Doshas. The relationship between the five gross elements and the three humors is as follows: Space and Air are predominant in Vata; Fire in Pitta; Water and Earth are predominant in Kapha.

2.2.1.1.4 Vital Organs/Tissues

There are seven vital organs derived from the functional units of the three humors; they include bone tissue, blood, body fluids, muscular tissue, adipose tissue, nerve tissue, and bone marrow. The generative organs include sperm and ovum.

2.2.1.1.5 Excretory Products

This concept deals with the waste products of the body. The foods consumed by the human body bring into existence and build further the seven vital organs/tissues. During the metabolic process, each organ produces a specific waste such as stool, urine, and sweat. Health according to Ayurveda is thus a balanced inter−intra state of all the three humors, vital organs, and excretory products. However, these are subjected to quantitative and qualitative alterations.

2.2.1.2 Pathogenesis of Diseases and Principles of Treatment in Ayurveda

An individual is said to be born with a predominance of a particular humor which contributes to his constitution. Apart from genetic influence, a person's constitution is also affected by age, environment, and diet. Thus, depending on the excess of any one of the three humors, a person would have one of the following constitutions: Kapha, Vata, or Pitta. Constitution is said to sometimes decide the susceptibility of an individual to a disease. For example, individuals with a Pitta nature are more prone to develop diseases with symptoms similar to peptic ulcer. Such a medical view is not unique to Ayurveda. The Yin/Yang theory and the Hippocratic theory of four humors in Greek medicine have a similar approach. The treatment is thus aimed at not only curing the disease but also enhancing the body vitality to minimize the chances of a relapse. The different modes of treatment offered by Ayurveda include dietary alterations, drugs, exercise, surgery, and stress management.

2.2.1.2.1 Diet

Ayurveda has laid great emphasis in the diet both for its direct effects on the physiological state of an individual and also for its influence on drug action. A proper assimilation of the dietary constituents is essential for the maintenance of good health. Improper assimilation or formation of intermediate products of digestion has toxic properties and they are treated as foreign compounds by the body. Specific diets have been prescribed for psychiatric disorders. Recent evidence indicates that brain levels of neurotransmitters such as serotonin, catecholamine, and acetylcholine can be influenced by dietary constituents. Consequently, it has been suggested that normal brain functions and mental diseases can be altered by diet. Ayurveda prescribes certain diets during drug therapies as dietary constituents are believed to influence drug action.

2.2.1.2.2 Drugs

Drug therapy is well-developed in Ayurveda. There are at least 70 books containing up to 8000 recipes for the preparation of drug combinations. The drugs used are derived from a wide range of plant materials, animals, and minerals. Rig Veda is the oldest literary document that presents the knowledge about medicinal herbs. Ayurvedic pharmacology explains the qualities and properties of drugs in terms of its taste, potency, metabolism, specific potency, and the other properties. Detailed guidelines are available on locating, collecting, and identifying medicinal plants for the preparation of drugs.

Many formulations ranging from simple distillates, decoctions, linctus, and powders to elaborate pharmaceutical preparations like pills, fermented products, and medicated oils are available. Ghee medicated with herbs and medicated oils are attractive techniques which use the process of incorporating drugs in oily particles to their site of action. Another practice in Ayurvedic medicine is that of administering drugs in combination in order to reduce toxicity and increase efficiency.

Drug therapy in Ayurveda is highly individualized. Thus the choice of drugs as well as their doses is not only influenced by the disease state, but also by the constitution of the patient and the environmental conditions which are likely to affect the balance of the humors and hence the response to the drugs. *Piper longum* and *Zingiber ofiicinale*, e.g., can increase the Pitta humor and must be used cautiously in individuals with a Pitta constitution.

Many plant preparations are prescribed to strengthen the general host resistance. These drugs are called Rasayana, Jeevanya, and Balya drugs, all of which increase tissue resistance to disease. For the promotion and maintenance of positive health and prevention of disease, Ayurveda prescribes the observation of certain principles: daily routine, nightly routine, seasonal routine, and the ethical routine, and also emphasizes that one must follow a regulated diet, sleep, and regulated gratification of sex. Thus, Ayurveda is not merely a medical science but a way of life.

2.2.1.3 Ayurvedic Concepts in Modern View

Experimental studies on some of the concepts of Ayurveda have pointed to the assumption that Vata, Pitta, and Kapha are neuron-humors liberated by the brain and its nerve endings. For example, Vata has been equated to acetylcholine liberated by the cerebral cortex and peripheral and parasympathetic nerve endings; Pitta with catecholamines liberated by the hypothalamus, sympathetic nerve endings and the adrenal medulla; and Kapha with histamine secreted by the brain stem. The drugs, when administered, can act by promoting or destroying the respective humors. It was also observed that a person with Vata humor is lean with an excess of acetylcholine, that of a Pitta humor is muscular with an excess of catecholamines, and Kapha humor has a heavy body with an excess of histamines.

However, such studies lead to the path of Western medicine leaving the much acclaimed holistic approach to total neglect. Hence, if Ayurveda has to sustain itself, it is essential to retain its concepts and plurality of outlook on health and disease in its original form.

2.2.2 Siddha

Siddha is a system of medicine that is of truly Indian origin. It is practiced in the Tamil-speaking areas of southern India. The principles and doctrines of the Siddha system have a close similarity to Ayurveda with specialization in iatrochemistry. Mercury, sulfur, iron, copper, gold, bitumen, white-, yellow-, and red-arsenic, and other minerals as well as vegetable poisons, marine and, animal products are extensively used in Siddha tradition.

Even though Siddha bears a close resemblance to Ayurveda, the evolution of this medicine system in a different environment led to its own merits. Many diseases not mentioned in the Ayurvedic texts and many potent remedies are included. While Ayurveda does not make use of plants like *Acalypha indica*, *Dichrostachys cinerea*, and *Mukia maderaspatana*, they are profusely employed in Siddha medicine for a variety of purposes.

2.2.3 Other Classical Indian Medical Systems

The *Unani* medicine system was introduced to India about a thousand years ago by the Muslims and became indigenous to the country. It is now practiced in the Indo-Pakistan subcontinent. The Unani physicians who settled in India have added new drugs to the system and therefore the Unani system practiced in India is somewhat different from the original Greek form.

The *Amchi* system of medicine, also known as the Tibetan System, is practiced in northern India and some other regions of the Himalayas particularly by the Buddhists. This system traces its origin to Ayurvedic system to include

treatments by herbs, minerals, animal organs, spring and mineral waters, puncturing of veins, mysticism, and spiritual powers.

Naturopathy (natural cure) is not only the system of treatment but a way of life. It is often referred to as drugless treatment of diseases. Naturopathy is based on the ancient practice of simple laws of the nature. This system is closely allied to Ayurveda as far as the fundamental principles are concerned.

2.2.4 Tribal Medicine and Local Health Traditions

The Indian subcontinent is inhabited by over 53 million tribal people belonging to 550 communities that come under 227 linguistic groups. They inhabit varied geographic regions and climatic zones and live in perfect harmony with the ecosystem. Magico-religious beliefs predominate their way of living. The tribal people have acquired unique knowledge about the flora and fauna by empirical observation and reasoning. They have perfected simple but effective remedies to treat common ailments as well as methods to improve vigor and vitality. The resource base of their traditional remedies is mainly plants. Animals and minerals also find their use to certain extent.

Apart from the tribal communities, the rural population of India, living in the villages, make use of a large number of plants found in their neighborhood for treatment of a variety of ailments. This knowledge system mainly consists of household remedies and finds application especially in the mother and child care and in the treatment of simple ailments such as fever, cough, diarrhea, cut wounds, and sprains.

The Local Health Tradition system is widespread in India. The carriers of these traditions are housewives, traditional birth attendants, bone setters, practitioners skilled in acupressure, ophthalmic physicians, dental physicians, veterinary physicians, and village herbal medicine workers.

2.2.5 Current Status of Medicinal Plant and Natural Product Research in India

In India there are over 300,000 registered Ayurvedic medical practitioners, over 20,000 dispensaries, 2000 hospitals, 187 undergraduate teaching Institutions, 51 postgraduate departments, and 8400 pharmacies manufacturing Ayurvedic medicines.

Several government and private institutions have been established exclusively for research on medicinal and aromatic plants. Over the years, these institutions have generated a wealth of information on the medicinal properties of a large number of plants. In addition, they have been successful in introducing a number of scientifically validated herbal remedies into the Indian market. Representative medicinal plants on which significant research leads have been obtained in the Indian laboratories are listed below:

Picrorhiza kurroa—Antihepatotoxic
Phyllanthus amarus—Antihepatotoxic
Andrographis paniculata—Antihepatotoxic
Curcuma longa—Anti-inflammatory, anticancer
Withania somnifera—Adaptogenic
Acorus calamus—Tranquilizer
Sida rhombifolia—Anabolic
Albizia lebbeck—Immunomodulator
Trichopus zeylanicus—Immunomodulator
Valeriana wallichii—Tranquilizer

Some important plant-derived drugs manufactured in India include etoposide, tenoposide, vincristine, vinblastine, quinine, taxoids, ajmaline, ajmalicine, morphine, codeine, papaverine, thebaine, emetine, digoxin, caffeine, hyoscyamine, hyoscine, xanthotoxin, psoralen, rutin, colchicine, berberine, strychnine, brucine, ergot alkaloids, senna glycosides, artemisinin, etc.

2.2.6 The Way Forward

With India being a melting pot of different cultures—Dravidian, Aryan, Greek, and Islamic—each ethnic group developed and nurtured its own system of medicine. Thus in the Indian subcontinent, we can trace the origin and development of four classical systems of medicine, namely, Ayurveda, Siddha, Unani, and Amchi, each having its own theoretical and philosophical back grounds and therapeutic regimes. However, since these systems have coexisted in India

for several centuries, they have liberally drawn and assimilated the best from each other, thus promoting the harmonious growth and popularity of each system. After independence, the Government of India promoted the Indian systems of medicine through the Department of AYUSH (Ayurveda, Yoga and Naturopathy, Unani, Siddha, and Homeopathy). This Department was created in 2003 with a view to providing focused attention to the development of education and research, quality control and standardization of drugs, in providing the availability of medicinal plants, and research and development using state of the art scientific and technological knowledge and tools. Thus, Indian systems of medicine are growing in a big way gaining popularity not only in the Indian subcontinent but also internationally.

2.3 AFRICAN TRADITIONAL MEDICINE

African traditional medicine is a diverse and multifaceted knowledge system, which remains largely transmitted from generation to generation in oral form. As much as Africa is a large continent uniting very different cultures and ethnic groups, traditional medical knowledge combines many global and local elements.

This section highlights the more common hallmarks of traditional medicine, explains the context and the different types of healing practices, and discusses some aspects of African traditional medicine having its rightful place in primary health care and health innovation.

2.3.1 The Context of African Traditional Medicine

Across Africa, healing and care are placed in the context of "Ubuntu" or "Botho" as it is called in Botswana. Both terms describe a philosophy that emphasizes human relatedness, socially, with the natural environment, and with the spiritual world. As a consequence of this relatedness, "Ubuntu" is based on certain values, such as generosity, hospitality, loyalty, honesty, and respect for elders, ancestors, nature, and God.

The implication of "Ubuntu" for healing is that there has to be a holistic approach to human conditions and health restoration, an approach that considers not only symptoms of a condition and medication to address them, but also the root cause of disease and preventative measures encompassing social or pathogenic aspects of diseases.

The example in Box 2.1 illustrates this point. Health seeking is often coded and the traditional healer has to decode before addressing physical issues. At the same time a social dimension has to be considered before health can be fully restored. This social dimension recognizes that health matters are sometimes private and complex, hence the need to negotiate in codes in order to avoid stigma and embarrassment—a critical aspect of "Botho."

BOX 2.1 Backache—How are things at home?

Dikaelo Ndozi, an old healer in Maun, Northwestern Botswana, who has been a healer in the seventh generation of his family, told us about a certain medicinal plant he uses for treatment and he mentioned backache as indication. He explained: "When a man comes to me and complains about backache, I first ask him 'how are things at home?' He most likely does not suffer from backache. A man will use backache as a code that his love life is not intact. He will need something that heals impotence or if he has a sexually transmitted infection (STI) things are a bit tricky. His partner also needs treatment. He needs advice on how to handle this situation."

2.3.2 The Principles of African Traditional Medicine

2.3.2.1 Concepts of Diseases

The basic concept of disease in African healing is that no one becomes sick without a reason. Conditions are seen as consequences of an imbalance either in social relations, human/nature relations, or human/ancestral relations. Therefore, measures for treatment and cure need to address not only symptoms but also the imbalances themselves.

Often one can distinguish two dimensions of disease causality: a proximal cause which addresses *how* a specific disease has been contracted, and secondly an ultimate cause which accounts for *why* a disease has inflicted a particular person. A mother may accept that her child has diarrhea because flies settled on his food (proximal cause), but she also want to establish who sent the flies to harm her child (ultimate cause).

A good healer is therefore a person who knows effective medicines, but at the same time has an intimate understanding of the relation of his/her client and the world around him/her. More concretely, three general categories of causes leading to diseases can be distinguished, which are often related to each other.

2.3.2.2 Contact With Pollutants

It is well understood that people are exposed to pollutants originating in their environment, which can cause infection and contagion. That understanding of proximal causes actually predated the arrival of Western biomedical concepts. Pollutants are seen to accumulate in body fluids, such as semen, menstrual blood, blood in general, secretions, and discharges. Therefore much attention is given to practices whereby such body fluids are exchanged. Additionally, death is also considered as a form of pollution affecting the social environment of the deceased. Protection is sought through sets of behavioral codes of conduct ("taboos") which need to be observed and by taking protective measures in the form of taking cleansing medicines or undergoing cleansing rituals.

2.3.2.3 Violating Taboos

Taboos are sets of rules to control exposure to pollutants and to avoid imbalances. In the African context taboos are often related to sexual conduct, food, and taboos to maintain maternal health.

Sexual taboos, for example, include that no sexual intercourse should happen if a woman has her menstruation, has had an abortion, or has recently miscarriaged. Here the intention is to reduce exchange of body fluids which could be sources of pollution and therefore lead to infections. Other sexual taboos seek to prevent sexual intercourse with a person who has recently become a widow or a widower. The rationale is to maintain psychological stability by allowing grief to occur and to become ready for new relationships.

Food taboos include abstention from consumption of certain foods, which might have hygienic or cultural reasons. For example, communities in Botswana are often aligned to a "Totem" animal, such as a crocodile or a type of antelope. Members of that community are not allowed to eat the meat of their "Totem." Maternal health taboos can include restrictions on food intake in order to ensure the normal development of the baby. Other taboos prescribe a confinement of the mother for a period of time to the house in order to give mother and child the opportunity to adjust to the new situation and also to reduce exposure of the newborn to potential infective agents as a consequence of a highly interactive environment.

Essentially, taboos mediate basic hygiene procedures and help to avoid psychological disturbances that may lead to mental conditions. Obedience to taboos strengthens the life force and health, while disobedience weakens it. Diseases inflicted through violation of taboos require treatment regimen that involve general and specific cleansing procedures performed by traditional healers.

2.3.2.4 Imbalance With Ancestral World

Good health and well-being is related to appropriate behavior in accordance with the values of "Ubuntu" that emphasize respect for other human beings, nature and ancestors. When ancestors are disrespected, neglected, or forgotten they can punish with diseases. Mitigation can only be achieved by prescribing medicines, specific foods, or beverages thought to restore the lost connection to the ancestral world. The domain of mental conditions often falls in this category.

Concepts of infection and contagion, often perceived as sole domains of Western biomedicine, do exist in African traditional medicine and are incorporated in the fabric of proximal and ultimate causes. An example illustrating this point is a condition, which is called "Seswagadi/Boswagadi" in Setswana, the local language of Botswana (Box 2.2).

BOX 2.2 The condition of "Seswagadi/Boswagadi"

According to traditional healers of North Western Botswana "Seswagadi/Boswagadi" is an STI. Its symptoms include stiffness of the neck, severe headache, and patients often cannot walk upright. As these symptoms do not constitute the classical hallmarks of STIs, and even children can be affected, we asked healers for clarification. They explained that the disease is a consequence of a violation of certain sexual taboos, such as having had intercourse with a woman who had her menstruation or with a woman whose partner had recently died or had a miscarriage. A person who has violated the taboos might get "Seswagadi/Boswagadi," which can be fatal if left untreated. According to the healers the condition is highly contagious. If one sits on the

(Continued)

BOX 2.2 (Continued)

same chair as the "Seswagadi/Boswagadi" patient, the person will suffer from the condition as well. Even children might acquire conditions as a consequence of exposure to "Seswagadi/Boswagadi," with its own set of symptoms. Treating this condition is considered as a domain of traditional healers who will prescribe in most cases a combination therapy consisting of herbal medicines taken orally and for bathing with the purpose of general cleansing. Often all immediate contact persons of the "Seswagadi/Boswagadi" patient are included in the therapy.

Interestingly, HIV/AIDS was initially considered by many healers as a form of "Seswagadi/Boswagadi." Its cause was considered as a violation of sexual taboos by being too promiscuous. The condition was seen as contagious and being transferred through exchange of body fluids and treatment was recommended urgently. Even though healers might not have had a concept of viral infection, important aspects of HIV/AIDS were grasped and could have been incorporated in a comprehensive health response.

This condition is not an STI in the sense of the biomedical concept where a pathogenic organism is the disease-causing agent. Nevertheless, traditional healers subsume "Seswagadi/Boswagadi" under this category of diseases as the condition arises through sexual intercourse in a context where sexual taboos have been violated. The condition is contagious.

Having explained the connection of disease causes and imbalance in social and ancestral relations, it does not mean that all conditions require a systemic approach to health restoration addressing the spiritual/ancestral domain. Concrete physical procedures, such as blood-letting, small operations, healing of bone fractures, opening abscesses, treating wounds, managing cough and asthma, treating headache/migraine, heart palpitations, stress-related conditions, and high blood pressure, are daily practices of traditional healers. Furthermore, traditional birth attendants are very active in various communities providing valuable services to pregnant women, before, during, and after delivery.

2.3.2.5 Witchcraft

During colonialization and with the arrival of Christian missionaries, African traditional healers were derogatorily labeled as "Witch doctors" practicing "Witchcraft." Most serious traditional healers would outrightly reject such an association. However, generally no one would object to the fact that witchcraft exists. For example, illness can be inflicted by people (relatives, neighbors, other community members) who have been offended by a victim's behavior. "Witchcraft" is therefore seen as a possible ultimate cause for ill health, which can only be counteracted by certain rituals that deflect witchcraft and strict obedience to the norms of appropriate behavior.

2.3.3 Different Types of Traditional Healers

Given the above discussed concepts of diseases it follows that traditional healers in Africa need to unite a range of skills which include a good understanding of nature and its resources, excellent social skills, and the ability to relate with the ancestral world in order to exercise their profession.

However, during colonialization with the advent of missionary churches and the establishment of formal education, traditional healers have evolved into internally differentiated groups. The spectrum includes herbalists, who only use medicinal plants for healing practices. There are "diviners"—healers who use a set of bones which allow communication with ancestors and which is used for diagnostic purposes. Diviners often act on the basis of dreams during which communication with ancestors occurs. Finally there are faith/prophetic healers.

The example of British-colonialized Botswana may help to understand why these separate groups of healers have emerged. Early missionaries in Botswana considered traditional healers who communicated with ancestors as sorcery, un-Christian and evil. These "diviners" were regarded as heathens and their practice was associated with witchcraft. Herbalists on the other hand, since they used natural herbs only, were not seen as interfering or contravening the concept of God. Efforts to eliminate traditional healers who are able to communicate with ancestors culminated in the Witchcraft Proclamation Act of 1927 in Botswana, which banned all divining activities, while herbalism was permitted. This separation undermined the totality of belief systems which considered diseases as a consequence of an overall imbalance between the natural and the spiritual world.

However, even after centuries of efforts to eliminate the spiritual aspects from traditional healing, the proportion of "diviners" is still relatively high. But the banning of their activities has created new spaces of healing practices for churches and faith/prophetic healers to come into and compete for health-seeking choices. In difficult socioeconomic

conditions the demand for traditional healing interventions are increasingly sought by workers, the unemployed, the poor, but also by the upper class to secure protection and promotion.

In spite of separate groups of traditional healers, there also exists a continuum of practice. Faith/prophetic healers use sometimes herbs, "diviners" use bones and dreams for diagnosis but prescribe herbal treatment. Herbalists are often guided through dreams which medicinal plants to use.

Apart from the historical differentiation of traditional healers, a specialization of healing (professionalization) has also emerged. Traditional healers can be general practitioners, who are mostly treating routine conditions, such as headache, coughs, respiratory ailments, colds, minor and/or often occurring infections, stress-related conditions, pains, high blood pressure, diabetes, gout, diarrhea, stomach complains, and fever. Other healers are specialists. These include traditional midwives/birth attendants; healers who treat only children (traditional pediatricians), traditional surgeons who perform circumcisions, removal of growths (e.g., piles), tooth extractions or scarification, traditional healers who are very skilled in treating mental conditions ("Sangomas"), and a class of healers who exclusively treat bone fractures or dislocations in humans and animal (bone setters).

2.3.4 Medicinal Plants, Mixtures, and Combination Therapies

African traditional medicines largely remain plant-based, although some preparations include animal parts and minerals. The mode of administration of medicines varies and includes oral ingestion, topical applications, inhalation of steam, sniffing, and smoking or exposure of affected areas to smoke.

Mostly a mixture of different medicinal plants is prescribed. A reason for this is that in most cases patients present to traditional healers with conditions expressing themselves through multiple symptoms. Therefore medicinal plants are mixed in a way that all symptoms are addressed. For the same reason often combination therapies are applied which consist of two or more different treatment regimen. For example, if a patient suffers from a boil, the patient might be prescribed a generally "cleansing" medicinal plant preparation for oral intake, and another mix of medicinal plants for applying topically onto the boil or for washing the affected area.

2.3.5 African Traditional Medicine in the 21st Century

Traditional medical knowledge systems are not a thing of the past. African traditional healing forms a lively part of pluralistic primary health care systems. People prefer to consult traditional healers for certain conditions, while they will visit local clinics for others. Looking at health-seeking choices of patients there should be mutually respectful and trustful coexistence of traditional health practices and biomedicine.

As traditional medical knowledge has accumulated over generations there are valuable health data from patient observations that can meaningfully contribute to drug discovery processes.

African traditional medicine has found its global market often masked by shiny presentations and packages. Particularly for chronic diseases cosmopolitan, health-conscious people prefer more balanced therapeutics to the "magic bullets" of biomedicine. An example is Devil's claw (*Harpagophytum procumbens*), which has a huge market in Europe and the United States as an over-the-counter medicine for arthritis and rheumatic conditions. The Devil's claw tubers originate from Botswana and Namibia and are exported to the health industry in Europe which adds value by manufacturing pills and ointments. Another example is *Pelargonium sidoides*, a medicinal plant which is indigenous to South Africa. A tincture of this plant is widely sold, for example in Germany under the name "Umckaloabo" for treatment of upper respiratory conditions. Other important African medicines are compiled in Table 2.3.

However, Africa needs to get a better share of the health sector than just being the supplier of raw materials. Value addition to African traditional medicines should be realized on the continent itself so that African natural resources can lead to better means to respond to health challenges and to create revenue and employment in a lively health industry.

2.3.6 The Way Forward

Health solutions can come out of Africa. But there are problems and challenges to be overcome. Firstly, the profession of traditional healers should be formally and legally recognized by Governments through policy and regulatory frameworks that can also ensure a transparent system for obtaining qualifications of this profession. This is needed to reduce the number of fake healers and business seekers.

Secondly, the persisting negative attitude to traditional medicine by Western biomedicine needs to change in order to facilitate fruitful collaborations. What unites the two spheres of knowledge is a deep concern for the well-being

TABLE 2.3 Important African Medicinal Plants

Plant species/ Family	Origin/ Distribution	Traditional Medical Use	Pharmacological Properties	Health Products
Ancistrocladus korupensis	Cameroon	Malaria	Alkaloids Michellamine A–C possess anti-HIV properties, particularly Michellamine B is active against HIV-1 and HIV-2.	Development suspended as toxicity too close to desired antiviral activity dose.
Catharanthus roseus	Madagascar/ all over Africa	Blood cancer treatment; hypotension; diabetes; hepatitis	Dimeric alkaloids vincaleukoblastine and vincristine have antitumor and antileukemic properties. Reserpine and ajmalinine have hypotensive characteristics. Leurosine sulfate and vindolinine have hypoglycemic properties.	Vinblastine and vincristine for treatment of Hodgkin's and other forms of lymphoma, leukemia, Wilm's tumor in children and breast cancer.
Harpagophytum procumbens	Botswana/ Namibia/ South Africa/	Allergies, analgesia, arteriosclerosis, neuralgia, myalgia, migraine	Anti-inflammatory, analgesic, and antioxidant properties. Some clinical studies demonstrated efficacy in treatment of rheumatic symptoms and osteoarthritis.	Devil's claw in the form of tinctures capsules, and ointments
Pelargonium sidoides	South Africa	Diarrhea, dysentery, sexually transmitted infections	Immune stimulation/immune modulation; antibacterial and antiviral effects. Clinical studies have demonstrated shortening the duration and severity of acute bronchitis.	"Umckaloabo" tincture for treatment of upper respiratory conditions particularly for children
Prunus africana	Tropical and subtropical parts of Africa	Genitourinary complaints, inflammation, kidney problems	Contains inhibitors of prostatic 5α-reductase. Clinical studies revealed efficacy and safety of *Prunus* extracts in treatment of mild to moderate benign prostatic hyperplasia.	Extracts and capsules for treatment of benign prostatic hyperplasia
Piper guineense, *Pterocarpus osum*, *Eugenia caryophyllus*, and *Sorghum bicolor*	Nigeria	Sickle cell anemia	Niprisan (also known as Nicosan) was found in clinical trials to be effective in reducing severe painful crises of sickle cell anemia.	Marketed in capsules for treatment of sickle cell anemia.
Warburgia salutaris	Eastern and Southern Africa	Important tonic; treatment of fever, colds, pains, headache, chest infections	Drimane sesquiterpenoids are highly active against *Candida* (yeast) infections and can serve as adjunct to antibiotic treatment of microbials that have poor membrane permeability.	Freeze-fried *Warburgia* leaf tablets for treating vaginal candidiasis

Source: Modified from African Herbal Pharmacopoeia, Association for African Medicinal Plants Standards, Graphic Press: Baie du Tombeau, Mauritius (www.aamps.org).

of humans. On the side of traditional healers there is the need to "professionalize" their practice. For example, record-keeping of both patient's cases and medicines is necessary. This would help in changing entrenched attitudes that associate traditional healing with witchcraft and superstition.

Thirdly, new forms of participatory research need to be developed to make most out the synergy of scientific investigation and patient observation ("clinical data") by traditional healers. Only if knowledge holders and their communities are part of a research process, just access- and benefit sharing models can be negotiated, where scientifically evaluated traditional medicines in local production indeed lead to revenue and employment opportunities and to a better health for all. In this respect it is significant to mention the contribution of the World Health

Organization Regional Office for Africa (WHO-AFRO). Through many interventions in the framework of their first (2001−10) and second (2011−20) Decade of African Traditional Medicine many countries have now adopted national policies on traditional medicine, formulated legal frameworks for the practice of traditional medicine, and promoted codes of ethical conduct for traditional healers.

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Chapter 3

Areas of Science Embraced by Pharmacognosy

Constituent Sciences of Pharmacognosy

S. Badal[1], G. Byfield[2], M.C. Brown[3], Y. Lawrence[4], S-A. Hartley[5], D.K. Daley[6,7] and K.N. Smith[8]

[1]University of the West Indies, Kingston, Jamaica, [2]North Carolina A&T State University, Greensboro, NC, United States, [3]University of Technology, Kingston, Jamaica, [4]Comfort Medical, Coral Springs, FL, United States, [5]Biotech R&D Institute, Kingston, Jamaica, [6]University of Technology, Jamaica, Kingston, Jamaica, [7]Biotech R&D Institute, Kingston, Jamaica, [8]University of North Carolina at Chapel Hill, Chapel Hill, NC, United States

Chapter Outline

Teaching Goals

Understand the various fields of science that are incorporated in Pharmacognosy.
Gain exposure to these areas with an appreciation on how they contribute to the field.
Develop an integrated view of Pharmacognosy.
Consider aspects of the sciences of Pharmacognosy for further development.

3.1 BOTANY

Plants have been used to treat a variety of maladies for thousands of years, long before there was documentation of the plant part, the process, or knowledge of the specific bioactive compounds. As pathogenic organisms become more resistant to conventional drugs, there is growing interest in plant-based medicaments; therefore the science of plants, also known as botany and plant life becomes an important and integral aspect of Pharmacognosy.

Plant taxonomy provides an essential, systematic approach to the global classification of plants. The organizational hierarchy is based on the morphology, histology, physiology, and ecology of a particular species. It is estimated that only about 30,000 of the approximately 250,000 botanical species of higher plants have been documented for their medicinal properties [1]. Morphology refers to the gross form or external features of a plant, while histology describes the internal structure. All physical and chemical processes occurring within the plant constitute the physiology, and the ecology relates to the external environment in which the plant grows. Groups responsible for the classification of plants include the International Association for Plant Taxonomy, the International Code of Nomenclature, the International Code of Nomenclature for Cultivated Plants, the International Plant Names Index (www.ipni.org), and Tropicos (www.tropicos.org).

Pharmacognosy. DOI: http://dx.doi.org/10.1016/B978-0-12-802104-0.00003-2

TABLE 3.1 A List of Common Phytochemicals, Their Sources, and Medicinal Uses

Metabolite Class	Source	Medicinal Uses
Alkaloids	Amaryllis, buttercups, fungi, nightshades, poppy, periwinkle	Cancer, cardiac stimulant, pain relief, vasoconstriction, sedative
Glycosides	Almonds, apples, apricots, buguzhi, cherries, peaches, plums, raspberries	Angina, antioxidant, antiseptic, expectorant, heart failure,
Phenols	Conifer wood, carnation, citrus fruits, coriander, eucalyptus, lavender, lemon grass, lilies, peppermint species, rosemary, sage, thyme	Antioxidants, flavoring agents, fragrances
Phytosterols	Cereals, fruits, nuts, legumes, seeds, vegetables, vegetable oils	Lowering cholesterol

Both the vegetative (leaves, roots, bark, stem) and reproductive (flowers, fruit, seeds) parts of plants have been used as medicinal agents. These structures are known to contain phytochemicals, including alkaloids, glycosides, phenols, tannins, terpenes, and sterols. Phenolic compounds found in medicinal and dietary plants include phenolic acids, flavonoids, stilbenes, curcuminoids, coumarins, lignans, and quinones, among other metabolites. Alkaloids belong to a class of naturally occurring organic nitrogen-containing metabolites, and are often classified on the basis of their chemical structure [2] (Table 3.1). There are more than 20,000 different alkaloids that have been identified in numerous plant species [3]. Morphine, the potent active phytochemical of opium poppy latex, was the first alkaloid to be isolated and crystallized in the early 19th century. Some alkaloids are illicit drugs and poisons with severe negative effects on human health and society [2].

Glycosides comprise another group of medicinally important phytochemicals in plants. Inactive glycosides contain a sugar and a nonsugar component and once separated, the nonsugar portion can exert its chemical effects on the body. Tannins are bitter plant polyphenolic compounds which are widely distributed in many plant species; their astringency is thought to provide protection against predators. Quebracho, chestnut, and mimosa are some common plant sources of tannins. Terpenes and terpene-like compounds (terpenoids) are a class of plant secondary metabolites with several different roles. These compounds are well-known for their pleasant smells, spicy taste, or specific pharmacological effects [4]. Many plants produce volatile terpenes to attract insects for the purpose of pollination, and also serve as natural fragrant and flavoring agents. Other bitter-tasting or toxic terpenes act as antifeedants by preventing some plants from predation by animals and insects. Investigations have also shown that terpenes may play a role as signaling compounds and as plant growth regulators (phytohormones) [4].

Phytosterols encompass plant-derived sterols and stanols. Plant sterols are naturally occurring components of plant cell membranes with similar structure and function to cholesterol in animal cells. The three most abundant plant sterols are β-sitosterol, campesterol, and stigmasterol. Legumes, nuts, whole grains, and unrefined vegetable oils are food sources rich in sterols. It is well-established that a high intake of plant sterols or stanols can lower serum total and LDL cholesterol concentrations in humans [5–7]. Early human diets were rich in phytosterols, providing as much as 1 g/day; however, the typical Western diet today is relatively low in phytosterols. Foods and beverages with added plant sterols or stanols are now available in many countries throughout the world, and in some instances allow health claims for such commercial products. In the United States, plant sterols and stanols added to a variety of food products are generally recognized as safe by the U.S. Food and Drug Administration (FDA) [8].

Phenolics are ubiquitous secondary metabolites in plants and have substantial antioxidant properties. Plants require phenolic compounds for growth, pigmentation, reproduction, and resistance to pathogens. As these compounds are deposited in the plant, fruits, and vegetables are the major sources of phenolic compounds in the human diet. In addition, spices, tea leaves, roasted coffee or cocoa beans, and red wine are also high in phenols. These phenolic substances are mainly deposited in leaves or bark [9].

3.2 CHEMISTRY

Chemistry offers a comprehensive study of the building blocks of organic and inorganic matter at the atomic level in terms of molecular complexity and diversity. This area of science is essential to Pharmacognosy given the abundance

of chemical components from natural sources that are used toward a medicinal or biological outcome. These natural compounds can be categorized as primary and secondary metabolites. Primary metabolites are substances produced by the plants which are needed for survival, development and reproduction. Secondary metabolites are by-products of primary metabolism which are not necessarily needed for the plant's survival, and are often specific to a family or genus of plants based on the evolved secondary metabolite enzymology. These compounds confer supporting properties, including defense mechanisms against predators and structural or functional properties, such as attraction of insects towards pollination [10].

The traditional uses of plants and natural preparations derived from them over the millennia have paved the way for investigations of these materials as sustainable medicinal agents. Applications of chemistry are used to deduce the structures of the active principles, and a number have shown health benefits, and are thus currently on the market in pure form. These include the powerful pain medications morphine and codeine from the poppy plant, salicylic acid from the willow plant, anticancer alkaloids from the periwinkle plant, digitalis from foxglove, atropine from nightshade plant, and physostigmine from the Calabar bean. Care in medicinal use should be taken however, since some secondary metabolites have exhibited high toxicity at varied concentrations.

Delineation of many pathways for the production of secondary metabolites has been examined by use of the tracer techniques. This is a quantitative method that utilizes radioactive (e.g., ^{3}H and ^{14}C) and nonradioactive precursors (e.g., ^{13}C, ^{15}N, and ^{18}O) to monitor the fate of the precursors in metabolic pathways. A plethora of metabolic pathways (plant, fungal, and bacterial) have been deduced using this technique [11]. These include terpene producing nucleoside diphosphate sugar pathway or C_5 precursor isopentenyl diphosphate, phenolic producing shikimate-cinnamate/malonate acetate pathway and alkaloid-producing pathways [10].

As mentioned previously, several classes of phytochemicals have been classified based on their chemical structures and properties, including: alkaloids, glycosides, flavonoids, phenolics, saponins, tannins, terpenes, anthraquinones, and steroids. Overlapping information exists on the biosynthetic pathways and classification of these phytochemicals, and so this section will discuss three key classes; polyphenols, alkaloids, and terpenes [12].

Phenols are also referred to phenolics. They are compounds containing a hydroxyl group (or a derivative thereof) that is bound to an aromatic hydrocarbon nucleus (Fig. 3.1). Phenolic compounds are described as simple phenols or polyphenols based on the number of phenolic units in the compound. Carbolic acid is the simplest phenolic compound, while resveratrol is an example of a simple polyphenol derivative found in wine (Fig. 3.2). The antioxidant activity of phenolic compounds is largely determined by the number and positions of the hydroxyl groups, as well as the substitutions on the aromatic rings. Such biological activity is ubiquitous in higher plants.

Alkaloids are secondary metabolites usually derived from a select group of amino acids, and frequently containing one or more basic nitrogen atoms in their structure. There are several classifications that are used to categorize these compounds and this is due to the wide and vast diversity. Numerous classifications of alkaloids have been proposed; the classification described below is one of the earliest, and for further review see Waller and Nowacki [13]. This classification system dictates that if the nitrogen is derived from an amino acid and is positioned in a heterocyclic ring,

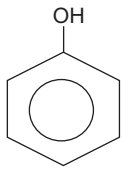
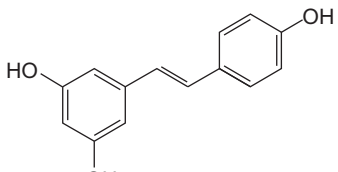
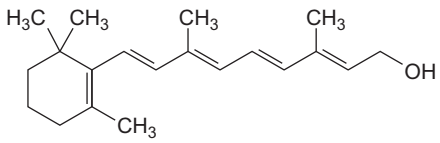


FIGURE 3.1 Phenol (formerly carbolic acid), the simplest phenol.



Resveratrol

FIGURE 3.2 The simple polyphenol resveratrol.



Vitamin A

FIGURE 3.3 Vitamin A, a terpene.

then it is classified as a true alkaloid, if the nitrogen is from another source and also positioned in a heterocyclic ring, then it is classified as a pseudo-alkaloid, as is the case with caffeine. Proto-alkaloids contain a nitrogen from an amino acid source, however, the nitrogen atom is located in a position other than on the heterocyclic ring; hormones such as adrenaline and ephedrine fall into this category. Alkaloids exhibit a wide array of pharmacological properties, such as: morphine (powerful analgesic), cocaine (anesthetic), caffeine (CNS stimulant), berberine (antibacterial), and vincristine (anticancer).

Terpenes are a class of secondary metabolites which derive their name from turpentine. Vitamin A is a well-known example of a terpene (Fig. 3.3). Monoterpenes are frequently volatile compounds that have a hydrocarbon structure or possess simple oxygenation. They are quite similar in structure; however, they have modifications in their functional groups.

The biological properties of monoterpenes, such as menthol and camphor, include conveying a strong odor. They are present in various commercial essential oils that are used in aromatherapy and for medicinal purposes. Further agricultural uses include the potential to reduce parasitic attacks on plants.

For the commercial use of secondary metabolites, it is important that an understanding of the methods of separation of these compounds is garnered. To access pure quantities of these metabolites, the methods mostly used include isolation procedures involving solvent extraction, super critical fluid extraction, steam distillation, solvent partition, various chromatographic separation techniques, and crystallization.

Extracting the maximum yield and therefore biological activity is dependent on the physical and chemical properties of the compound, including polarity, and as such, isolation of the active components is dependent on the extraction conditions, for which a number of solvents can be utilized. These range from nonpolar to polar, and include petroleum ether, hexane, chloroform, dichloromethane, ethyl acetate, methanol, ethanol, and water. To extract a nonpolar compound, a nonpolar solvent, such as hexane, is used. This solvent selectively extracts compounds that are fatty or waxy in nature, such as long-chain aliphatic hydrocarbons. At the other extreme, the more polar solvents, such as methanol and water, will extract the more polar compounds, including glycosidic compounds with numerous hydroxyl groups. Structure analysis can also assist in optimizing the extraction conditions by revealing the number of hydroxy groups, as these typically determine the polarity of the compound.

Typically, when the solvent extraction is carried out, a pure compound is not obtained. A next step at that point may be a solvent partition against graduated solvent polarities to effect a partial separation of a complex mixture. Further separation usually involves various chromatographic techniques. Several chromatographic options are available, with the final choice dependent on the nature of the compound. The simplest chromatography technique is thin layer chromatography (TLC), while more advanced technical methods include high pressure liquid chromatography (HPLC), gas chromatography, and ultrapressure liquid chromatography, as well as high pressure counter current chromatography. Data from these advanced chromatography techniques, which reflect the sequence of elution of the metabolites, are summarized in a chromatogram. An example of such is seen in Fig. 3.4.

The basic principle of these separation techniques is the same. Chromatography involves a stationary phase and a mobile phase; these two phases are used to separate the extract of interest by manipulating their polarities. Another solvent extraction method that is more environmentally friendly is supercritical fluid extraction (SFE). It utilizes liquid carbon dioxide at high pressure which expels different types of analytes (polar or nonpolar) at different pressures. The extract derived from SFE is still complex and has to be further separated, as with solvent extraction.

Once the compounds are separated, the structures of the secondary metabolites can be elucidated by nuclear magnetic resonance (NMR) techniques, such ^{13}C NMR, to identify the carbon frameworks and their attached protons and certain functional groups or determine symmetry and in combination with ^{1}H NMR to identify molecular fragments. The NMR technique causes the nuclei within the compounds to absorb and emit electromagnetic radiation. The patterns of emission obtained enable a trained chemist to elucidate the structure of the compound. Infrared and mass spectrometry techniques are also used to aid in compound identification.

After compounds have been identified, in silico methodology can be used to further assess the compounds. In silico technology is a computerized methodology that utilizes the knowledge of different proteins to evaluate the responses of

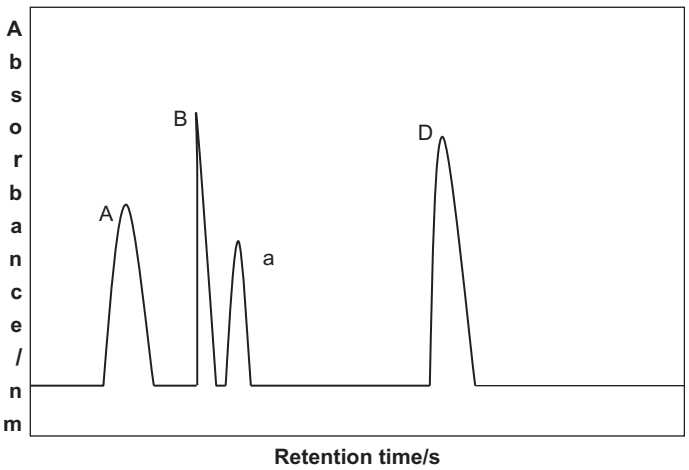


FIGURE 3.4 An example of a chromatogram.

compounds identified and hence could postulate the compounds uses and efficacy. This technology requires a massive amount of database input and is somewhat limited by the available information on protein structures related to disease state inhibition. Nonetheless, this method is advantageous in that it reduces the time needed to assess a compound, and its potential synthetic derivatives, in comparison with that taken for in vitro, in vivo, and clinical studies.

3.3 ENZYMOLOGY

Enzymes constitute a major fraction of all known proteins, and serve the function of catalysts in the formation of secondary metabolites. Additionally, enzymes are active in numerous biochemical processes which contribute to the synthesis and breakdown of metabolites from plant and animal sources, and in the biological functions of medicinal agents. Therefore, having a comprehensive understanding of enzyme chemistry and biochemistry is an essential aspect of Pharmacognosy.

The four primary families of plant enzymes are proteases, amylases, lipases, and cellulases; the former three families are also found in animals. When consumed, these enzymes are involved in the breakdown of macromolecules. Specifically, proteases are responsible for the breakdown of long protein chains into smaller chains and eventually into single amino acid units through the catabolism of peptide bonds. Amylases are vital for the breakdown of polysaccharides to disaccharides (lactose, maltose, and sucrose) through the cleavage of α-1,4-glycosidic bonds. Lipases are critical for reducing triglycerides to individual fatty acids and glycerol through hydrolysis. Cellulases breakdown certain carbohydrates found in plant fibers through the hydrolysis of the 1,4-β-D-glycosidic bonds.

Numerous families of animal enzymes exist. Those of special interest to Pharmacognosy are the phase I and phase II drug metabolizing enzymes, with particular interest in the phase I class. Phase I drug metabolizing enzymes are also referred to as cytochrome P450s (CYP450). Of the 69 encoded by the human genome, approximately 15 are involved in the metabolism of drugs and other xenobiotic chemicals [14]. CYPs are also termed "monooxygenases" based on their primary mechanism of action. By using reducing agent cofactors, including nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate [15], these enzymes are able to incorporate one oxygen atom from atmospheric dioxygen into a substrate. The other oxygen atom is reduced to water as shown in the scheme below.

$$\mathrm{NADPH} + \mathrm{H}^{+} + \mathrm{RH} + \mathrm{O}_2 \rightarrow \mathrm{NADP}^{+} + \mathrm{H}_2\mathrm{O} + \mathrm{ROH}$$
$$(\mathrm{RH} = \text{substrate},\ \mathrm{ROH} = \text{product/metabolite})$$

Most CYPs were once believed to be liver-specific enzymes, however, their extrahepatic expression is now well-established [16]. Among the CYPs identified, 11 are expressed in a typical human liver (CYP1A2, 2A6, 2B6, CYP2C8/9/18/19, CYP2D6, CYP2E1, and CYP3A4/5), while the others are extrahepatically expressed (Fig. 3.5).

Based on their monooxygenase activity, these enzymes are able to modify xenobiotics, chemicals that are foreign to the organism. This can lead to: (i) inactivation of the xenobiotic, resulting in the excretion of the drug; (ii) activation, that is conversion to a more bioactive state, as in the case of prodrugs; or (iii) conversion to a reactive state, which includes the formation of activated carcinogens. With this in mind, the FDA requires the elucidation of the metabolic profile of each drug candidate. This entails that they be screened against the major CYP450 drug-metabolizing

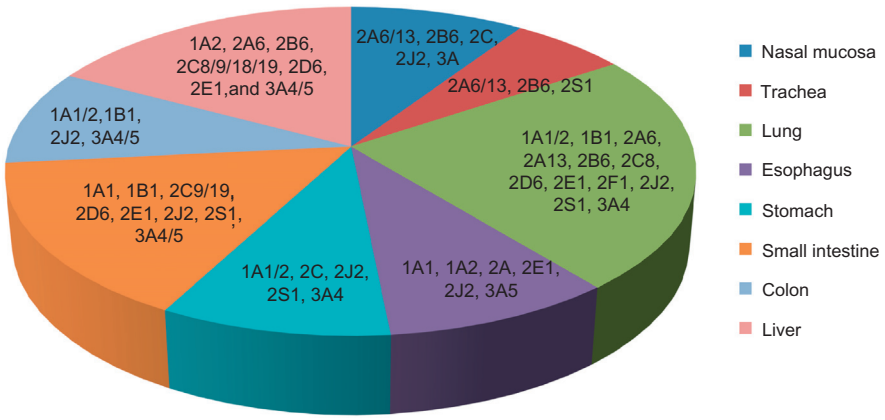


FIGURE 3.5 Human cytochrome P450 genes expressed in different organs. *Adapted from Ding X, Kaminsky, LS. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. Annu Rev Pharmacol Toxicol 2003;43:49–173 [17].*

enzymes, including CYPs 3A4, 2D6, 2C19, 2C9, and 1A2. This provides information on the ability of the drug candidate to inhibit, or to induce, the CYP450s, or to act as a substrate of the enzymes. Such information offers insight into possible drug–drug interactions that could ensue, to the detriment of the patient. Adverse drug reaction reports total over 2 million per year in the United States [18], therefore there is interest in obtaining the metabolic profiles of drug candidates, and also of plant and animal extracts. This is of particular importance given the possible concomitant use of prescription, over-the-counter and plant-based medicines [19,20], often referred to as polypharmacy.

On the other hand, phase II drug metabolizing enzymes participate more in conjugative type reactions, and as such they are also known as transferases. These include UDP-glucuronosyltransferases, sulfotransferases, *N*-acetyltransferases, glutathione *S*-transferases, and various methyltransferases, including thiopurine *S*-methyl transferase and catechol *O*-methyl transferase (COMT) [21]. Frequently, such reactions lead to the formation of inactive hydrophilic metabolites which are easily excreted through the urine. Based on their inherent ability to participate in such reactions, phase II drug metabolizing enzymes are termed "detoxifying enzymes." They also play a role in the biotransformation of metabolites that are formed from phase I drug metabolism, a property that is important in the arena of chemoprevention [22].

3.4 GENETICS

Genetics is the branch of biology that is focused on the study of genes, heredity, and genetic variation [23]. The genetic blueprint of a living organism is encoded in its DNA sequence which contains information to build and maintain the cells of the organism, and its particular physical and metabolic traits. These traits are passed on to offspring to dictate their phenotype, properties which may be easily seen (leaf shape, petal color, eye color) or unseen (blood type). Typically, the offspring of animals inherit characteristics from two parents, and therefore are genetically unidentical to the parent, while plant offspring can either be identical or nonidentical by way of asexual or sexual reproduction, respectively.

Plants produce an extensive array of natural products, comprising photosynthetic pigments, signaling molecules, and secondary metabolites. In many instances, the secondary metabolites are also a defense mechanism for the plants, as in the case of tannins whose concentration levels tend to be higher in unripened fruits relative to ripe ones; fruit palatability therefore parallels a reduction in tannin concentration. Given the astringency of tannins especially in high amounts, higher levels act as a deterrent to herbivores and pests to give fruit an opportunity to ripen successfully.

Plant metabolites are typically produced as complex mixtures of different structural types, ostensibly fashioned by selective pressure over evolutionary timescales. Some of these metabolites have been critical to the drug discovery arena [24] and were mentioned in earlier sections. Thus, a comprehensive understanding of the genetic make-up and variations of living organisms, especially plants across generations, is necessary for reaping the full benefits of current and future medicines.

A better understanding of the relevant pathways involved in the synthesis of plant metabolites can open doors for increased yields through molecular breeding or metabolic engineering, and can also enable reverse genetic approaches toward an improved understanding of their physiological function [24]. A current challenge faced by the drug discovery community is the minimal yields of biologically active secondary metabolites, which ultimately impedes research aimed at acquiring a better understanding of the safety and efficacy of these entities, and ultimately their transition to drugs.

While synthesis is a potential alternative route, this is rarely a solution for structurally and stereochemically complex secondary metabolites.

Of particular interest is the use of molecular breeding toward increased levels of the monoterpene menthol, a cream of which is widely used to alleviate minor pain caused by arthritis, bursitis, tendonitis, muscle strains, backache, bruising, and cramping. Enzyme assays with plant cell-free extracts have led to genetic/metabolic engineering in peppermint [25]. Identification of the terpene synthases (TPSs) and CYP, limonene-3-hydroxylase (CYP71D13) [26] paved the way for modification of the menthol synthesis pathway in heterologous microorganisms. Such modifications involved the recombinant expression of TPS, 4*S*-limonene synthase in *Escherichia coli* [27], which showed robust activity upon truncation of the *N*-terminal plastid targeting sequence [28]. Functionally expressed *N*-terminally modified versions of CYP71D13 were achieved in *Saccharomyces cerevisiae* (yeast) and *E. coli*, with activity observed upon reconstitution with a plant CYP reductase [29]. Such genetic manipulations of these enzymes and their biochemical pathways led the way to genetically engineered mint plants that produce elevated and more reliable yields of menthol [30]. The principle of this approach can potentially be employed with other organisms (animals, fungi, and bacteria) toward improving the yields of secondary metabolites with promising efficacy. Some examples are discussed further in Section 3.8.

The use of genetics is beneficial toward increased yields of plant metabolites, and toward increasing the yields of the plants themselves in terms of biomass, their variations, and their resistance to viral and microbial infections, as well as metazoan pests. Such increased resistance translates to reduced pesticide requirements [31]. It is believed that the use of pesticides weakens the innate defense mechanism of the plant through impaired secondary metabolite production and function [32]. Consequently, the efficacy and nutritional content of the plant is compromised. In some instances, the use of genetically modified plants provides a counteractive solution.

3.5 PHARMACOLOGY

In order to fully reap the potential of these naturally derived medicinal agents, a thorough understanding of the mechanisms by which they exert their biological effects is required. This critical aspect relating to the utilization of the numerous biologically active compounds that arise from natural sources is often a limitation preventing more detailed biological profiling and use.

Pharmacology requires the phenotypic screening of natural products, crude extracts, or more well-defined pharmaceuticals. This is an initial step in the determination of the beneficial or adverse effects of the material on the organism. One key consideration that dictates the pharmacological activity is the product formulation, which is based on an understanding of the composition and derivation of the active compounds, a definition of their chemical and physical properties, and the potential route of administration. As previously mentioned, the bioactive pure compound may be difficult to identify and/or purify. Optimally, medicinal chemistry protocols are used to develop a method for a standardized preparation with the aim of producing consistent products, with uniform quality, safety, and efficacy profiles, bearing in mind the possible presence of additional compounds which are not of interest. Consideration of the toxic effects of the undesired compounds is paramount, with various systems in place to examine potential central nervous system, hematopoietic system, kidney, and liver toxicity according to standard protocols.

The preferred pathway for the administration of natural medicines is the oral route for most conditions. Topical delivery may be also be used depending on the condition. The dose at the target site is dependent on the bioavailable fraction, which is the amount of compound that is absorbed or enters systemic circulation. On liberation of the active ingredient, the pharmacokinetic properties of absorption, distribution, metabolism, and excretion come into play; and these are key considerations which define the dosing regimen for the use of natural medicinal agents. Various bioassays are utilized in these determinations.

Pharmacodynamics, which examines the effects of a drug on the body, is dictated initially by the compound's interaction with biological receptors. Particular systems of interest include the gastrointestinal tract, metabolic tissues, and the central nervous system. Well-defined mechanisms of action, and the study of the physiological effects will begin to define potential medical applications, which may or may not match the traditional use of the plant material.

As mentioned previously, secondary metabolites are used for several purposes, including functioning as narcotics, stimulants, anesthetics, hallucinogens, poisons (which may serve as pesticides, including insecticides, rodenticides), essential oils for cosmetics, antimicrobial and antiparasitic agents, household products, resins for preservation, phenolics for tanning animal skins, dyestuffs, rubber, and in chemotaxonomy. Specifically, the medical applications of secondary metabolites are far-reaching, and involve support of bodily responses to pathogenic organisms, in addition to counteracting lifestyle and degenerative diseases, including neurological disorders, skin conditions, cancer, and diabetes.

A variety of natural products have demonstrated efficient activity against various pathogenic bacteria and dermatophytic fungi including *Bacillus* sp., *Enterobacter aerogenes*, *Erwina* sp., *E. coli*, *Klebsiella pneumoniae*, *Proteus* sp., *Salmonella* sp., *Staphylococcus* sp., *Xanthamonas campestris*, *Pityrosporum ovale*, *Trichophyton rubrum*, and *Trichophyton tonsurans*. One key mechanism of action is the inhibition of a central bacterial antitoxicity mechanism, that is the inhibition of the efflux pump [33–35]. Such antimicrobial and antifungal activity has disease relevance in skin conditions and dental maladies, and in diseases such as dysentery and typhoid.

Natural compound biological effects extend to antiviral activity, with impact on HIV and hepatitis C replication [36,37]. Pertaining to antimalarial activity, there is an emergence of strains that are resistant to the commonly used treatments, including atovaquone, chloroquine, mefloquine, quinine, and artemisinin, although the latter two natural products currently show the least resistance and serve as therapeutic replacements for strains resistant to common treatments [38]. These direct effects are further strengthened by immunomodulatory activity, with these compounds having demonstrable immunostimulating potential [39]. There are various systems that may be utilized to examine the medicinal effects on the immune response including monitoring the survival rate postinfection, as well as delayed type hypersensitivity response monitoring. Effects on the immune system can be monitored through neutrophil counts and phagocytic response.

The antimicrobial effects of plant extracts may also prove useful in wound healing. Extracts with wound healing capacity may have significant levels of antioxidants, saponins, and flavonoids. In these models, wound healing can be assessed by monitoring the rates of resurgence of epithelialization and wound contraction over time. In the laboratory, various animal models exist to investigate reparative potential.

Natural medicines may also favorably regulate the digestive tract. As such, these compounds and extracts have found a role in the treatment of various digestive ailments, including ulcer treatments and antidiarrheal remedies. Ulcer formation can occur as a result of various conditions, including bacterial infection and excessive alcohol consumption. To counteract this, the use of natural compounds and extracts can aid by optimizing gastric juice volume and pH, as well as by positively impacting gastric mucosa and prompting healing through improved microcirculation and precipitation of microproteins [40,41]. Phytomedicines also have a history of use in combating diarrheal activity, which may have varying causations, including inflammatory, osmotic, or secretory mechanisms. Some improvements seen include improvement in the amplitude and frequency of gut contractions, in addition to a reduction in stool moisture content.

More broadly, numerous natural formulations are apparent anti-inflammatories, as well as analgesic agents, properties conferred by various alkaloids, flavonoids, glycosides, phenolics, steroids, and tannins. These compounds combat oxidative species and free radicals, and counteract inflammatory prostaglandins.

Natural antioxidants have also been shown to positively support normal physiology in a number of conditions, and demonstrate antidiabetic, antiatherosclerotic, antiarthritic, and anticancer properties [42]. These diseases, in addition to the primary defect, also relay a host of additional pathologies. For instance, in addition to the direct effects due to diabetes, a number of secondary maladies occur as a result of this hyperglycemic condition including nephropathy, retinopathy, and limb ischemia. In these cases, compounds and extracts counteract these pathologies through action in various insulin-sensitive tissues to regulate glucose levels. Numerous compounds have been shown to reduce elevated serum glucose, total cholesterol, and triglycerides associated with these conditions. In cancer therapy, phenolics can enhance the body's immune system to recognize and destroy cancer cells, and may inhibit the development of new blood vessels (angiogenesis) that is necessary for tumor growth [43].

The best practice for incorporation of natural products as therapies, particularly for novel bioactive metabolites is by way of placebo-controlled clinical trials. Current trials are underway to monitor the efficaciousness of numerous natural-derived compounds for treatments in many therapeutic areas [44]. Preclinical studies that utilize animal models pave the way for clinical trials by providing evidence of safety, toxicity, and efficacy in laboratory animals. To complement these types of preliminary studies, in vitro assays play an important role; such assays involve various approaches including enzymatic studies, receptor assays, and experiments on cultured cell lines that are related to the in vivo assay or the anticipated therapeutic outcome [45].

3.6 HORTICULTURE

The ability to cultivate and improve the growth of medicinal plants is imperative for isolating active components. This area of study is referred to as horticulture, where plants may be propagated and cultivated for various reasons, including those for medicinal purposes. Horticulture is the art and science involved in growing, grooming, and marketing plants, and differs from agriculture as it incorporates smaller plots, often with a variety of plants. The word "horticulture" is derived from the Latin words "hortus" for garden and "cultus" meaning culture or cultivation [46], and is within the

field of agriculture that deals with optimizing plant growth, overall quality, and yield through plant propagation and appropriate cultivation techniques, with esthetics playing a major role in plant care.

Horticultural plants are grown for a particular purpose, including for food, medicine, as ornamentals, aromatic properties, or the wine industry. As a result, this leads to various fields within horticulture, such as floriculture, viticulture, olericulture, arboriculture, pomology, and enology which are the study, cultivation, and marketing of flowers, grapes, vegetables, trees, fruits, and wine, respectively. Each area of science involves the landscaping of crop fields which adds to the esthetics, but more importantly, the required growth guidelines are followed in order to maximize the production of the respective plants for their specified use in these industries.

The use of plants for medicinal purposes has significantly increased [47]. As a result, horticultural practices have expanded to incorporate plant pathology and entomology, where diseased or pest-infected plants may be treated quickly, as well as the soil science in order to improve plant production by reducing environmentally stressful conditions [48]. This field has also evolved to include various techniques that have been used to obtain new cultivars, including genetic engineering. For example, these cultivars may have an increased concentration of the active component, thus enhancing the medicinal value, or they may have increased nutritional value for use in the food industry.

For pharmacognosists, plants are grown for their healing properties as medicinal agents, also as natural alternatives used as pesticides and insecticides, in the form of crude drugs and/or for the subsequent isolation of active components. Many factors play a significant role in plant development, such as the type and quantity of the plant regulators/hormones used, which vary with the type of plant and the variety within a plant group [48,49]. The optimization of the growth conditions of various plants as a source of active components, whether in the form of an unrefined crude drug, which includes resins, essential oils, or balsams, or as an organized crude drug, such as the leaves of *Digitalis purpurea* or *Eucalyptus* species, could ultimately lead to the production of high quality nutraceuticals or pharmaceutical products [47]. Based on the bioactivity of the medicinal plant of interest, knowledge of horticulture therefore aids in plant productivity.

The horticultural care of a particular plant is dependent on the type of crude drug that is to be produced and the desired yield. A plant crude drug is a vegetable drug prepared from plant organs or exudates from plants, without extensive modifications being carried out. Also, the active components and the isolation techniques will create the framework for the cultivation practices being performed, such as obtaining essential oils, resins, gum, oleoresins, or balsams [50]. For example, *Tagetes erecta* L. (African marigold) is not cultivated solely for landscape and garden management; it is often grown for the extraction of the oleoresins present. Subsequently, pure carotenoids, such as lutein, may be isolated and marketed. Lutein is used as a food additive, and can also be used as a standard for cancer treatment research [51,52]. An important growth factor is the soil requirements for *T. erecta*, as it should be relatively neutral pH loam with irrigation done once a week based on the soil moisture content [53]. Water stagnation may reduce the plant yield and thus produce inferior exudates or organized crude drug, and significantly increase the risks of infections by pathogens. Altogether therefore, the cultivation practices of horticulture are important in providing food, shelter, improved soil and watershed management, and in obtaining a quality crude drug in significant quantity from various types of plants.

3.7 QUALITY CONTROL

Given the rich history of natural product usage for the treatment and prevention of various ailments, there is often the long-held perception by end users/patients and nonmedical practitioners that recommend them, that they are safe and effective and have comparatively fewer significant side effects than pharmaceuticals. The World Health Organization (WHO) has compiled quality control guidelines and techniques to help ensure the safety and efficacy standards of these natural products.

In the case of plant-based natural products, quality control in Pharmacognosy begins in the field. The quality of the plant constituents is dependent on the geologic soil conditions, water supply, temperature, and solar radiation. In addition, adherence to proper cultivation and harvesting methods, correct identification, and suitable handling of a plant specimen is critical. Quality control further includes expert extraction of the potentially therapeutic constituents, sampling, storage conditions, precise measurements, and use of temperature and calibrated equipment that are all in accordance with best laboratory practices to meet the criteria of quality assessment methods. Trained quality control personnel in Pharmacognosy tend to have experience in different branches of science, and their aim is to achieve purity, safety, efficacy, and consistency of manufactured phytotherapeutic supplements and the exploration of potentially new beneficial plant compounds.

Factors that negatively affect quality include variability in plant constituents, especially within plants of the same genus and species [54]. Plant constituents may also vary due to seasonal variation and/or the level of soil nutrient supply [55]. Unidentified active ingredients, lack of synergy of complex mixtures, and interferences in the production of formulations can also lead to phytomedicines that do not meet quality requirements. If these substandard drugs/supplements become available to patients it can lead to adverse events that may even be potentially life-threatening. Quality control is therefore critical in Pharmacognosy studies to reduce degraded and substandard natural products and preparations. Substandard products may also result from adulteration; substituting similar looking substances with those that are inferior or in deteriorated condition, or that do not contain the desired therapeutic compounds. Adulteration may also arise from the addition of biologically active synthetic compounds.

Quality assurance guidelines, such as Good Agriculture Practice (GAP) for medicinal plants also safeguard against contaminants that may lead to poor quality in natural products at its earliest stages: seeds and plant cultivation. Contaminants include parasites, microbial contaminants, fumigants, pesticide residues, and manure fertilizers. Other contaminants that need to be reduced to its lowest minimum include: toxic reagents, solvents, toxic metals and non-metals, and radioactive contamination.

For phytomedicines, one pertinent aspect of quality control is the preparation of the plant specimen to be examined. This includes basic steps such as simply clearing away foreign matter and contaminants before attempting classification. Proper identification, through macroscopic and microscopic examination, is key in ensuring reproducible quality, purity, and uniformity of drugs of plant origin that are recommended or prescribed to patients. A macroscopic description includes the morphological and other qualitative properties, including descriptions of the leaf, root, rhizome, bark, shape, color texture, plant base symmetry, as well as quantitative properties (e.g., fraction) which were also briefly mentioned earlier. These sample properties are derived primarily from the unaided senses (sight, smell, taste) of the pharmacognosist. Further, a hand lens with $4-20\times$ magnification may be used for plant samples that are whole or uncut. Plants not in whole form (powder or extract) may be more challenging to identify and authenticate. In such cases, positive identification of the plant or its constituents may include a comparison of the sample to pharmacopeia references or analytical test that can help to confirm botanical ingredients. To maintain quality, various methodologies are used as described below; all tests and examination methods used, applicable test limitations and test results are documented and reviewed for approval by the quality control personnel.

Quantitative testing to determine content or quality of plant constituents such as titration methods and UV spectrophotometry can be used where acceptable biomarkers are available.

Identification of the less obvious physical and chemical characteristics of the plant includes two commonly utilized tests: HPLC and TLC for ensuring adequate quality of medicinal plants. These technologies are able to define chemical profile characteristics based on an authenticated sample, and may increase testing speed and efficiency through automation.

These analytical methodologies, tests and observations are undertaken in a reliable and reproducible manner in order to identify potentially beneficial or toxic constituents in a sample. Further key pharmacognostic quality control aspects ensure uniformity in manufactured products, whether they are complex mixtures in crude or processed forms [56]. Altogether, this is to ensure that products contain the same amount of ingredients as indicated on the label in order to satisfy the purpose for which it is recommended, and to meet regulatory standards. Further elements for consideration include quality as it pertains to product stability and shelf-life. These criteria ensure the safety and efficacy of the natural products to end users and patients.

A reference database containing useful plant material information and their possible adulterants became necessary in keeping with quality control efforts to authenticate plant ingredients used for natural based remedies. This database also helps to ensure uniformity, efficacy, and safety. It provides short DNA sequences or organelle genomes, i.e., DNA barcodes, which are unique identifiers of the plant species. This is a more reliable means of identifying and screening than macroscopic, microscopic, and chemical profiling technologies [57]. The use of PCR and DNA sequence analysis also helps to reduce the incidence of adulteration and the proliferation of substandard natural products. This is crucial as the efficacy of medicinals decreases, and may even be toxic if the product is adulterated.

There has been a notable increase and resurgence in the production, availability, and use of phytomedicines in the United States spurred by Federal legislation in 1994, the Dietary Supplement Health Education Act, "DSHEA." Unfortunately, this has been coupled with an increase in poorly regulated nutraceuticals and phytonutrients with health promoting claims. These claims are often made by mass producers trying to meet the demands of e-commerce and often solely with marketing and profitability in mind, reaffirming the impetus for quality control for the benefit of the patient. In response, national regulatory bodies have developed pharmacopeias; official compilations of quality standards for pharmacological active constituents and drug products. The WHO, European Scientific Cooperative on Phytotherapy

(ESCOP) monographs, and Physicians' Desk Reference (PDR) for Herbal Medicines produce publications that provide information on medicinal characteristics, including usage, standardization, identity, purity (physically and chemically), and the analysis of plant compounds. The ultimate goal of these bodies is to safeguard the health and welfare of the patient. To this end, these publications are readily available through workshops/conferences and electronic media.

3.8 BIOTECHNOLOGY

So far, the emphasis in this chapter has been on the value of plants as a source of natural products for drug discovery. Important considerations arise when the demand for such products places a strain on plant populations leading to species depletion or extinction, loss of genetic diversity in some habitats, as well as habitat degradation. Plant collection and extraction may be unable to meet the high demand and is unable to provide sustainably high yields and be cost effective. Therefore, the area of biotechnology involves the use of organisms to develop or make useful products. Biotechnology offers the advantage of protecting plant species and habitats through the sustainable production of higher yields of plant compounds without impacting species diversity. Such an area goes hand in hand with the precepts of ecopharmacognosy.

Plant biotechnology involves genetic engineering, an area previously introduced and discussed, where genes are introduced into plants or plant cell lines by electroporation, gene gun, *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* followed by micropropagation, which is used to multiply plant stock material. This propagation method involves the rapid multiplication of plant species from existing plant material. Micropropagation has several advantages, including obtaining disease-free plants, production of large numbers of plantlets, and allowing genetically modified plants to be regenerated [58].

In order to increase the yield of plant metabolites, genes can be suppressed or silenced; for example in taxol biosynthesis, taxoid 14β-hydroxylase catalyzes C-14 oxygenated taxanes which are formed as side routes, but are not precursors to taxol synthesis in *Taxus* media TM3 cell lines. Therefore, this enzyme was suppressed through antisense RNA inhibition by the introduction of antisense cDNA for the 14β-hydroxylase gene to decrease substrate competition, thereby increasing taxol production [59]. The role of terpenoids in plants includes communication, plant−insect, and plant−animal interaction; alteration of their production can affect disease resistance, enhancement of flavor or fragrance, and increase pollination [60]. Discoveries of the role of the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway in the biosynthesis of plastidial terpenoids, such as the carotenoids, have led scientists to silence geranylgeranyl diphosphate synthase in tobacco which has led to improved insect resistance [61].

Plant tissue culture allows compounds that are normally produced by the parent plant to be synthesized by cells that are biochemically totipotent; this is the ability to form all the differentiated or specialized cells of the entire organism [62−64]. This methodology also allows for biotransformation—the regioselective and stereospecific chemical transformations that are catalyzed by biological systems through their effective enzyme systems. Biotransformation allows the modification of plant secondary metabolites to create novel compounds which may not be feasible by semisynthetic processes. Reaction types carried out by such cultures include acetylation, esterification, glycosylation, glucosylation, hydrogenation, hydrolysis, hydroxylation, isomerization, methylation, and oxidoreduction of various exogenous substrates [62−64].

Some secondary metabolites are only produced in organ cultures, such as by hairy roots; *A. rhizogenes* induces hairy root production and has been exploited to obtain these compounds. Transformation of plant roots with *A. rhizogenes* allows for high growth rate, high proliferation in media free of plant growth regulators, high branching of roots, genetic and biochemical stability, and long-term secondary metabolite production. This has been employed with *Pharbartis nil* which produces commercially important coumarins, flavonoids, and phenolic acids at significantly high concentrations [62,64].

Plants or in vitro cell lines can also be used to produce pharmaceutical proteins, such as blood proteins (human serum albumin), cytokines (interleukin-12), growth factors (human epidermal growth factor), growth hormones (human growth hormone), monoclonal antibodies (CaroRx, prevents bacterial adhesion to teeth thus preventing tooth decay) and anti-West Nile virus mAb (Hu-E162), vaccine antigens (transgenic tobacco produced against Newcastle disease virus which affects birds), therapeutic enzymes (glucocerebrosidase), and industrial enzymes (cellulase and trypsin) in a process known as molecular farming [11,63,64].

Molecular farming is desirable as there is greater consistency of protein products, the rapid proliferation of cell cultures, a lower risk of pathogen contamination, unlike mammalian and bacterial systems, and fewer regulatory environmental and safety compliance mandates [63,64].

When the innate homeostatic balance of the body is affected, many disease conditions can and do arise, conditions that affect the disease-bearer and the caregivers as well. Such conditions have steered the directional approach of the research community for quite some time. Such direction has resulted in the genesis of many drugs, natural and synthetic, though most are only effective on 30–50% of persons who take them. As such, there is a current transitioning of the existing way of thinking and performing among research communities and drug industries from the lock and key paradigm to a synergistic one, like network pharmacology. Such an area is very important given the numerous genetic points of impact of various disease conditions, areas that would all need to be addressed to achieve safer and more efficacious outcomes. While natural products have been known to be relatively safer than their synthetic counterparts, as research continues to evolve and new discoveries are made, other paradigms reveal further expanses of needed attention.

3.9 CONCLUSION

Naturally derived medicinals are broadly utilized worldwide to treat numerous health concerns. Each of the areas described in this chapter are critical components that contribute to best usage of such products. Obstacles remain in the field of Pharmacognosy; these include inaccuracies in identification, poor quality control and protocol variations, substandard identification of metabolites, and a low yield of bioactive compounds. Fortunately, technological advances, including biotechnological methods and high throughput approaches, will facilitate advances in Pharmacognosy including uncovering new and underexploited targets.

3.10 REVIEW QUESTIONS

1. Explain the basis of the organizational hierarchy of plant taxonomy.
2. Define phytochemicals and discuss the role of any example described in the chapter.
3. List the classes of medicinal phytochemicals.
4. Discuss methods for the separation of metabolites.
5. Enzymes play a universal role in the catalysis of numerous reactions: discuss the importance of these to drug metabolism and excretion.
6. How do prodrugs exert their intended efficacy?
7. Discuss the role that enzymes play in adverse drug reactions.
8. Discuss the use of genetics in plants toward increasing the yields of pharmaceutical products.
9. How can the area of genetics be used toward assisting an individual who has a mutant form of the CYP34A, and is therefore unable to take a needed prescription medicines?
10. a. Describe a disease that can be treated from naturally derived medicinal agents.
    b. What is the mechanism of action?
11. What bacterial system is overcome by natural products to mediate an antimicrobial effect?
12. Natural compounds have shown efficiency against several species of bacteria and fungi. List five.
13. Define horticulture, as it pertains to Pharmacognosy.
14. What is a plant crude drug?
15. What is quality control in Pharmacognosy?
16. What are the goals of quality control in Pharmacognosy?
17. What are some factors that affect the quality of phytomedicines?
18. Which are the national/international regulatory bodies and organizations that support quality control and how?
19. What is involved in the identification of medicinal plant constituents?
20. What are the advantages of biotechnology over plant harvesting for obtaining plant compounds?
21. Why is species conservation important?
22. What is the role of *Agrobacterium* in biotechnology?
23. How is molecular farming of benefit to human health?

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Chapter 4

Plant Anatomy and Physiology

F.B. Lopez[1] and G.F. Barclay[2]

[1]*The University of the West Indies, Cave Hill, Barbados,* [2]*The University of the West Indies, St. Augustine, Trinidad and Tobago*

Chapter Outline

Learning Objectives

At the end of this chapter you will be able to:

- Describe the structure and function of the different types of cells and tissues found in plants.
- Illustrate the internal and external organization of stems, leaves, and roots.
- Explain how leaves can be modified for support, protection, nitrogen acquisition, and a xeromorphic environment.
- Describe the structure of a flower and how it is modified.
- Explain the steps involved in the process of photosynthesis.
- Differentiate between the C3, C4, and the CAM photosynthetic pathways.
- Explain the Pressure-Flow theory of translocation in plants.
- Review the processes by which water moves through the soil–plant–atmosphere continuum.

Plant anatomy refers to the detailed structure of the plant: leaf, stem, roots, flowers, and fruits, while plant physiology is concerned with the processes that occur within the plant that account for it being alive and productive. A living plant must collect a few simple materials from the environment: water, oxygen, carbon dioxide, and several minerals. From these simple materials, the plant uses light energy to synthesize more complex substances, e.g., carbohydrates, which are in turn used to synthesize other complex substances such as proteins and lipids. Simple materials and more complex substances need to be transported within the plant to locations where they are needed. Plants utilize these materials to produce cells and organs, which leads to growth and development. There are control systems within the plant to ensure that growth and development occur in an ordered way. In this chapter, we first examine details of plant structure and architecture, and then proceed to look at the important physiological processes of photosynthesis, translocation, and transpiration.

4.1 PLANT STRUCTURE

4.1.1 General Structure of Flowering Plants

Higher plants are comprised of roots that provide them with water, nutrients, and anchorage; stems, for support; and leaves, which carry out photosynthesis for growth and development. These organs can have tissues that are protective, supportive, vascular, or meristematic in nature and organs can be modified to perform different functions, dramatically so in flowers, which are simply assemblies of leaves specialized for reproduction.

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4.1.2 Meristems

Because plants are rooted to one spot they need to adapt to changes in their environment to stay alive. They are able to replace parts that are damaged or lost while continuing the reproductive cycle as they grow. Plants continually renew themselves with restricted growth and development through the activity of meristems, groups of undifferentiated, genetically sound cells. Meristems can be determinate and produce organs of a definite shape and size such as leaves and flowers. In contrast, indeterminate meristems exhibit continual growth in roots and stems, so plants can grow in length from their apices or in breadth from cambial activity. The ground meristem produces pith and cortex while procambium produces primary phloem and xylem. Axillary buds forming in axils of leaves normally produce branches or flowers. These are all referred to as primary meristems since they establish the primary growth pattern of plants.

Stems and roots increase in girth from the activity of vascular cambium and cork cambium, lateral meristems arising during secondary growth in eudicotyledons. Vascular cambium normally creates more xylem inwards and more phloem outwards by periclinal cell divisions and is essentially indeterminate (Fig. 4.1A,B). Monocotyledons such as corn (*Zea mays*) lack true secondary growth (Fig. 4.2).

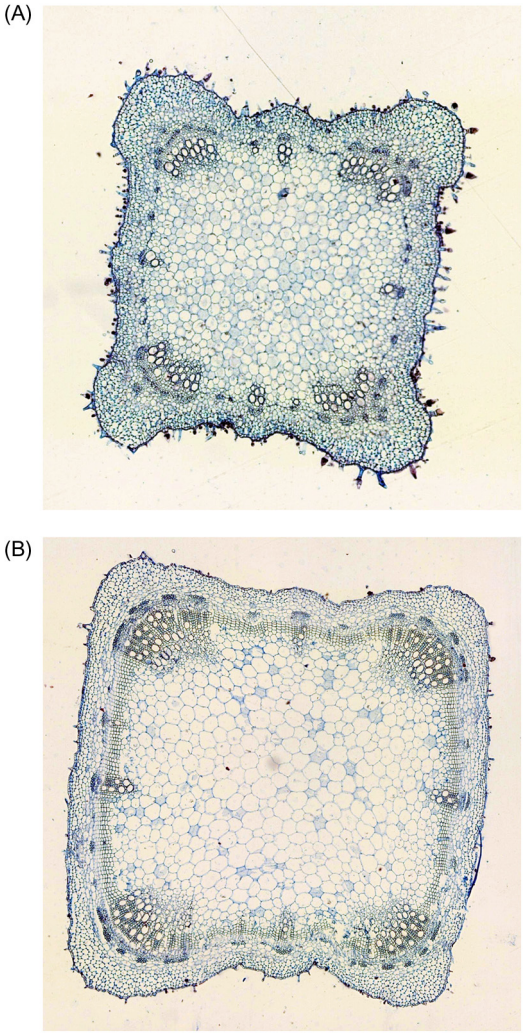

FIGURE 4.1 Transverse section of stem of Coleus (*Plectranthus scutellarioides*) showing (A) late primary growth with vascular tissues organized in discrete bundles and (B) early secondary growth, with a ring of vascular cambium giving rise to phloem outwards and xylem inwards.

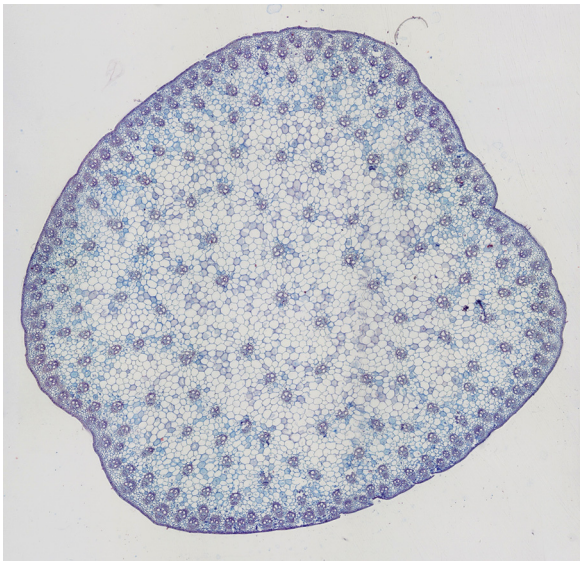

FIGURE 4.2 Transverse section of corn stem (*Zea mays*) with vascular tissues in discrete bundles.

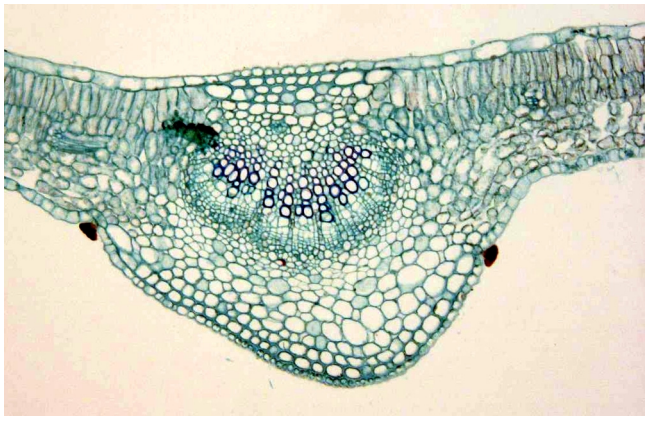

FIGURE 4.3 Midrib region of lilac leaf (*Syringa vulgaris*) with thick walled collenchyma tissue beneath the upper epidermis and above the lower epidermis.

4.1.3 Simple Tissues

4.1.3.1 Parenchyma

Thin-walled, isodiametric parenchyma cells occupy the bulk of the cortex, the area between the epidermis and the vascular tissues, and the pith, the area to the inside of the vascular tissues, of stems and roots. Parenchyma cells can function as storage sites for starches, proteins, oils, and so on, and they contribute support to the plant if they are turgid. There is evidence that pressure exerted by parenchyma in the stem contributes to its growth.

4.1.3.2 Collenchyma

Parenchyma cells may be modified with the addition of primary cell wall material, deposited mainly in the corners of the cells, to form collenchyma. Collenchyma grows with the plant and provides support to elongating stems, where it occurs in ridges under the epidermis and in midribs of leaves (Fig. 4.3). Its cell walls lack hydrophobic components, so collenchyma tissue is relatively cheap for the plant to make, but like parenchyma, it helps support the plant only if it is turgid.

4.1.3.3 Sclerenchyma

Sclerenchyma cells have thickened lignified walls, which make them strong and waterproof. They are commonly classified into support types and conducting forms.

Support sclerenchyma is comprised of sclereids and fibers. This tissue reduces wilting, but it is energetically costly for the plant to create. Sclerenchyma matures with the surrounding tissues and provides more permanent support than collenchyma, maintaining the established morphology of the plant. Fibers have tapered ends, can be many centimeters long, and comprise the bundle caps and sheaths characteristic of vascular bundles, especially in monocotyledonous plants. The bundle sheath may form bundle sheath extensions by spreading to the epidermis, especially in grass leaves.

Sclereids are roughly isodiametric, and clumps of these "stone cells" (brachysclereids) give the Bartlett pear (*Pyrus communis*) its distinctive grittiness. Testas (seed coats) of many plants, especially legumes, are made of two layers of sclereids while sclereids comprise the thick dense layer forming the shell (endocarp) of the coconut. Star-shaped or branched astrosclereids make water lily leaves (*Nymphaea* sp.) tough but pliable, allowing them to withstand the tearing forces of waves and currents.

The conducting types of sclerenchyma are the tracheids and vessel elements of the xylem, the tracheary elements of plants.

4.1.4 Complex Tissues

4.1.4.1 Vascular Tissues

Vascular tissue is comprised of the xylem and the phloem, the main transport systems of plants. They typically occur together in vascular bundles in all plant organs, traversing roots, stems, and leaves.

Xylem is responsible for the transport of water and dissolved ions from the roots upwards through the plant. Phloem transports metabolites (mainly sugars, amino acids, and some ions) in solution from "sources" of production, mainly fully expanded leaves, to "sinks," including developing roots, leaves, and fruits. Mason and Maskell carried out fundamental research on transport in plants at the St. Augustine Campus of the University of the West Indies in the 1920s and 1930s.

The phloem is comprised of sieve tubes, companion cells, parenchyma cells, and fibers. Sieve tubes are separated into sieve tube members, commonly referred to as sieve elements, by thickened end walls, termed "sieve plates," pierced by sieve pores.

Sieve elements contain little cytoplasm, mainly filamentous proteins and amyloplasts (starch-filled plastids). Mature sieve elements lack nuclei and tonoplasts, but possess a plasmalemma, and are living cells, despite lacking nuclei. Abundant plasmodesmata connect sieve elements to associated companion cells that surround them. Companion cells are characterized by dense cytoplasm with abundant organelles and vacuoles and large nuclei; they function to load and unload metabolites into the sieve elements.

Xylem consists of tracheids, vessels, parenchyma, and fibers. Vessels consist of vessel elements joined together in files by large perforation plates, large gaps in the end walls between successive vessel elements, while tracheids have tapering ends that overlap with adjacent cells, and lack perforation plates. Various states of lignin distribution occur in vessel elements, ranging from annular, helical, scalariform, reticulate, to pitted.

Transport through vessels can occur longitudinally through their perforation plates, as well as laterally, among adjacent vessels, through pits, areas of their sidewalls that are not covered by the secondary cell wall. Lateral transport is the greatest between vessels with annular thickenings, the least between pitted vessels, but there is no longitudinal transport between tracheids, and so lateral transport is restricted to pits.

4.1.4.2 Protective Tissue Layers

Epidermis is the outermost layer of cells of the primary plant body, covering all external surfaces of herbaceous plants and forming an interface between the plant and its environment. It is coated with cuticle, which is very impermeable to water, making it indigestible by most pathogens and thus keeps water in the plant and pathogens out. The epidermis is usually covered with epicuticular wax, which enhances these qualities. Because the cuticle is clear, as are the epidermal cells it covers, light easily penetrates to photosynthetic tissues underneath, while protecting the plant from mutagenic ultraviolet radiation from the sun.

Stomata in the epidermis of leaves and stems permit gas exchange between the plant and the atmosphere. Each stoma (or stomate) is made up of a pair of guard cells that can bend to form a stomatal pore. Dicot guard cells are

typically kidney-shaped, with the inner walls that separate the guard cells being thicker than their outer walls. The ends of the cells are attached to each other, and cellulose microfibrils in the cell walls are arranged in hoops around the guard cells. When guard cells absorb water they expand, but the restraining hoops of cellulose force them to get longer instead of wider, so they are forced to bend apart because of their thicker inside walls, creating a pore for gas exchange. Monocots have guard cells that are dumbbell-shaped. The walls of the middle part of the cells are relatively thick, and those of the bulbous ends are relatively thin. The bulbous ends of these guard cells expand when they absorb water, again creating a pore for gas exchange.

Trichomes are extensions of epidermal cells that normally divide to form files of cells. Trichomes have various functions, including protecting plants from insects and herbivores, either passively (simply hindering access to the plant surface) or actively, by secreting toxins. They shade the plant surface from sunlight and reduce air flow, reducing desiccation. Trichomes can be cheaper and faster for the plant to make than cuticle, hence they are seen in great abundance on young, elongating stems, before extensive cuticle layers are deposited on the epidermis.

Leaves and stems of the stinging nettle (*Urtica dioica*) bear trichomes with brittle silica tips that easily break off to inject passing hikers or grazing animals with irritating histamines. The insectivorous sundew (*Drosera* sp.) bears leaves with perhaps the most highly modified trichomes of all plants. These produce sticky nectar that attracts and grips insects, then bend over while the leaf folds to trap them, and then excreting enzymes to digest their prey.

Root epidermis has different functions and is less complex in structure than shoot epidermis. While shoot epidermis shields plants from desiccation, root epidermis allows plants to extract water from the soil. Root epidermis has a cuticle that is thin and impregnated with relatively short chain waxes, compared to those of shoot epidermis, which interfere little with water uptake. Roots often secrete mucigel, a hydrophilic carbohydrate that absorbs water to help lubricate the passage of the root through the soil. Root hairs are delicate outgrowths of epidermal cells that significantly increase the volume of soil that plants mine for nutrients.

Periderm replaces epidermis more substantially in plants during secondary growth. It is comprised of phellogen (cork cambium) producing an inner phelloderm layer (contributing to the cortex) and an outer layer of phellem (cork cells). Cork cells are dead and their walls have many layers of suberin and sometimes lignin, which impart great resistance to insect and pathogen attack, and desiccation. Gaps in the phellem called lenticels permit gas exchange in the periderm. Periderm can form in roots in secondary growth, normally arising from the pericycle.

"Bark" refers to the tissues from the vascular cambium outwards: secondary phloem, phloem fibers, cortex, and periderm layers. Bark exhibits various patterns since periderm arises at different places and rates on the maturing stem. Breaks appear in the periderm as the stem increases its circumference, splitting the cork apart. New cambia arise under old ones, pushing those outwards to create patterns in the bark. In most plants, cork cambium does not live for the life of the plant. The cork oak (*Quercus suber*) has one layer of cork cambium that is continuously active, with seasonal variation in growth rate reflected in growth rings visible in bottle corks.

Roots, stems, and sometimes leaves have subdermal protective cell layers. The endodermis, characteristic of roots, is the most prominent such layer, and forms the innermost cell layer of the cortex, separating it from the stele (vascular cylinder). Its walls contain suberin, the same material that makes cork cells waterproof. In the endodermis, it is usually confined to a thin strip, the Casparian band, running around the middle of each cell in its radial walls. It prevents uncontrolled uptake of water and ions from the soil by the stele via the apoplast (the "free space" of the cell walls and intercellular spaces). Instead, the Casparian strip directs transport selectively through the cytoplasm of the endodermal cells.

Many plants have endodermal cells with highly lignified walls, forming characteristic U-shaped or "phi" thickenings (Fig. 4.4). The endodermis is primarily a feature of the root, but it does (rarely) appear in stems, where it usually lacks a Casparian band or phi thickening. A few aquatic plants have an obvious endodermis in their stems and in their leaves. Exodermis occurs in the roots of some plants, especially monocots, directly beneath their epidermis. It is distinct from the cortex and frequently lignified. The exodermis can have a Casparian band and may occur in stems (where it may be called a hypodermis). Perhaps, it functions like a second endodermis.

4.1.5 Plant Architecture

Plants adopt a bewildering variety of forms, designed primarily to optimize survival in various habitats so that they proliferate and ultimately perpetuate the species through the production of seed. Plants are inherently branched: a branching root system mines the soil and anchors the plant, a branched stem holds leaves in an efficient way to intercept sunlight, and clusters of flowers attract pollinators to fertilize them.

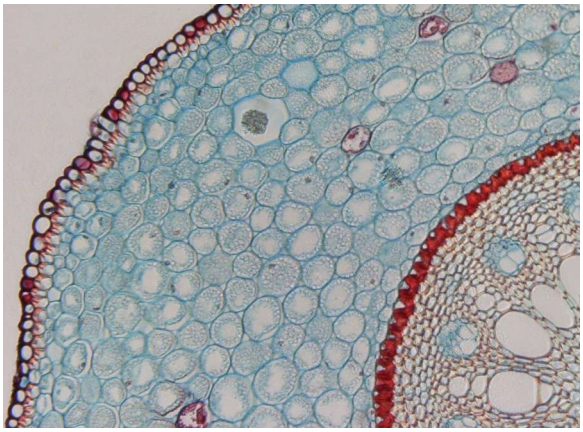

FIGURE 4.4 Transverse section of root of carrion flower (*Smilax herbacea*) with prominent exodermis beneath the epidermis and phi thickenings in the endodermis.

4.1.5.1 Leaf Form and Arrangement

The leaves on a plant usually have fixed shapes: all of their leaves are essentially identical. Some plants exhibit heterophylly, having more than one shape of leaf. The aquatic monocot arrowhead (*Sagittaria* sp.) produces long strap-like leaves under water and characteristic arrowhead shaped leaves above water. The narrow submerged leaves allow water to flow around them, and the broad aerial leaves, a liability in a strong water current, are designed to intercept light.

A simple leaf has one undivided surface while compound leaves have laminas divided up into leaflets (*pinnae*). Leaves may be palmately compound, with the leaflets attached to the end of the petiole, or pinnately compound, with the leaflets attached to the sides of the petiole (which then becomes a *rachis*). If the rachis is unbranched, it is *unipinnate*, with secondary branches, *bipinnate*, and with tertiary branches, *tripinnate*.

It is advantageous for plants to have compound leaves rather than simple leaves. They flutter more easily in a breeze, aiding cooling as well as allowing them to capture more CO_2 from the air surrounding them because diffusion of gases is not great enough to replace that absorbed through the stomata. Also, fluttering makes it more difficult for fungal spores and pests to establish themselves on compound leaves.

Phyllotaxy, the sequence of leaves on a stem, is usually fixed and affects how much sunlight each leaf can intercept without shading its neighbors. Monocots generally have one leaf per node and dicots have one or more leaves per node. The fundamental terminology is:

A. One leaf per node (alternate):

Distichous (a diagnostic feature of grasses.): Leaves are all arranged in two rows seen from above, usually with 180 degrees between rows.

Spiral: (very common) Applies if three or more longitudinal rows are present, e.g., five or eight. Successive leaves in the spiral are separated by an angle that in most plants approaches 137.5 degrees. This same Fibonacci angle also appears in flowers (sunflower) and fruit (pineapple) and occurs in many plant species in many families.

B. Two leaves per node (opposite):

Two leaves 180 degrees apart at each node forming two rows.

Opposite decussate: Successive pairs oriented 90 degrees to each other, forming four rows.

C. Three or more leaves per node (whorled):

A fixed or variable number of leaves arise at each node. Leaves in successive whorls may or may not form discrete rows when seen from above.

4.1.5.2 Stem Branching

The pattern of stem branching can be fixed, like leaf arrangement, but it is more commonly so in lower plants than in higher ones. While branching is a complex topic it is convenient to recognize four basic patterns:

Monopodial: The shoot apical meristem remains rhythmically active throughout the life of the plant. Axillary shoots are secondary and are controlled by the apex of the terminal shoot (many conifers, e.g., *Araucaria* sp.).

Sympodial: The shoot apex either aborts or becomes reproductive. Axillary shoots grow outwards and then turn upward to produce clusters of leaves. One shoot of these shoots grows upward to become the primary stem, then its shoot apex aborts or becomes reproductive, and so on. The pagoda shape characteristic of the seaside almond (*Terminalia catappa*) results from this growth pattern.
Dichasial: A type of sympodial branching in which the terminal bud gives rise to two axillary buds on opposite sides. These grow at similar rates then branch again, resulting in a repeatedly forked pattern. Examples include pink poui (*Tabebuia pentaphylla*), frangipani (*Plumeria* sp.), and mango (*Mangifera indica*). True dichasial branching is extremely rare.
Adventitious: While shoots normally grow from apical or axillary buds, they may arise endogenously from any organ. The scrambling habit of sweet potato (*Ipomea batatas*) results from new stems arising from roots, creating disorganized looking plants.
Endogenous shoots on tree trunks and branches give rise to cocoa pods on the cocoa tree (*Theobroma cacao*). In the cannonball tree (*Couroupita guianensis*), shoots that produce flowers and fruits ring the trunk a few meters off the ground, a condition termed "cauliflory."

4.1.5.3 Floral Branching (Inflorescence)

Plants may have solitary flowers borne singly in the axil of a leaf, as in species of *Hibiscus*, or possess branched systems of flowers forming inflorescences. A great variety of these exist, but in general they can be divided into two types. The main axis of a racemose inflorescence is not terminated by a flower, so it is capable of indefinite growth. It displays monopodial branching and is indeterminate. A cymose inflorescence is terminated by a flower on the main axis and is therefore capable of limited or determinate growth. Additional flowers must arise from sympodial branching.

4.1.5.4 Root Branching

Root branching is poorly understood because roots grow out of sight and are inherently difficult to study. Although some general types of root systems have been identified, root growth is extremely plastic because it is strongly governed by soil homogeneity, composition, moisture, and fertility. They never seem to approach the geometric patterns that stem systems can exhibit. Roots lack axillary buds, flowers/fruits, leaves, and any real apical dominance. Under the influence of gravitropic factors, the primary root from a seedling normally penetrates downwards through the soil, giving rise to lateral secondary roots (often roughly in rows, usually four). Secondary (lateral) roots arise from the pericycle of the primary root. (These lateral roots may arise as seminal roots from primordia in the embryo before germination.) Lateral roots are anatomically identical to the primary root and produce tertiary roots. Their arrangement is less well ordered than the laterals. The tertiaries may themselves be branched, and the branching process may continue on. Roots are indeterminate and continue growing in patches, seeking regions of soil rich in nutrients or water.

4.1.5.5 Flower

A flower is a shoot system comprised of concentric rings of four types of leaves arising from a determinate axis and modified for sexual reproduction: sepals, petals, stamens, and carpels. Sepals and petals are not required for reproduction and are thus called accessory parts; therefore stamens and carpels are essential parts. Together the sepals comprise the flower's calyx while its petals comprise the corolla; both whorls combined form its perianth. The male parts (androecium) are the stamens (consisting of filament and anther) and the female parts (gynoecium) are the carpels (consisting of stigma, style, and ovary).

Flowers form in leaf axils which are often tiny and are termed "bracts." Some flowers have large conspicuously colored bracts that can supplement or completely replace them (*Poinsettia* sp.). A flower is borne on a stem called a pedicel while its attachment point is a receptacle, and the stem of an inflorescence, a branching floral system, is called a peduncle.

4.1.5.5.1 Floral Modifications

Flowers are modified in many ways, chiefly because many of them coevolved with pollinating insects. Four kinds of floral modifications are briefly described here.

4.1.5.5.2 Loss of Floral Parts

Complete flowers possess all four whorls of floral leaves while incomplete flowers lack one or more whorls. Perfect flowers possess carpels and stamens, but lack petals, sepals, or both of these parts. An imperfect flower lacks either carpels or stamens, whereas carpellate (female) flowers have only carpels and staminate (male) flowers have only stamens, no carpels.

Botanically the ear of a corn plant (*Z. mays*) is a carpellate (female) imperfect inflorescence. The silks are conspicuously elongated styles, with exposed and adhesive stigmatic surfaces that can catch airborne pollen. The tassel is a staminate (male) imperfect inflorescence. Corn is a monoecious plant, with both female and male reproductive organs; many cucurbits are likewise monoecious. Papaya (*Carica papaya*) is dioecious, with female and male flowers produced on separate plants.

4.1.5.5.3 Fusion of Floral Parts

Fusion within floral whorls creates a gamosepalous flower if the sepals are involved (many legumes) and a gamopetalous flower if it involves the petals (morning glory, *Ipomea* sp.). Flowers with petals that are not fused together are polypetalous. The most common type of fusion between whorls involves stamens and sepals; in this case the stamens are said to be adnate to the petals, producing a flower that is epipetalous.

Fusion of the filaments of the stamens into a tube creates a connate structure with the resulting androecium being *adelphous*. Fusion of the carpels produces a syncarpous gynoecium, while an apocarpous gynoecium has free carpels; a gynostegium results from fusion of the gynoecium with the androecium. The flowers of many legumes and all of the Compositae have their stamens fused into tubes.

4.1.5.5.4 Position of the Ovary

Hypogynous flowers have convex receptacles that make their ovaries superior to the rest of the flower (in other words the rest of the floral parts are "below the gynoecium"). Perigynous ("around the gynoecium") flowers have concave receptacles so their ovaries are below the rest of the floral parts, thus making them half superior. The ovary of an epigynous flower ("above the gynoecium") is inferior and completely surrounded by the receptacle.

4.1.5.5.5 Floral Shape

If the parts in each whorl of a flower are all the same size and shape, the flower will display radial or actinomorphic (multilateral) symmetry. Any longitudinal cut through its central axis will make halves that are mirror images (*Hibiscus* sp., *Cucurbita* sp.). If there are differences in the shape or size of the parts, or some parts are missing then flower will have bilateral (zygomorphic) symmetry, such that just one particular longitudinal cut results in halves that are mirror images (Orchidaceae, many Leguminoceae). The flowers of some plants exhibit a lack of any recognizable pattern in the arrangement of their parts and are thus asymmetric (*Canna* sp.).

4.1.5.6 Fruits

Fruits are botanically different from what are often distinguished as being fruits or vegetables. The common green bean (*Phaseolus vulgaris*) is classified as a type of fruit called a legume, rather than a vegetable, while coconuts are not really nuts (though coconuts and true nuts are both fruits). Avocados (*Persea americana*), bananas (*Musa acuminata*), and tomatoes (*Solanum lycopersicum*) are examples of berries.

True fruits are mature ovaries of angiosperms. When the ovule(s) develop to form seed(s) in the ovary, the ovary wall generally becomes thicker and forms three layers that constitute the pericarp. The exterior layer is the exocarp, which can be just an epidermis (blueberry, *Vaccinium* sp.) while mesocarp, the middle layer, is often soft, like the flesh of the mango (*M. indica*). The interior layer (endocarp) can range from soft (cucumber, *Cucumis sativus*) to stony (the "stone" or "pit" of the plum (*Prunus* sp.).

A fruit is commonly classified by the number of flowers and ovaries involved in its development, whether the mesocarp is soft and fleshy, rather than dry and hard, and whether or not the fruit splits open (dehisces) when mature.

4.1.5.7 Seed

Seeds are the mature, fertilized ovules of flowers that contain dormant embryos and food stores awaiting germination. Protection is provided by a seed coat (testa) that develops from the layers of integument cell(s) of the ovary inside the nucellus (megasporangium). The micropyle forms a pore at the apex so that the pollen tube can reach the ovule.

Near the micropyle is the hilum, a scar formed when the seed separates from the funiculus, or seed stalk. Albuminous seeds store food while exalbuminous seeds do not. Food reserves, which are usually carbohydrates but can be protein or lipids can be either perispermous (enlarged nucellus tissue), endospermous (originating just from the principal nucellus cell), or both of these.

Most eudicotyledonous seed embryos use up their food reserves long before they germinate; exceptions include sunflower (*Helianthus annuus*) and rapeseed (*Brassica* sp.), which have oily seeds. In contrast, the seeds of many monocotyledons, including the crops wheat, oats, and corn, have conspicuous endosperms.

4.1.5.8 Accessory and False Fruits

False fruits arise from inferior ovaries. In apples (*Malus domestica*) and pears (*Pyrus communis*) the mesocarp merges together with the receptacle with no discernible exocarp, because the carpels fuse with receptacle tissues during fruit development. There are two groups of vascular bundles visible in the flesh of these fruits. One group led to the sepals and the other one to the petals of the flowers from which the fruits formed. Strawberry (*Frageria* sp.) fruits are really tiny achenes, a kind of dry indehiscent fruit that resemble seeds on the swollen red receptacle. The pericarp can be bolstered by other structures that protect the seeds, including the proliferated receptacle of soursop (*Annona muricata*) and perianth of breadnut (*Artocarpus camansi*), and the scale-like bracts of pineapple (*Ananas comosus*).

4.2 PLANT FUNCTION

4.2.1 Photosynthesis

All life forms on earth require energy for maintenance, growth, and productivity. This energy is derived directly or indirectly from the sun for most organisms, and the first step in the use of solar energy is the process of photosynthesis. Photosynthesis can be defined as the synthesis of organic compounds (primarily sugars) from carbon dioxide (CO_2) and water using light energy. It can also be defined as the process by which light energy from the sun is converted into chemical energy of carbohydrate molecules. The main products of photosynthesis are carbohydrates (of general chemical formula $C_6H_{12}O_6$) and oxygen gas (O_2). A summary of the overall process is as follows:

$$6CO_2 + 6H_2O = C_6H_{12}O_6 + 6O_2$$

Photosynthesis is the most important characteristic of plants, and all agriculture is dependent on this process directly or indirectly. There are three major steps in the process of photosynthesis: the CO_2 diffusion step, the Photochemical step, and the Biochemical step.

In the diffusion step, CO_2 diffuses from the air (which has about 0.04% CO_2) into the plant tissue to the site of photosynthesis (chloroplasts). There is first a vapor phase diffusion of CO_2 largely through the stomata to the intercellular spaces, followed by liquid phase diffusion to the chloroplasts. We will now examine with greater detail the photochemical and biochemical steps of photosynthesis.

4.2.1.1 The Photochemical Step

The photochemical step of photosynthesis consists of what are called light-dependent reactions that occur within the chloroplasts. During this step, light energy is absorbed by pigments within the chloroplast and used to produce a high-energy compound (adenosine triphosphate, ATP) and a strong reducing agent (reduced nicotinamide adenine dinucleotide phosphate, NADPH). The water molecule is also split during this step and oxygen is released.

4.2.1.1.1 Chloroplasts

A leaf cell may contain 40−50 chloroplasts, and there may be hundreds of thousands of chloroplasts per square millimeter of leaf surface. The chloroplast is surrounded by a double membrane and contains a dense solution called stroma. Within the stroma another membrane system forms flattened sacs or vesicles called thylakoids. The green chlorophyll pigments and other pigments are located on the thylakoid membranes. Groups of thylakoids are often stacked together on the flat surfaces to form grana (singular = granum). Within the thylakoids there is a lumen filled with a solution that is different from the outside stroma.

4.2.1.1.2 Photosystems

On the thylakoid membranes, certain pigments and associated proteins are packed together to form units called photosystems. There are two types of photosystems, designated Photosystem I (or PSI) and Photosystem II (or PSII). Each photosystem has a critical pigment for photosynthesis (called chlorophyll *a*) and accessory pigments, e.g., chlorophyll *b* and carotenoids. The critical chlorophyll *a* pigment is designated P700 for PSI and P680 for PSII, indicating the light wavelength that is absorbed most efficiently by these pigments. The chlorophyll pigments absorb mainly red and blue light wavelengths, and reflect and transmit green wavelengths, so leaves are green. The accessory pigments absorb light of slightly different wavelengths than chlorophyll *a*, and channel this energy to chlorophyll *a*. In higher plants, both photosystems must cooperate in carrying out photosynthesis.

4.2.1.1.3 The Photosynthetic Z-Scheme

To understand the light-dependent reactions of photosynthesis, it is best to start with reactions in PSII. Light energy is trapped by PSII causing an electron from P680 to be promoted to a higher energy level (an excited state). This excited electron is rapidly transferred to a primary electron-acceptor molecule that is closely associated with P680. If this transfer does not occur immediately, the excited electron falls back to its ground state in P680, giving-off energy (fluorescence) in the process. From the primary electron acceptor, the electron is transferred from one acceptor to another within PSII. In this electron transfer chain, the energy of the excited electron is utilized to move protons (H^+) from the stroma to the lumen of the thylakoids. Finally, a mobile electron acceptor carries the electron to PSI where it is transferred to P700. The photochemical reactions can be illustrated in the Photosynthetic Z-scheme (Fig. 4.5).

As electrons move on to Photosystem I, the pigment P680 (in Photosystem II) is depleted of electrons and becomes a powerful oxidizing agent capable of stripping electrons from water, thereby splitting the water molecule as follows:

$$2H_2O \rightarrow 4H^+ + 4e^- + O_2$$

Protons released from this reaction accumulate in the lumen of the thylakoids.

P700 (in PSI) also absorbs light energy and in so doing one electron (supplied by PSII) is promoted to the excited state. Again this excited electron is immediately transferred to a primary electron acceptor closely associated with PSI. From the primary electron acceptor, the excited electron moves across an electron transfer chain and is finally transferred to $NADP^+$ resulting in the formation of NADPH as follows:

$$NADP^+ + 2e^- + H^+ \rightarrow NADPH$$

This reaction occurs on the stroma side of the thylakoid membrane. NADPH is a powerful reducing agent, which means that it has a strong ability to force its electrons and hydrogen on to other molecules.

4.2.1.1.4 Photophosphorylation

The above reactions result in the accumulation of protons (H^+) in the lumen of the thylakoids and a depletion in the stroma, which leads to a large gradient in proton concentration across the thylakoid membranes. Protons flow out to

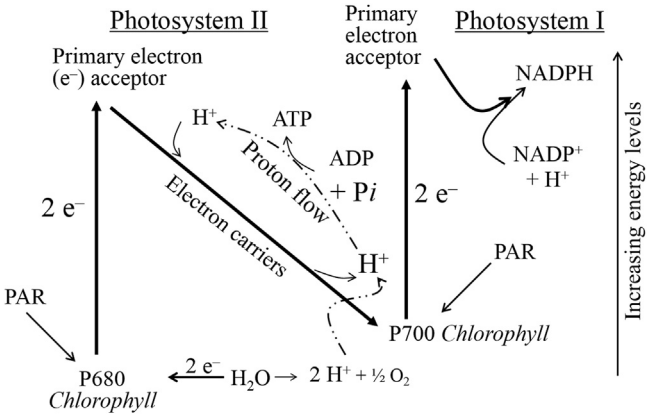

FIGURE 4.5 Diagrammatic representation of the photochemical reactions (the Photosynthetic Z-scheme).

the stroma through specialized membrane proteins (called ATP synthase) that utilize the energy of this electrical flow to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) as follows:

$$ADP + P_i + \text{energy} \rightarrow ATP$$

The plant can utilize energy stored in the triple bond of ATP at a later time for any type of activity that requires energy. Therefore, ATP serves as energy currency in the plant. The entire process that utilizes light energy to produce high-energy phosphate bonds is referred to as photophosphorylation.

4.2.1.2 The Biochemical Step

The biochemical step of photosynthesis consists of what are called light-independent reactions that can occur in darkness. During the biochemical step of photosynthesis, the products of the photochemical step (ATP and NADPH) are used to incorporate carbon dioxide into organic compounds. The energy of solar radiation becomes stored in these organic molecules as energy associated with chemical bonds. During respiration, organic molecules are broken down to release CO_2 and the stored energy is made available for various activities.

4.2.1.2.1 The Calvin Cycle and C3 Plants

The light-independent reactions can proceed once products (NADPH and ATP) of the light-dependent reactions are present in the chloroplast. The actual fixation of carbon dioxide, which diffuses into the leaf from the atmosphere, occurs by a cyclic series of reactions called the Calvin cycle (named after one of the pioneer researchers in this area) A summary of the Calvin cycle is illustrated in Fig. 4.6.

There are three major steps in the Calvin cycle: Carboxylation, reduction, and regeneration. The initial incorporation of CO_2 (carboxylation step) is catalyzed by an enzyme called rubisco (ribulose bisphosphate carboxylase-oxygenase) which occurs in relatively large quantities in photosynthetic tissues. In this reaction, CO_2 (a molecule containing one carbon atom) combines with a five-carbon compound (ribulose bisphosphate, or RuBP) to form an unstable product that immediately breaks down to give two molecules of a three-carbon compound (phosphoglyceric acid or PGA).

$$\text{RuBP } (5-\text{carbon}) + CO_2 \ (1-\text{carbon}) \rightarrow 2 \text{ molecules of PGA } (3-\text{carbon})$$

Plants in which the first detectable product of photosynthesis is a three-carbon compound are called C3 plants (Fig. 4.7). Energy of ATP and the reducing power of NADPH are then used to reduce PGA to phosphoglyceraldehyde (PGaldehyde) in the Reduction step. ATP is also needed in the Regeneration step to regenerate molecules of the original five-carbon compound (RuBP) from molecules of the three-carbon compound (PGaldehyde).

$$\text{For example, } 5 \times 3-\text{carbon} \rightarrow 3 \times 5-\text{carbon}$$

However, with every turn of the cycle an additional carbon atom is incorporated, so that there is an accumulation of three-carbon compounds, which are used to synthesize hexose (six-carbon) sugars.

$$\text{For example, } 2 \times 3-\text{carbon} \rightarrow 1 \times 6-\text{carbon}$$

The plant can use the basic building blocks of hexose sugars to build all other substances required, e.g., sucrose, starch, cellulose, amino acids, fatty acids.

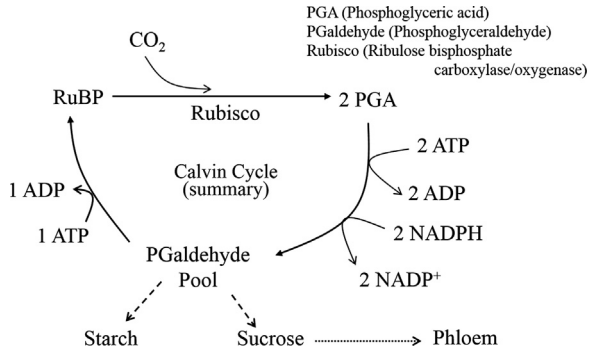

FIGURE 4.6 Summary reactions of the photosynthetic biochemical step.

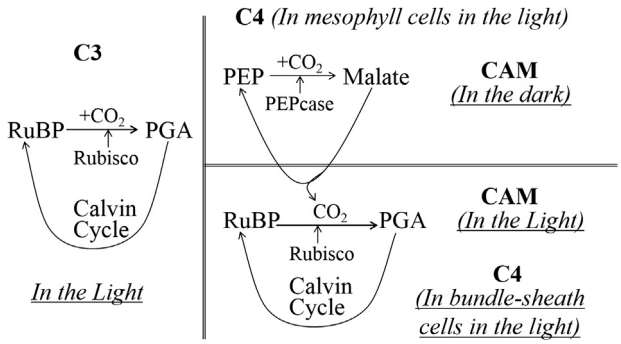

FIGURE 4.7 Photosynthetic biochemical pathways in C3, C4, and CAM plants.

4.2.1.2.2 Photorespiration

Photorespiration can be defined as the evolution of CO_2 during photosynthesis. It arises because rubisco can act as either a carboxylase (incorporating CO_2) or as an oxygenase (incorporating O_2). In the presence of relatively high CO_2 levels, rubisco acts mainly as a carboxylase. However, when oxygen levels are high, rubisco acts as an oxygenase and incorporates O_2 into the five-carbon compound (RuBP) as follows:

$$\text{RuBP (5 - carbon)} + O_2 \rightarrow \text{PGA (3 - carbon)} + \text{glycolate (2 - carbon)}$$

The PGA formed continues along the Calvin cycle, while glycolate is metabolized in the glycolate pathway involving chloroplasts and other cell organelles. In the glycolate pathway there is a net loss of CO_2, and ATP and NADPH are required. Therefore, photorespiration appears to be a very wasteful process in which net CO_2 fixation is reduced and energy is also used up in the process. Any factor that reduces the availability of CO_2 or increases the availability of O_2 to rubisco will increase the levels of photorespiration.

4.2.1.2.3 C4 Plants

Under hot dry conditions, stomata tend to close which serves to restrict water loss. However, stomatal closure restricts the diffusion of CO_2 to the chloroplasts and O_2 levels also build up, which encourages photorespiration. Some plants have evolved mechanisms to reduce the levels of photorespiration under these conditions. In many of these plants two enzymes are involved in the incorporation of CO_2.

In some plants the first detectable product of photosynthesis is not a three-carbon compound but a four-carbon compound. These plants are called C4 plants, and in addition to rubisco they have another enzyme (phophoenolpyruvate carboxylase, or PEPcase) capable of capturing CO_2. PEPcase is present in the mesophyll cells of the leaf, while rubisco occurs only in cells (called bundle sheath cells) immediately surrounding the transport tissue (vascular bundles). PEPcase first incorporates CO_2 into a three-carbon compound (phosphoenolpyruvate, or PEP) to form a four-carbon compound (oxaloacetic acid, OAA). OAA is not very stable and is quickly converted to either malic acid or aspartic acid (four-carbon compounds, Fig. 4.7).

The four-carbon compound formed from the activity of PEPcase diffuses to the bundle sheath chloroplasts, where the newly fixed CO_2 is released for incorporation by rubisco in the Calvin cycle. After releasing CO_2, the resulting three-carbon compound returns to the mesophyll cells and the process is repeated. However, some additional energy (ATP) is required for PEP to be regenerated. The C4 mechanism serves to concentrate CO_2 into the bundle sheath chloroplasts, thereby reducing the levels of photorespiration. Therefore, photorespiration tends to be negligible in C4 plants.

4.2.1.2.4 Crassulacean Acid Metabolism Plants

In some plants adapted to very dry (desert) conditions, the stomata are closed during the daytime and open at night. These plants are said to show the crassulacean acid metabolism (CAM) pathway, which was first discovered in members of the plant family Crassulaceae. Photosynthetic tissue of these plants contains both PEPcase and rubisco. During the night, when stomata are open, CO_2 is fixed by PEPcase to form malic acid, which accumulates in plant tissues. During the day, stomata close and malic acid breaks down to release the fixed CO_2, this is then incorporated by rubisco in the Calvin cycle (Fig. 4.7). Rubisco is activated by light, and has a lower affinity for CO_2 than PEPcase. A relatively high CO_2 concentration is likely to exist within the photosynthetic tissue when rubisco is active, so that photorespiration is generally lower than that of C3 plants.

4.2.1.2.5 Blackman's Law of Limiting Factors

Several environmental factors influence photosynthesis, and the actual rate of photosynthesis at any instant will be limited by the factor that is either in short supply or furthest from its optimum value. This is known as Blackman's Law of limiting factors (or Liebig's law of the minimum), which can be restated as follows:

> *When a chemical process depends on more than one essential condition being favorable, its rate is limited by that factor that is nearest to its minimum value.*

For example, photosynthesis is likely to be limited by light under heavy shade conditions and according to Blackman's law cannot be increased by changes in any other factor besides light e.g., temperature, water supply, or carbon dioxide concentration. When light levels are increased to the photosynthetic light saturation point, another factor becomes the limiting factor. A good analogy to help explain Blackman's law is a barrel containing holes at different points on the sidewalls. The maximum height to which water can rise in the barrel is determined by the height of the hole nearest to the base of the barrel.

4.2.2 Translocation

The transport of products of photosynthesis (also called assimilates or photosynthates) from Source to Sink in plants is called translocation. The Source is a net exporter of assimilates (e.g., leaves), while the Sink is a net importer of assimilates (e.g., tubers, fruits, roots, stems). A storage tissue can become a Source when stored material is mobilized and exported.

4.2.2.1 Translocation Mechanism

Translocation occurs in phloem tissue via sieve elements (with associated companion cells) and metabolic energy is required for this process. Rates of movement in the phloem can sometimes exceed 1 m per hour and substances can move in different directions at the same time. Movement by diffusion is much too slow to account for such rapid rates of movement observed in the phloem.

Some movement in individual sieve elements may be explained by cytoplasmic streaming—this is a rotational movement of the cytoplasm around the periphery of many cells due to the action of microfilaments. Cytoplasmic streaming is readily observed in young but not in mature sieve elements, and observed rates of movement are still too slow to explain the rapid rates of movement that can occur in phloem tissue.

4.2.2.1.1 Munch's Pressure-Flow Theory

The most widely accepted explanation of the translocation mechanism is given by Munch's Pressure-Flow theory. This theory suggests that movement in the phloem is due to mass flow along a turgor (hydrostatic) pressure gradient.

Assimilates enter the sieve tubes of the phloem by active transport (phloem loading) at the Source (e.g., leaf). The osmotic potential falls as solutes accumulate in the sieve elements at the Source. Water is then dragged in by osmosis from surrounding tissue and ultimately from the xylem. The pressure increases as water enters the sieve tube leading to the mass flow of water and dissolved substances along the sieve tube under a hydrostatic pressure gradient.

Assimilates are removed from the sieve tubes (unloading) at a Sink (where assimilates are utilized). The water potential of the solution in the sieve tube increases as dissolved substances move out and the solution becomes more dilute. Water moves out when the water potential of the solution in the sieve tube becomes higher than that of the surrounding cells. This leads to a fall in the hydrostatic pressure at that location in the sieve tube, which serves to bring more phloem sap toward the active Sink. Water flowing out of the sieve tubes at the Sink will ultimately return to the xylem. Translocation is therefore linked to water flow in xylem (Fig. 4.8).

There are two mechanisms that can prevent uncontrolled loss of phloem sap in cases where the sieve tube is damaged:

1. Formation of P-protein (phloem protein) plugs. P-protein filaments form a fine network next to the plasma membrane of sieve elements. If the sieve tube becomes damaged, the P-protein (along with other contents of the phloem) surges toward the cut end due to the internal hydrostatic pressure. The tangled mass of protein filaments and protein bodies form a "P-protein" plug, which helps to seal the cut end of the sieve tube. However, not all flowering plants have P-proteins.

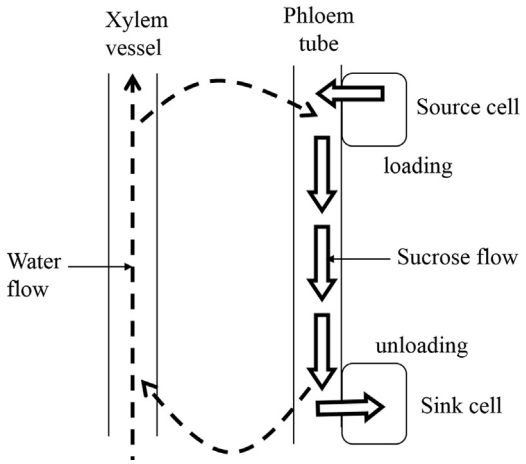

FIGURE 4.8 Diagram illustrating the Pressure-Flow theory.

2. Proliferation of callose. This is a carbohydrate polymer that is synthesized by the plasma membrane especially under stress conditions. Callose is deposited into the tangled mass in the sieve pores of damaged sieve tubes, which serves to seal off the damaged sieve elements. Callose proliferates when there is a pressure drop, which helps to seal the sieve pores.

4.2.3 Transpiration

Living plant tissues can contain about 95% water, and apart from being an essential constituent for life, water also has several specific roles in the plant. Water is a very good solvent and dissolves many organic and inorganic solutes, which facilitates the transportation of these substances around the plant. Certain properties of water molecules are beneficial for the buffering of temperature changes in plants, allowing plants to operate under more stable temperature conditions. Hydrostatic pressure in plant tissue (due to water content) is very important for plant form, movement, leaf display, and growth. Water also takes part in many chemical reactions within the plant, such as hydrolysis reactions and photosynthesis. However, most (98%) of the water that enters plant roots is lost by evaporation from the shoot system. The evaporative loss of water from plants is called transpiration, and this process is important for transport of substances within the plant as well as for evaporative cooling of leaves. We will now look at the pathways for water movement from the soil through the plant to the atmosphere and the forces that drive water flow.

4.2.3.1 The Soil–Plant–Atmosphere Continuum (SPAC) and Water Potential

There is a continuous pathway (called the SPAC) for the movement of water from the soil through the plant to the atmosphere. Water is present in a continuous system in the SPAC, and water movement in this system can occur by two major processes:

a. Diffusion—the net movement of a substance from one point to another due to the random (or thermal) motion of individual ions or molecules.
b. Mass flow—the simultaneous movement of groups of ions or molecules in one direction due to a hydrostatic pressure gradient.

In plants, mass flow occurs in the vascular tissue (e.g., xylem vessels) as a result of pressure gradients created by the diffusion of water into roots or out of leaves. What is the driving force for water movement by diffusion? To answer this question, we need to introduce the concept of water potential. Water potential of any system is a measure of the capacity of that system to give out water. The word "system" here can refer to a cell, solution, soil, atmosphere, or any part of the SPAC. Water moves spontaneously from a point of high water potential to one of low water potential in any system or across systems, once there is a pathway for water movement. The symbol commonly used to denote water potential is "Ψ" (the Greek letter "Psi").

Water potential is actually a measure of the free energy of water in the system, and free energy can be defined as the energy available to do work. However, absolute values of free energy are very difficult to determine and it is much

easier to calculate relative values. Water potential is defined as the difference in free energy of water at any point in a system and that of pure unconfined water at the same temperature. Therefore, the free energy of water in any system is determined relative to that of pure water, which is assigned a water potential of zero as a consequence of our definition. Water potential has units of pressure and is commonly expressed as MPa (Megapascals). Water potential has components due to the effects of dissolved substances, surfaces that attract water, hydrostatic pressure and gravity. There is usually a gradient in the water potential across the SPAC with values being high in the soil (−0.01 to −1.5 MPa) and low in the atmosphere (−10 to −200 MPa). This gradient in water potential provides tremendous driving force for water movement in the SPAC.

4.2.3.2 Water Uptake From Soil

Water is not held very strongly in the large pore spaces of the soil due to the reduced matric potential forces as the diameter of the pore spaces increases. Water in the largest pore spaces drains away readily under the influence of gravity, and this water is not available to plants. As plants take up water from the soil, water is first removed from the largest pore spaces that contain water. The water menisci then recede to smaller and smaller pore spaces and uptake becomes increasingly difficult as the soil dries. Additionally, the conductance of the soil to water declines sharply as the soil dries.

Water is held most strongly in clay soils since these soils are likely to contain the smallest capillary pore spaces. The maximum water uptake per unit root surface area occurs in the Root Hair Zone, which occurs a short distance (2−20 cm) from the root tip. As roots age, the outer surfaces become increasingly suberized and water uptake rates decrease. However, older roots can still take up a substantial amount of water since they generally make up the bulk of the root system. Water uptake is increased by the presence of Mycorrhizae fungi that form a symbiotic relationship with the roots of most higher plants. The fungal hyphae infect the root and penetrate the soil increasing the surface area for the absorption of water and mineral nutrients. In return the plant supplies the fungus with carbohydrates.

4.2.3.3 Plant Available Water

At "Field Capacity" (FC) the soil is wet and contains all the water it can hold against gravity. At the "Permanent Wilting Point" (PWP) the soil is dry and the plant can no longer extract any more water. The difference in the water content of soil between field capacity and the permanent wilting point gives the amount of soil water available for uptake by plants. The plant available water is expected to be greater for clayey and organic soils compared to sandy soils. If we know the plant available water and the rate at which this water is being depleted by crops then we can determine the necessary frequency of irrigation. Apart from irrigation scheduling, this information can also be useful in the modeling of crop growth and prediction of yields.

4.2.3.4 Water Flow Pathways in Plants

There are two major pathways for water flow in plants, the apoplastic and the symplastic pathways. In the apoplastic pathway, water moves in the free spaces (apoplast) within the plant that are unbounded by membranes and includes movement along cell walls and in intercellular spaces. In the symplastic pathway, water moves across the symplast, which consists of the cytoplasm and plasmodesmata (minute connections between the cytoplasm of adjacent cells). The resistance to water flow is higher in the symplastic pathway, largely due to the flow restriction imposed by the plasma membrane.

4.2.3.5 Water Flow Across Root Cortex

Water flows from the soil across the root epidermal layer and cortex to the root xylem, with movement occurring both in the apoplastic and symplastic pathways. The apoplastic pathway is blocked at the innermost layer of cortical cells, the endodermis. The radial and transverse cell walls of the endodermis are suberized (embedded with a wax-like material) forming the Casparian strip, which prevents further movement of water along the apoplastic pathway. Both water and dissolved mineral ions from the apoplast must enter the symplast at the endodermis in order to proceed further into the plant.

4.2.3.6 Driving Forces for Water Flow From Roots to Leaves

The driving forces for water flow from roots to leaves are root pressure and the transpiration pull. Root pressure is the lesser force and is important mainly in small plants at times when transpiration is not substantial, e.g., at nights. Root pressure requires metabolic energy, which drives the (active) uptake of mineral ions from the soil into the root xylem. As ions accumulate in the root xylem, the osmotic potential of the xylem solution falls causing the passive uptake of water from the soil by osmosis into the xylem. As pressure builds up within the xylem due to osmotic water

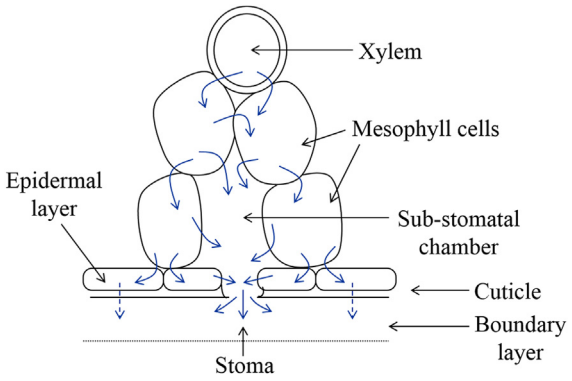

FIGURE 4.9 Diagram illustrating water diffusion out of a leaf.

uptake, the xylem solution is forced upward to the leaves by mass flow. Root pressure can result in the loss of liquid water from the leaves during times of low transpiration. This process is called guttation and specialized structures (hydathodes) in the leaves are involved. The maximum root pressure that develops in plants is typically less than 0.2 MPa, and this force for water movement is relatively small compared to the transpiration pull.

The transpiration pull is explained by the Cohesion−Adhesion Theory, with the water potential gradient between the leaves and the atmosphere providing the driving force for water movement. The water potential of the atmosphere is dependent on the relative humidity and temperature of the air, and can typically range between −10 and −200 MPa. Leaf water potential typically ranges between −0.2 and −3.0 MPa. Water evaporates from the leaf surface into the atmosphere along this steep water potential gradient (no metabolic energy is required). The water potential of surface cells falls as these cells lose water and water is pulled from successively deeper cell layers along the water potential gradient created, until eventually water is pulled from the xylem vessels (Fig. 4.9).

Water columns in the xylem vessels are pulled upward by mass flow as water is removed by leaf cells. Strong attractive forces between water molecules (cohesion) and between water molecules and the walls of the xylem vessels (adhesion) allow the water columns to stay intact. The typical tension (pulling force) that develops within the xylem vessels ranges between −2 and −3 MPa, which is about 10 times the force that develops under root pressure. Cavitation can occur under water stress, which results in a snapping sound as air enters the xylem forming an embolism that blocks further water flow in that particular xylem vessel. Air embolisms may be temporary in some cases as air can redissolve in the xylem sap or be expelled by root pressure.

4.3 PRACTICE QUESTIONS

1. Explain the functional significance of the cells that comprise the vascular tissues.
2. Describe the cells and tissues that provide support in plants.
3. Outline the major differences between the C3, C4, and CAM photosynthetic pathways.
4. List three crop examples for each photosynthetic pathway (C3, C4, and CAM) based on information available on the Internet.
5. Explain the mechanism by which products of photosynthesis are transported in the plant.
6. Distinguish between root pressure and transpiration pull.
7. What environmental factors are likely to influence transpiration rates?

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Chapter 5

Plant Constituents: Carbohydrates, Oils, Resins, Balsams, and Plant Hormones

L.L. Dilworth[1], C.K. Riley[2] and D.K. Stennett[1]

[1]*University of the West Indies, Kingston, Jamaica,* [2]*Scientific Research Council, Kingston, Jamaica*

Chapter Outline

Learning Objectives

- To provide an overview of carbohydrate classification, highlighting medicinal uses of selected polysaccharides.
- To give an overview of essential and nonessential oils, highlighting their contributions to science, medicine, as well as general health and well-being.
- To highlight the classification and main applications of plant hormones, resins, balsams, and growth factors.

5.1 CARBOHYDRATES: CLASSIFICATION, FUNCTION, AND USES IN MEDICINE

5.1.1 Nomenclature and Definition

Carbohydrates belong to a group of complex biomolecules commonly regarded as the "staff of life." They are the most abundant organic compounds in living organisms and are among the four major classes of biomolecules. During photosynthesis, carbohydrates are produced from the reaction of carbon dioxide with water. Carbohydrates go by several common names, including sugars, starches, saccharides, and polysaccharides. In chemical terms, the word carbohydrate refers to a "hydrate of carbon." They are often represented by the chemical formula $C_x(H_2O)_y$, where the numerical values of x and y range from 3 to 12. Glucose, e.g., has the chemical formula $C_6(H_2O)_6$ and is commonly written as $C_6H_{12}O_6$.

The chemistry of carbohydrates closely resembles that of alcohols and carbonyls (aldehydes and ketones). Advancements in modern chemistry revealed that several carbohydrates such as deoxyribose ($C_5H_{10}O_4$) and gluconic acid ($C_6H_{10}O_7$) do not match the required hydrogen to oxygen ratio proposed by the general formula (Fig. 5.1A). In addition to this, other carbohydrates are known to possess other elements such as nitrogen, sulfur, and phosphorous (Fig. 5.1B). As a result, modern conventions now define carbohydrates as polyhydroxy aldehydes or ketones and their derivatives. The classification goes even further to include substances that yield polyhydroxy aldehydes or ketones on hydrolysis.

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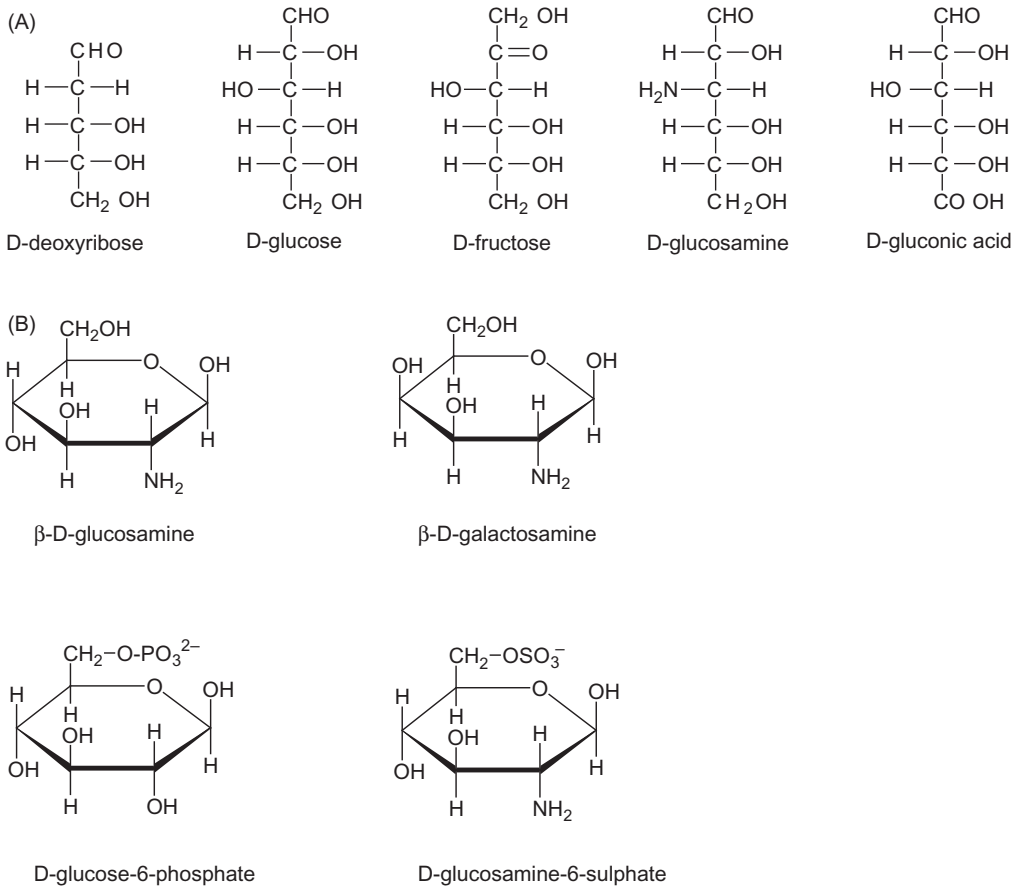

FIGURE 5.1 (A) Structural variation among sugars. (B) Sugars containing N, S, and P.

Advancement in chemistry revealed disparity in existing carbohydrates to general structures. Further some carbohydrates contain N, S, and P. Modern conventions now refer to carbohydrates as: polyhydroxy aldehydes, ketones, substances that yield polyhydroxy aldehydes, or ketones on hydrolysis.

Carbohydrates may exist as straight chain or cyclized molecules. Additionally, each carbon atom in most cases has a hydroxyl group bound to it.

5.1.2 Classification of Carbohydrates

Carbohydrates are classified based on the complexity and diversity of their structures. Simple sugars, commonly referred to as monosaccharides, possess only one sugar residue, while oligosaccharides are made up of two to ten sugar residues and polysaccharides have eleven or more.

5.1.2.1 Monosaccharides

Monosaccharides are classified as aldoses (aldehyde functional group) or ketoses (ketone functional group). They are further classified according to the number of carbon atoms in the backbone, commonly designated with prefixes such as tri-(3), tetr-(4), pent-(5), hex-(6), etc. in the chemical name of the sugar. Glucose, e.g., with six carbons and ribose with five carbons are classified as hexose and pentose, respectively.

Monosaccharides are further classified stereochemically as D (dextro) and L (levo) based on the configuration of the asymmetric carbon farthest away from the carbonyl group in straight chained compounds. If the farthest hydroxyl

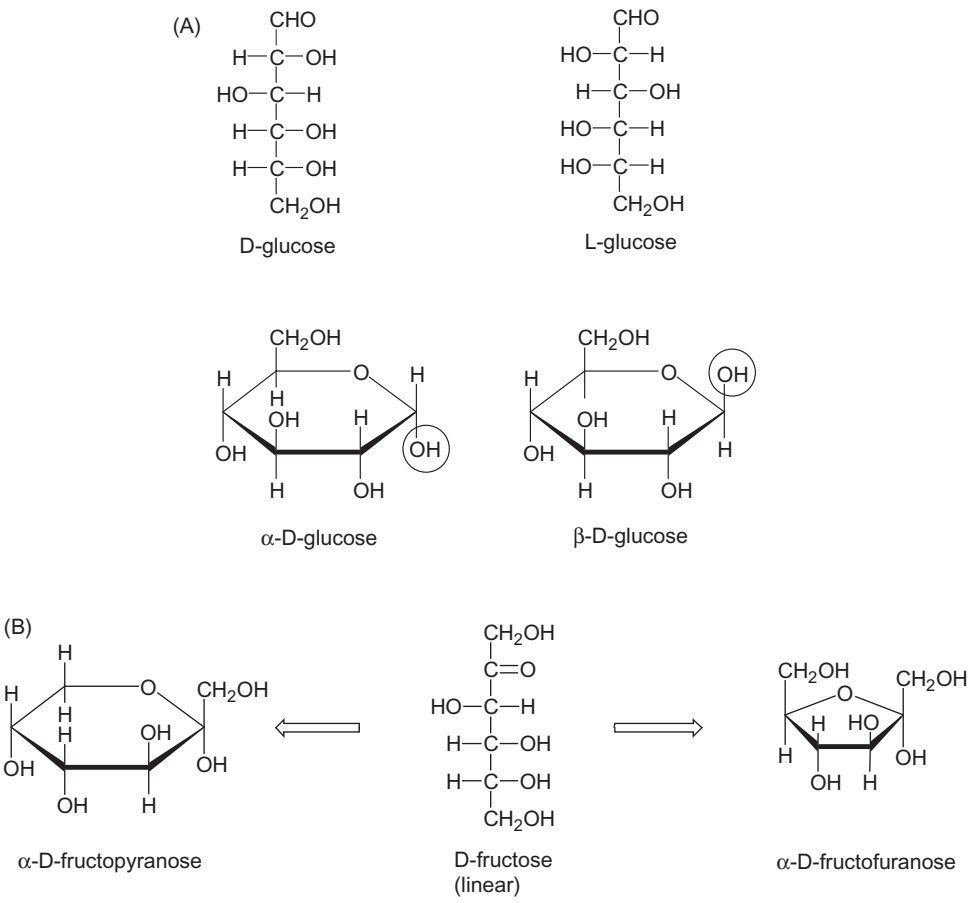

FIGURE 5.2 (A) D, L, α, and β Glucose formations. (B) Open chain and cyclic forms of fructose.

(−OH) group on the carbon atom next to the last CH_2OH is on the right as represented in the Fisher projection, it is classified as ***D*** and if on the left, classified as ***L*** (Fig. 5.2).

Aldoses such as ribose and glucose can exist in three structural forms: the open chain, the alpha (α) cyclic form, and the beta (β) cyclic form (Fig. 5.2A). Ninety-nine percent (99%) of glucose molecules exist in the cyclic form (66%β and 33%α) and 1% in the open chain form. Upon cyclization, α-D-glucose is formed if the hydroxyl group on carbon-1 is pointed in the opposite direction to the CH_2OH group in Haworth projection, while the β-D-glucose is formed if the hydroxyl group is pointed in the same direction as the CH_2OH group (Fig. 5.2B). Cyclic pentoses are referred to as furanoses, while hexoses are referred to as pyranoses. The cyclic formations are more thermodynamically stable than their open chain counterparts. Ketopentoses and ketohexoses such as ribulose and fructose can also exist in the open chain or cyclic forms. Fructose, the most common ketohexose, can cyclize to form either a furanose or pyranose ring depending on whether the C-2 keto group reacts with the hydroxyl group on C-6 or C-5 (Fig. 5.2B).

Carbohydrate Classification

Number of sugar units
Number of carbons
Functional groups
Stereochemistry
Straight chains vs. rings
Type of bonding
Structural vs. functional
Source (plant vs. animal)

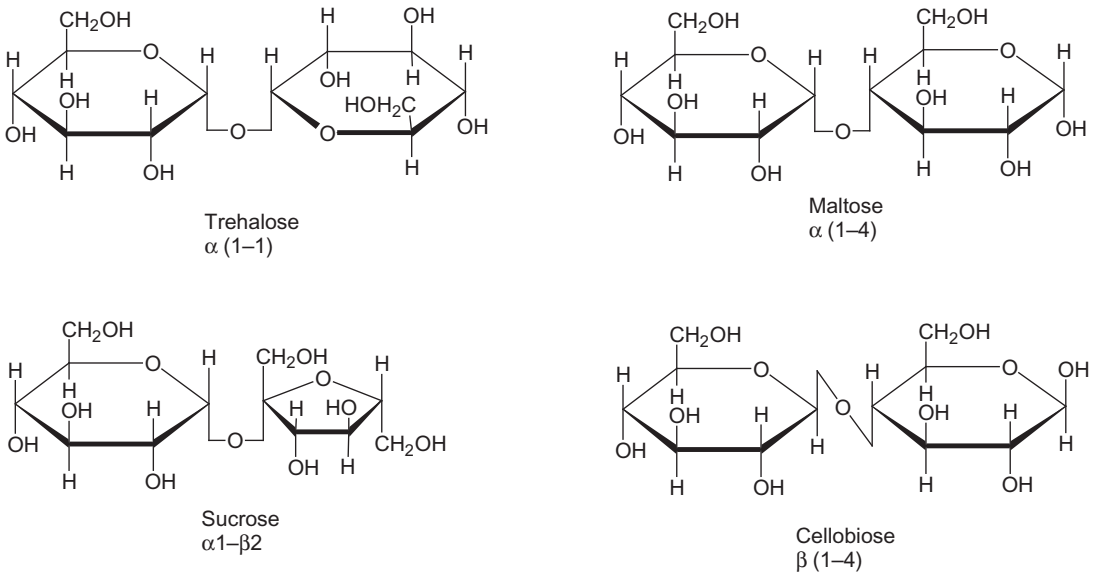

FIGURE 5.3 Structure of common disaccharides.

5.1.2.2 *Oligosaccharides*

Oligosaccharides are a class of carbohydrates possessing 2−10 monosaccharide units. The monosaccharide units may be linked via *O*-glycosidic or *N*-glycosidic bonds. *O*-glycosidic linkages may be formed from α 1−1 (trehalose), α 1−4 (maltose), α 1−β 2 (sucrose), β 1−4 (cellobiose) glycosidic bonds. The most abundant oligosaccharides are those possessing two monosaccharide residues, commonly referred to as disaccharides. These include sucrose, maltose, lactose, cellobiose, and trehalose. Sucrose, a disaccharide of glucopyranose and fructofuranose (Fig. 5.3), is the most important disaccharide in plants and is found in large amounts in crops such as sugarcane, sugar beets, and sweet sorghum [1]. Oligosaccharides are also commonly bound to lipids and amino acids by way of *O*-glycosidic and *N*-glycosidic bonds to produce glycolipids and glycoproteins.

5.1.2.3 *Polysaccharides*

Polysaccharides are high molecular weight carbohydrates which yield monosaccharides or related compounds upon hydrolysis [2]. D-Glucose, L-fructose, and L-rhamnose are the most abundant residues found in polysaccharides. Other monosaccharides such as D- and L-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid, D-glucosamine, and D-galactosamine also occur as constituent molecules in polysaccharides [2]. Chemically, polysaccharides are classified according to the nature and diversity of the constituent monosaccharides. A polysaccharide, such as starch or cellulose, which yields a single monosaccharide upon hydrolysis is classified as a homopolysaccharide. Conversely, heteropolysaccharides, such as pectins, yield a mixture of monosaccharides after hydrolysis. Functionally, polysaccharides are classified based on their biological purpose as structural or nutrient. Nutrient polysaccharides serve as metabolic reserves in plants (starch and inulin) or animals (glycogen), while structural polysaccharides form rigid protective structures in plants (cellulose, pectins) and animals (chitins).

It is important to note that polysaccharides differ from each other not only by type or diversity of the individual monosaccharide residues but also by the molecular weight of the polymer, type of chains formed (branched, linear), type of glycosidic bond (α or β), and position at which condensation occurs (1−2, 1−1, 1−4, 1−6). A typical example is starch and cellulose which are both made up of glucose units but are structurally different. Cellulose is a linear molecule formed from β (1−4) glycosidic bonds with molecular weights ranging from 200,000 to 2,000,000 Da, while starch is a branched molecule possessing α (1−4) and α (1−6) glycosidic bonds and molecular weights up to 1,000,000 Da.

5.1.2.3.1 Starch

Starch, the major carbohydrate reserve in higher plants, accounts for over 85% of the dry weight. Starches possess two primary chains, a linear portion formed from α 1−4 (amylose) glycosidic bonds and a branched region consisting of α 1−4 and α 1−6 (amylopectin) glycosidic bonds. Amylose (15−20%) is generally a minor component of most

starches while amylopectin is the major constituent accounting for 80−85% of the starch molecule [2]. Amylopectin residues are characterized by the presence of α 1−6 glycosidic bonds at every 24−30 glucose units.

Starch is found as granules (ovoid or spherical in shape) in the chloroplasts of plant leaves or in the amyloplasts of storage organs such as seeds and tubers [3]. Starch occurs as a white, soft, tasteless, and amorphous powder that is insoluble in polar solvents. The structure and functionality of starches can be readily altered through chemical, mechanical, or physical treatment. Physical treatment of natural starch by exposure to dry heat results in the formation of dextrins (dextrinization), while heating in water leads to gelatinization. During gelatinization, granules swell and burst, resulting in loss of the semicrystalline structure and leaching of amylose molecules from the granule. Starches are commonly modified chemically through acid hydrolysis, oxidation, esterification, phosphorylation, ethylation, and polarization.

Starch can be hydrolyzed by several enzymes, including α-amylase, β-amylase, and α (1−6) glucosidase to produce a mixture of glucose and maltose or completely hydrolyzed to glucose by mineral acids. As such, starches are commonly referred to as glucosans (yields only glucose residues on hydrolysis). Plant starches are a major source of digestible carbohydrates and by extension serve as a source of energy in animals.

5.1.2.3.2 Cellulose

Cellulose is the most abundant natural polymer in the biosphere. It is present in all plant systems but cannot be metabolized by animals. Cellulose, the major constituent of the cell wall, is a fibrous, tough, white insoluble solid which serves a structural function in plants. It is a linear polysaccharide formed from condensation of glucose residues yielding β (1−4) glycosidic bonds. The structure of cellulose closely resembles that of amylose, except that β-glycosidic bonds are formed as opposed to the α-glycosidic bonds in amylose. Cellulose molecules are usually larger than starch with molecular weight ranging from 200,000 to 2,000,000 Da. Cellulose is widely used as textiles (cotton and linen), excipients in pharmaceutical products, in thin layer chromatography and filtration systems, and in the corrugated industry.

5.1.2.3.3 Glycogen

Glycogen is the major carbohydrate reserve in animals. It is produced and stored in the liver cells and the muscles, and functions as secondary long-term energy storage. The structure of glycogen is similar to that of the amylopectin molecule in starch with glucose residues linked by α (1−4) and α (1−6) glycosidic bonds. Glycogen has far more branched points than amylopectin, with α (1−6) glycosidic bonds forming branch chains at every 10−20 glucose units.

Glycogen is a white amorphous powder, poorly soluble in water, and readily hydrolyzed by mineral acids to yield glucose residues. It may also be hydrolyzed by the enzyme glucoamylase (1,4-α-D-glucanmaltohydrolase) to produce a mixture of glucose and maltose.

5.1.2.3.4 Pectins, Chitins, and Xylans

Pectins are believed to be the most complex family of polysaccharides in nature. They account for 35% of primary cell wall of dicotyledons and nongraminaceous monocotyledons, 2−10% in grass, and up to 5% of woody tissues [4]. Most pectins (70%) are formed with α-1,4 glycosidic bonds following condensation of D-galacturonic acid residues. The structural classes of the pectins include homogalacturonan, rhamnogalacturonan, xylogalacturonan, and apiogalacturonan [4].

Chitin is one of the most abundant biopolymers on earth and the second most abundant natural polysaccharide, following cellulose. Chitins are found extensively in arthropods serving a structural function in the formation of exoskeletons. Chitin is a linear polymer consisting of *N*-acetylglucosamine residues joined together by β (1−4) glycosidic bonds. When hydrolyzed with mineral acids, chitin degrades to form glucosamine and acetic acid. *N*-acetylglucosamine residues are liberated when hydrolyzed by chitinase.

Xylans are a diverse group of polysaccharides formed from xylose residues linked by β-(1,4) glycosidic bonds and bearing side chains of 4-*O*-methyl glucuronic acid. The composition and distribution of the substitutions varies with plant cell species. Xylans containing arabinose residues are known as arabinoxylans and glucuronoarabinoxylans [5].

5.1.3 Carbohydrates and Medicine

Carbohydrates have been used extensively in modern medicine, either as agents for improving the delivery of active pharmaceutical ingredients to their sites of absorption or as therapeutics themselves. They have been shown to play numerous roles in binding to proteins and fats on cell surfaces aiding in cellular signaling and recognition [6,7],

the functioning of the immune system [8,9], cellular function [10], determining human blood grouping [11,12], improving and maintaining intestinal health, etc. Starch, cellulose, pectins, and their derivatives are extensively used in drug delivery systems, such as tablets and granules, to improve the delivery of therapeutic agents to their sites of absorption either in controlled or immediate release dosages. Starch, cellulose, and their derivatives are ranked among the top 10 excipients used in tablet delivery systems where they are used as disintegrants, binders, glidants, or fillers in tablets, granules, and capsule systems [13]. Some examples include delivery of acetaminophen, tramadol, alprazolam, oxycodone hydrochloride, and acetyl salicylic acid to the various sites of absorption.

Pectins have been shown to bind to cholesterol in the gastrointestinal tract of humans and retard glucose absorption by trapping carbohydrates. Consumption of pectin (at least 6 g/day) has also been shown to significantly reduce blood cholesterol [14,15]. Anticoagulant, antithrombotic, and antiviral activities have also been reported for xylan and its derivatives, primarily sulfated and phosphorylated derivatives [16].

5.2 NONESSENTIAL OILS

5.2.1 Definition

While essential oils are characterized by having a distinct and characteristic essence, nonessential oils by contrast are not characterized as such and can be any other oil or fat themselves comprised of saturated and unsaturated fatty acids. Fats tend to be solids at room temperature because of their relatively higher saturated fatty acid content compared to oils, which have more monounsaturated and polyunsaturated fatty acids. Nonessential oils can therefore technically be referred to as "plant lipids" inclusive of oils and fats. They may also be referred to as "fixed oils." Fixed oils and fats are made up of glycerol esters and complex aliphatic acids.

5.2.2 Plants Containing Nonessential Oils

Nonessential plant oils are normally extracted from various plant parts but the seeds are generally good sources of these products. Alternatively, oils not derived from seeds are called pulp oils and this group includes avocado oil and palm oil. Examples of nonessential oils include almond oil, peanut oil, olive oil, rice bran oil, sesame seed oil, canola oil, castor oil, grape seed oil, cotton seed oil, safflower oil, corn oil, soybean oil, and pumpkin seed oil [17]. Some nonessential oils exist as fats at room temperature including coconut oil, palm oil, cocoa butter, and palm kernel oil. This list is not exhaustive as numerous other plants serve as sources for other economically important nonessential oils. Plants store energy in seeds in the form of lipids, and it is well known that plant triglycerides serve as energy sources for mammals [18]. Nonessential oils also play important roles in plant protection serving as physical barriers against desiccation while also serving as signal molecules and plant hormones.

5.2.3 Extraction of Plant Oils/Lipids

Extraction with organic solvents is the preferred method. In this method, extraction of nonessential oils begins with a preliminary extraction using propan-2-ol followed by re-extraction with chloroform methanol (2:1 v/v), after which the solvent is evaporated. This method is used as opposed to the direct chloroform/methanol extraction method that results in extensive enzymatic degradation of lipids [19]. Contaminants are then removed from the resulting crude mixture with an organic wash as outlined by Folch [20]. Oilseeds may be extracted with petroleum ether but the main disadvantage is that the process is laborious. Other methods utilize the enzyme-assisted aqueous extraction method involving sonication as well as enzyme treatments [21]. Researchers have found that the efficiency of lipid extraction can be improved by enhancing conventional organic lipid extraction with an ultrasound-assisted extraction method. This method shows promise since it reduces the extraction time observed in oil extraction methods for commercial purposes [22].

Essential oils are not characterized as having a distinct essence.
Components: They are comprised of saturated and unsaturated fats.
Extraction: Solvent and most recent supercritical fluid extractions.
Bioactivities: Antimicrobial; antioxidant; anti-inflammatory; antiatherosclerosis, atrial fibrillation, and cardiovascular disease.
Nutraceuticals: Oils from walnut, flaxseed, *Nigella sativa*, *Eruca sativa* seeds, and hemp seed.

Organic methods of extraction are considered laborious and the solvents used are irritants and toxic. Researchers have developed a single-step procedure involving the same solvents but with results that are comparable to and in some cases better than traditional solvent extraction methods [23]. Other modern extraction methods aim to improve on this process further. An example of this is supercritical fluid extraction wherein organic solvent use is totally eliminated. This method utilizes supercritical fluids to extract one component from a complex matrix. The fluid generally used is carbon dioxide since it has ideal properties for the complex process, including its inert nontoxic nature along with low critical temperature (31.1°C) and moderate critical pressure of 7.39 MPa [24]. This method generates products with less chemical alterations and with less contaminants compared to what is obtained when traditional extraction methods are used.

Direct expression may also be used to extract nonessential oils from plant tissue. This is a physical extraction process wherein the plant tissue is first milled to small particles followed by a hydraulic process whereby mechanical power is used to remove the oil. Milling allows for increased surface area to facilitate removal of oils. Direct expression carried out below 27°C results in "cold pressed oil" which is highly regarded for its quality and quantity of nutritional content versus refined oils. The disadvantages are that this process is not efficient, gives low yields, and there are problems with deriving quality products consistently. Efficiency and yield may be improved by applying heat to the plant material before extraction, the resulting product being "hot pressed oil" [25]. The heating process employed during the hot method of extraction may degrade some oil components, thereby altering their antioxidant properties, hence products of hot pressing are lower in nutritional quality compared to cold press oils.

5.2.4 Bioactivity of Nonessential oils

5.2.4.1 In vitro Bioactivity

In vitro studies show that nonessential oils display a wide range of pharmacological effects including antimicrobial and antifungal properties. Nonessential oils extracted from the black cumin plant (*Nigella sativa*) displays effective antibacterial activity against *Mycobacterium avium and Mycobacterium tuberculosis, while also displaying antifungal activity against Candida albicans*, *Candida tropicalis*, and *Candida krusei* [26]. Nonessential oils extracted from *Pistacia lentiscus* L. also possess potent antimicrobial properties against *Staphylococcus aureus* and *Aspergillus niger* strains [27]. Enhanced neuronal survival by coconut oil was demonstrated in in vitro studies, indicating that coconut oil may be able to counteract neuronal deficits associated with neurodegeneration commonly seen in patients with Alzheimer's disease [28]. Other properties of this product including its antifungal activity have led to its increased use in complimentary and alternative medicine. Studies show that *Candida* isolates have greater susceptibility to coconut oil compared to conventional antifungal agents [29]. Its efficacy against *M. tuberculosis* is also well documented [30].

5.2.4.2 In vivo Activity and Clinical Trials Involving Nonessential Oils

In vivo studies highlight that nonessential oils from *Linum usitatissimum* (linseed/flaxseed) possess antimicrobial activity against five strains of bacteria including *Sta. aureus*, *Streptococcus agalactiae*, and *Escherichia coli* and was effective in treating bovine mastitis [31]. Further studies also show susceptibility of *S. aureus* and *Propionibacterium acnes* to coconut oil suggesting a role for the product in treating moderate infections of the skin [32]. Antineoplastic properties are also ascribed to nonessential oils from sesame seed, coconuts, and olives [33,34]. One proposed mechanism is the antioxidant properties of the oils, due in part to the polyunsaturated fatty acid content [35].

The efficacy of nonessential oils has been highlighted in numerous clinical trials. Evening primrose oil displays anti-inflammatory effects and may therefore be a potential targeted therapy for treatment of different types of arthritis, atopic dermatitis, etc. [36,37]. Parenteral nutrition studies with olive oil show good glycemic index control and low rates of infections in patients in intensive care units [38]. Clinical trials show that olive oil has numerous other benefits, including enhanced production of oxidized LDL, thereby reducing the probability of developing artherosclerosis, and also reduces the risk of atrial fibrillation as well as cardiovascular disease and mortality in susceptible individuals [39–41]. Nonessential oils from a number of herbs including *Ocimum sanctum* Linn., Black Seed (*N. sativa*) oil, olive oil, linseed oil (*L. usitatissimum*), etc., are widely utilized for their hepatoprotective, antidiabetic, antiasthmatic, hypocholesterolemic, antioxidant, and anticancer effects [42–45].

5.2.5 Nutraceuticals Containing Nonessential Oils

Some nutraceutical products rich in nonessential oils are important in maintaining organ integrity. Oils extracted from avocado, walnut, flaxseed, *N. sativa*, and *Eruca sativa* seeds are shown to maintain kidney integrity after a toxic insult, while prevention of liver damage has been attributed to nonessential oils extracted from olive oil and coconut oil [46–49]. Nutraceuticals containing nonessential oils have also been shown to possess cardioprotective properties while moderating the effects of dyslipidemia [50]. Consumption of hemp seed and flax seed oils are known to have positive impact on cardiovascular health [51]. Overall, research shows that protection of organ integrity and maintenance of good overall health status can be achieved by selective consumption of nutraceuticals rich in nonessential oils.

5.3 ESSENTIAL OILS

5.3.1 Definition

The term "essential oil" is misleading as these products are not essential for life. The name is derived from the mostly pleasant essences they produce when evaporated. As a result, these products are also referred to as "essences." In addition, they do not share similar structure to oils but are nonpolar and fat soluble. Essential oils can be defined as any volatile fragranced nonpolar organic plant extract that is responsible for a plants characteristic odor and they are reported to have therapeutic uses. These oils may be extracted from any part of the plant and are used in aromatherapy, yoga, etc. In plants, essential oils are normally found within impermeable granules located in glandular hair, cells, or in secretory granules [52].

5.3.2 Composition

Essential oils fall under the classification of secondary metabolites including terpenoids, shikimates, polyketides, and alkaloids. The most abundant essential oils are produced via three main pathways: mono- and diterpenes are made in the methyl-erithrytol pathway; sesquiterpenes are made via the mevalonate pathway; while phenylpropenes are produced via the shikimic acid pathway [53]. Other essential oils are continuously being discovered that may not be produced by these main pathways. Essential oils have been identified in the roots of numerous plants, e.g., *Ballota nigra*, *Valeriana jatamansi*, and *Zingiber officinale*. They are rich in sesquiterpenes and possess antimicrobial activity [54,55]. The stem of various plants are also rich in bioactive essential oils including those derived from the peteribi wood (*Cordia trichotoma*) and *Cinnamomum cassia* [56]. Important essential oils are also found in leaves of numerous plants including *Artemisia monosperma*, allspice (*Pimenta dioica*), and the eucalyptus tree (*Eucalyptus citriodora*) [57]. The flowers of various plants also serve as sources of essential oils, as seen in lavenders (*Lavandula officinalis*) and *Halimondendron halodendron*, while essential oils have been extracted from various citrus fruits [52,58–60]. Essential oils derived from seeds of various plants including *N. sativa* are also reported to have beneficial properties [61,62].

5.3.3 Preparation of Essential Oils

The preparation method depends on the characteristics of the product to be extracted. Numerous extraction procedures exist, including hydrodistillation, solvent extraction, maceration, microwave distillation, expression, controlled instantaneous decomposition, *enfleurage*, superfluid critical extraction, dynamic and static headspace techniques, etc., but only a few will be explored in this medium.

5.3.3.1 Hydrodistillation

Hydrodistillation represents a commonly used method of extracting essential oils from plant samples. This method may be further classified into the subcategories of steam distillation, water distillation, or a combination of water and steam distillation.

5.3.3.1.1 Steam Distillation

One of the most widely used methods of extracting essential oils from their sources is steam distillation, and it is one of the preferred methods due to its low cost. This is a low temperature distillation method which allows for separation

of nonvolatile, water immiscible substances at temperatures below the boiling point of individual constituents. This leads to preservation of components that may be disrupted at high temperatures. Steam distillation is utilized in extracting essential oils at temperatures near to 100°C, followed by subsequent condensation to form an immiscible liquid from which the essential oil can be separated in a clarifier. The operating principle is that the injection of steam results in the sample being heated while reducing the boiling point of individual components owing to the higher steam tension in water compared to that of individual volatile components of the sample [52]. Steam distillation is widely used and the process is easily controlled, however the startup capital tends to be higher than the other two forms of hydrodistillation.

5.3.3.1.2 Water Distillation

In water distillation the starting material is immersed in water and boiled using direct heat, steam coil, or steam jacket. This process requires the material to be kept in motion to avoid degradation of dense material that may settle to the bottom of the apparatus. Essential oil is collected along with steam then separated post condensation. The required products can be easily extracted from finely cut material or powders, which would be difficult to achieve with steam distillation as it results in formation of lumps which are partially impenetrable to steam. Another advantage of this method is the ease and relative low expense associated with setup. On the other hand, it is a slow process and there is degradation of required products via hydrolysis or polymerization of sensitive components due to prolonged heat exposure. This process is used if the other two distillation methods are unfeasible.

5.3.3.1.3 Water and Steam Distillation

Combined water and steam distillation is similar to water distillation, however the material in question is not in direct contact with water but rather placed on a solid support above the boiling water so that steam can directly pass through it. For best yields, the material should be evenly distributed so that there is efficient contact with the material by steam. Some setups increase their efficiency by addition of a cohobation tube which ensures that after condensation and separation of the essential oil, water is returned to the still to be reboiled, thereby ensuring that there is enough water in the system to ensure complete extraction. This method also ensures minimal loss of oxygenated components including phenols [63]. Compared to water distillation, this method gives greater yields, oil quality is more reproducible, and the process is quicker than water distillation.

5.3.3.2 Organic Extraction

Organic extraction results in higher yields compared to hydrodistillation hence it is heavily utilized on products that give low yields (e.g., rose plant). This process also keeps compounds intact that would otherwise be destroyed by heat or those water soluble ones that may be lost by distillation. Organic extraction setups are however more complex and costly than distillation setups. This technique also has limited application in extracting oils for consumption as some organic solvents are implicated in organ pathologies because even slight residues in the final extracted product may be toxic. Although some organic solvents are considered safe for consumption, products obtained by organic solvent extraction and supercritical fluid extraction may not be classified as essential oils although their profiles may be quite similar to the raw material from which they are derived [64].

5.3.3.3 Enfleurage

Enfleurage is an older, labor intensive, and expensive process wherein essential oils from delicate flowers, e.g., jasmine petals, are placed on glass supports impregnated with fat normally of animal origin. The perfume oils emitted are absorbed by the fat and continuous replacement of the raw material results in the fat retaining substantial amounts of the essential oil over time. At the end of this process, alcohol is used to extract the essential oil from fat followed by isolation.

5.3.3.4 Supercritical Fluid Extraction

This procedure employs lower temperatures than hydrodistillation and involves passage of pressurized carbon dioxide into specially designed chambers filled with the plant matter in question. At high temperatures the gas functions as a solvent thereby extracting the essential oil from the raw material. It is thought that products derived from supercritical CO_2 extraction are more representative of their natural states owing to maintenance of integrity of such compounds during the extraction process which is not as harsh as other methods of extraction.

Essential oils are volatile, soluble in organic solvents, and characterized as having a distinct essence.
Components: Terpenoids, shikimates, polyketides, and alkaloids.
Primary pathways: Methyl-erithrytol; mevalonate; shikimic acid pathway.
Extraction: Hydrodistillation; solvent; maceration; microwave distillation; expression; controlled instantaneous decomposition; *enfleurage*; superfluid critical; dynamic and static headspace techniques.
Bioactivities: Antimicrobial; dysbiosis; antidiabetic; antidyslipidemia; antioxidant; ameliorate menstrual cramps and dysmenorrhea; treat neurological disorders and migraines; alleviate nausea.
Primary clinical trials challenge: Standardization.
Crude drugs: Rosemary, peppermint, bay, basil, tea tree, celery seed.

5.3.4 Bioactivity of Essential Oils

5.3.4.1 In vitro Activity of Essential Oils

Bioactivity studies have largely focused on in vitro studies involving cell cultures. Studies on antimicrobial properties of essential oils are thought to have started in the 1800s where antibacterial properties of caraway oil, thyme oil, and thymol were investigated [65]. Follow-up studies over the decades have highlighted the pharmacological efficacy of a number of these products. More recent studies show that essential oils extracted from *P. dioica* (Jamaican pepper/allspice) display significant efficacy against *C. albicans* and are thought to be an effective treatment for different forms of candidiasis [66]. Essential oils from this plant also display antimicrobial properties against a wide range of gram-positive and -negative bacteria, some species of fungi, and also display anthelmintic activity [67]. Other in vitro studies have highlighted the antimicrobial properties exhibited by essential oils from numerous other plant species including thymus, citrus, lavender (*Lavandula stoechas*), cinnamon (*Cinnamomum verum*), turmeric (*Curcuma longa*), etc. [68,69]. Essential oils have been used as an effective treatment for dysbiosis, a disorder related to disruptions in gastrointestinal flora leading to altered metabolic status that often leads to other pathologies [70]. Dysbiosis is associated with numerous pathologies including irritable bowel disease arising from changes in the balance between protective versus harmful intestinal bacteria; disruptions in glucose and lipid metabolism; and breast cancers in women [71–73]. Essential oils derived from eight plant species (*Carum carvi*, *Foeniculum vulgare dulce*, *Illicium verum*, *Mentha x piperita*, *Trachyspermum copticum*, *Mentha arvensis*, *Lavandula angustifolia*, and *Citrus aurantium* var. *amara*) show efficacy in the treatment of dysbiosis [70]. Numerous other applications of essential oils in the in vitro setting exist with research continuously adding to the growing body of literature.

5.3.4.2 In vivo Activity of Essential Oils

In vivo applications of essential oils are well documented. In plant studies, essential oils are effective phytopathogenic agents, hence they may be used as alternatives to chemicals in treating fruits and vegetable in the postharvest stages [74]. This is important in light of the significant postharvest loss of food annually. Animal studies show that antidiabetic properties are attributed to essential oils derived from *Cymbopogon citrates* (lemon grass) in type II diabetic Wistar rats, while cardioprotective and nephroprotective activities are observed in essential oil derived from *Artemisia sieberi* in diabetic rats [75,76]. Essential oils derived from numerous other plants, including *Piper guineense* (black pepper), cinnamon, and *Pelargonium graveolens* (rose germanium), also possess antidiabetic activities [77,78]. Dyslipidemia is one of the main contributors to cardiovascular diseases worldwide leading to strains on health systems arising from high treatment costs. In vivo studies show that essential oils from several plant species including *Hoslundia opposita* are capable of reducing the metabolic disruptions caused by dyslipidemia [79,80]. It is thought that the beneficial effects of essential oils and their components may be attributed to their antioxidant properties. These oils are therefore included in many nutraceutical products as it is believed that they contribute to improved health. Essential oils from numerous plant species including *Cinnamomum osmophloeum*, *Thymus vulgaris*, *Syzygium aromaticum*, *C. verum*, *P. dioica*, etc. are currently utilized as nutraceuticals by humans and also find application in animal husbandry [81,82].

5.3.4.3 Clinical Trials Involving Essential Oils

Essential oils have been utilized in a variety of clinical trials. A significant portion of current clinical research has to do with the effects of essential oils used in aromatherapy on health, well-being, and general metabolism. Some of these uses are explored below. Essential oils from lavender, clary sage, and rose are effective in ameliorating the effects of

menstrual cramps and dysmenorrhea [83]. They are useful as relaxants in patients undergoing urodynamic assessments and are beneficial in treating neurological disorders inclusive of anxiety and depression [84]. In some studies, the oils are ingested, while in others the efficacies of topical applications are assessed. As with other topical applications, there may be side effects of the product including hypersensitive reactions, increased sensitivity to sunlight, etc. It is advised that consumers are made aware of the possible interactions of essential oils with other drugs/nutraceuticals being used and implement measures to minimize harm. Inhalation of lavender oil is useful in the treatment of migraines, while peppermint and lemon grass may have similar benefits [85]. Inhalation of essential oils, as commonly seen in aromatherapy, involves an initial inhalation and absorption of volatile molecules which may be converted to chemical signals. It is thought that the signals generated interface with the olfactory bulb as well as other parts of the limbic system where the resultant psychological effects are realized [86]. Essential oils derived from *Z. officinale* (ginger) are found to be useful in alleviating the symptoms associated with nausea and vomiting postoperatively, while other studies have outlined their potency against dermatophytic infections [87,88]. The main difficulty with the use of essential oils in clinical trials is that essential oils are not standardized. The concentration and potency of their components and by extension the essential oil itself varies based on prevailing conditions at the plants' locations, harvesting conditions, storage, extraction methods, etc. As such, they are still classified in some quarters as nutraceuticals as opposed to pharmaceuticals which are synthetically produced and standardized.

5.3.5 Crude Drugs Containing Essential Oils

Crude drugs are derived from any part or parts of plants and may contain one or more active ingredients. Many crude drugs (both regulated and unregulated) are in use worldwide. Research shows that antimicrobial properties of essential oils extracted from some of these drugs are attributed to phenols including terpenes and flavonoids [89]. It is however not far-fetched to ascribe these beneficial properties to other essential oil components. Crude drugs derived from rosemary, peppermint, bay, basil, tea tree, celery seed, and fennel are rich in essential oils with reported efficacy against numerous species of fungi and bacteria [90]. In addition to benefits to humans, essential oils in crude drugs are shown to be useful in plant protection with phytopathogen activity reported in varied crude drugs even after plant pathogens have developed resistance to conventional antibacterial agents [91]. The medicinal herb *Salvia officinalis* L is known to possess antibacterial activity attributed to 1, 8-cineol which is present in the essential oil extract [92]. Other studies have isolated essential oils as active ingredients in crude drugs that are commonly used to treat a wide variety of ailments.

5.4 RESINS AND BALSAMS

5.4.1 Resins

Resins are metabolic by-products of plant tissues, containing a mixture of volatile and nonvolatile terpenoid and/or phenolic secondary compounds and fatty substances, which exude naturally from plants (surface resins) or can be obtained by incision or infection (internal resins) [93]. A resin, known as lac, is also produced by a number of species of lac insects. The species of lac insects that is of most commercial importance is the Indian *Laccifer lacca* [94].

5.4.1.1 Properties

Resins play no apparent role in the primary or fundamental physiology of the plant. They are chemically stable and inert, hence they are not readily attacked by acids or alkalis. Resins are insoluble in water and inorganic liquids but exhibit solubility in organic solvents [95]. They have an amorphous structure (rarely crystalline), become sticky when heated, often at comparatively low temperatures and have no sharp melting points. When ignited, resins burn with a smoky flame. They have attractive properties such as adhesion and glassiness.

Resins are usually produced in specialized surface glands (glandular hairs) or internal ducts which are produced by both woody and nonwoody plants. They are more common in gymnosperms and dicotyledons than in monocotyledons [96]. Upon exposure to air, resins tend to harden and serve many purposes inclusive of sealing wounds and protecting plants from diseases and attacks from insects and microorganisms [97].

5.4.1.2 Resin Constituents

Resins can be classified as terpenoid or phenolic resins based on their constituents. Terpenoid resins contain terpenoid compounds such as the 10-carbon monoterpenes, sesquiterpenes (C15), diterpenes (C20), and triterpenes (C30) [93].

Phenolic resins contain compounds such as cinnamic acid, a simple C9 phenolic compound also referred to as phenylpropane, lignans, common dimeric phenylpropanes, and flavonoids [93]. The flavonoids are among the most bioactive groups and exist as water soluble glycosides or lipophilic aglycones. Di- and tri-terpenes are nonvolatile components of resins, while mono- and sesquiterpenes are found in the volatile fraction in most plant resins [98–100]. Conifers only produce internally secreted terpenoid resins, whereas angiosperms produce both terpenoid and phenolic resins, which may be secreted internally or on the surface of plants. Classification of resins is also aided by the fact that diterpenoids and triterpenoids are not found together in the same resins.

5.4.1.3 Synthesis of Resins

Resins occur predominantly in woody seed plants. They can be preformed and stored in secretory structures or spontaneously induced at the site of an injury.

Secondary compounds present in resins are derived from photosynthetically produced carbohydrates [93]. These carbohydrates are broken down to simpler compounds from which terpenoid and phenolic resins are formed. The metabolic pathways involved in the synthesis of the resins from the simple carbohydrates include the shikimic acid, phenylpropanoid, and malonic acid pathways, which are involved in phenolic resins synthesis, and the mevalonic acid and deoxyxylulose 5-phosphate pathways, which are responsible for the formation of terpenoids resins.

5.4.1.4 Types of Resins

Several different types of resins exist including oleoresins, balsams, varnish, and lacquer resins, and miscellaneous resins. Miscellaneous resins are resins that do not easily fit into the categories of the other resins. Oleoresins are relatively fluid terpenoid resins which contain a wide variation in the amount of volatile compounds. The volatile fraction consists of mono- and/or sesquiterpenes while the nonvolatile fraction, in conifers and most angiosperms, primarily contains diterpenoids [101]. Triterpenoids are primary in the Dipterocarpaceae [102].

Varnish resins such as dammar, sandarac, mastic, acaroid, and hard copal contain both volatile and nonvolatile terpenoids. The nonvolatile components are important because they form hard finishes. Lacquer is the name given to liquid resins applied directly to surfaces without the use of solvents. Resins categorized as miscellaneous resins have been used medicinally, as flavoring (*Humulus*), perfume fixative (*Cistus*), rubber substitute (*Parthenium*), or fuel (*Euphorbia*). Miscellaneous resins include umbelliferous resins (from plants of the *Dorema* and *Ferula*genera), convolvulaceous resins (in tuberous roots), hashish (marijuana plant), and hops resins (in plants of the *Humulus* genus), propolis, the allergenic anacard resins (plants of the *Rhus* genus), labdanum, and dragon's blood [93]. Some substances are confused or intermixed with resins. Gums for example are often confused with resins, the reverse of which is also true. Other substances confused with resins include mucilages, oils (fats), waxes, and latex.

Resins are amorphous by products of plant tissues
Components: Terpenoids; phenolics
Types: Oleoresins, balsams, varnish and lacquer resins and miscellaneous resins
Extraction: Plant wounding followed by steam distillation; kraft pulping
Bioactivities: Wound healing; cytotoxic;antimicrobial; spasmolytic properties; treat cough, ulceration, and genitourinary disorders; anti-inflammatory; hyperlipidemia; analgesic

5.4.1.5 Extraction of Resins

Resins can be obtained from trees or woody plants by a process known as tapping. This involves the wounding of the plant in the area where secretory tissue is located to ensure sustained yields and tree health maintenance. Extraction, through steam distillation, is then used to produce wood turpentine and wood rosin, when resin is obtained from tree stumps, and gum turpentine and gum rosin, when resin is from living trees. Resins are also extracted as by-products of the kraft pulping process in paper production. The by-product is referred to as sulfate wood turpentine.

5.4.2 Balsams

Balsams are not as fluid as oleoresins but are relatively soft and initially malleable which enables them to be used as an ointment in wound healing. Balsams consist primarily of cinnamic and benzoic acids and are therefore considered to be a phenolic resin [103]. The volatile fraction tends to be fragranced and is therefore used in perfumery and cosmetics,

and burned as incense. True balsams include the conifer balsams, leguminous balsams, storax, styrax, elemis, frankincense, and myrrh. Storax is a well-known balsam that has a significant phenolic components as well as terpenoid constituents [104,105]. The three triterpenoids acids identified in storax are oleanonic, 3-epioleanolic acids, and ambronovic acids [105]. Most elemis contain large quantities of the triterpenes α- and β-amyrin [106].

5.4.3 Medicinal Uses of Resins and Balsams

Resins from some plants have been reported to exhibit various medicinal properties. Oleo-resins from chirp, pine, etc., found in areas such as the Himalayas and Afghanistan, have been found to possess wound healing, cytotoxic, antibacterial, antifungal, and spasmolytic properties [107]. The plant also shows beneficial effects in the treatment of cough, ulceration, and genitourinary disorders [107]. It is also used in folklore medicine to treat inflammations, asthma, chronic bronchitis, piles, diseases of the liver and spleen, urinary discharges, toothache, tuberculosis, scabies, and epilepsy [107].

Balsams have been used in the treatment of a wide array of ailments including asthma, catarrh, rheumatism, hemorrhoidal pain, ulcers, bronchitis, laryngitis, and diarrhea [108]. Frankincense and myrrh, both classified as medicinal balsams, have been exploited for the treatment of rheumatoid arthritis and hyperlipidemia, as well as their anti-inflammatory, antibacterial, analgesic, antifungal, antiparasitic, and anticancer activities [109].

5.5 PLANT HORMONES AND GROWTH FACTORS

5.5.1 Definition

Plant hormones (phytohormones) are chemicals produced by plants that regulate their growth, development, reproductive processes, longevity, and even death. These small molecules are derived from secondary metabolism and are responsible for the adaptation of plants to environmental stimuli. Plants are subjected to an ever changing environment and require these phytohormones for appropriate responses. A single phytohormone can regulate many cellular and developmental processes, while at the same time multiple hormones often influence a single process.

5.5.2 Classification and Applications

The six major phytohormones identified are auxins, abscisic acid, cytokinins, ethylene gibberellin, and brassinosteroids. More recent additions to that list include the jasmonates and strigolactones.

Auxins function primarily in stem elongation by promoting cell growth. Indole-3-acetic acid (IAA) is the major naturally occurring auxin and one of the major growth factors in plants. They were the first group of plant growth hormones discovered. Auxins serve dual roles in plants depending on where they are produced. When produced by apical buds, they promote root growth and development but inhibit lateral bud growth thereby maintaining apical dominance [110]. They also stimulate ethylene production, cell division, and differentiation [111,112]. Auxins have been employed for rooting purposes in tissue culture and in stem cuttings [113]. As such, they have gained widespread commercial use in nurseries and in farming. Synthetic auxins have been utilized in greenhouses to promote fruit development and in preventing preharvest dropping of fruits, such as oranges, hence in the coordination of harvesting seasons [114].

Plant hormones regulate plant growth, development, reproductive processes, longevity, and death.
Six main types: Auxins, abscisic acid, cytokinins, ethylene gibberellin, and brassinosteroids.
Recent additions: Jasmonates and strigolactones.

Abscisic acid is a ubiquitous plant hormone which plays an important role in the inhibition of seed germination and budding. It is known as the plant stress hormone and is involved in the response of plants to weather stress, such as tolerance to cold and drought. Plant abscisic acid content tends to increase in stressful conditions resulting in the stimulation of various physiological processes which increase the plant's ability to cope [115]. Other regulatory functions of abscisic acid include embryo maturation, cell division and elongation, and floral induction [116]. Exogenous abscisic acid is currently employed in agriculture to delay wilting and allow plant survival during short periods of severe drought.

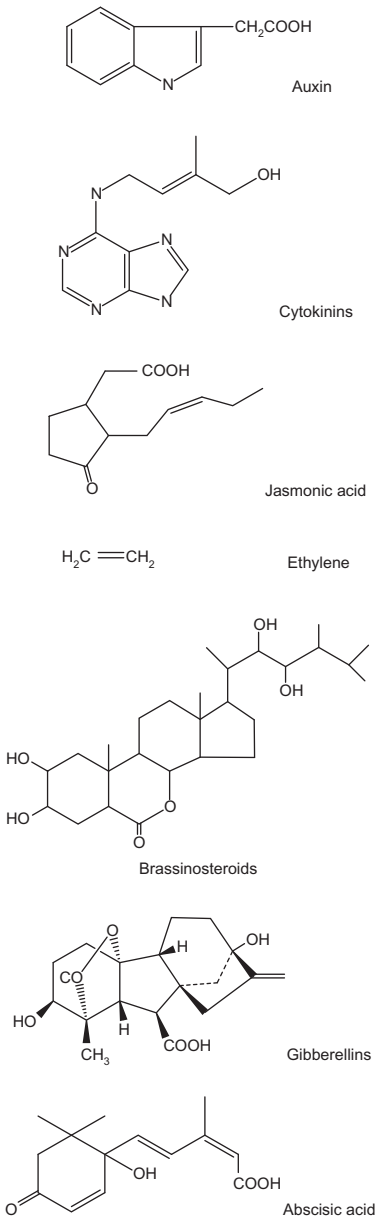

FIGURE 5.4 Molecular structures of varying plant hormones and growth factors.

Cytokinins regulate immunity in plants by modulating salicylic acid signaling and play a pivotal role in defense against pathogens and insects [117,118]. They also promote cell division and increase tolerance to drought stress [118]. Cytokinins are concentrated in root tips, the apical meristem region of the plant and in immature leaves and seeds [119,120]. The root tips are believed to be the major site of cytokinin synthesis. Environmental factors which affect the level of cytokinin production include both nutrient and water availability. Cytokinins are utilized in tissue culture to stimulate cell division, adventitious shoot formation, and embryogenesis [121]. Due to the ability of cytokinins to reduce the sensitivity to ethylene, they have also been used in the preservation of plants with high ethylene sensitivity [122] (Fig. 5.4).

Ethylene brings about various changes to developing plants. These include a thickening of the subapical portion of the stem and reduction in the rate of its elongation. The gas also enhances fruit ripening processes. The main cause of inhibition of stem elongation is cessation or retardation of mitosis in meristematic regions. Ethylene also promotes cell growth or expansion, regulates the stages of flower formation, and sex expression. The synthesis of the gas by plants requires the presence of both auxins and red light. The rate of production of ethylene is directly proportional to the concentration of IAA [123,124]. Ethylene can be utilized in agriculture to promote faster and more uniform postharvest ripening.

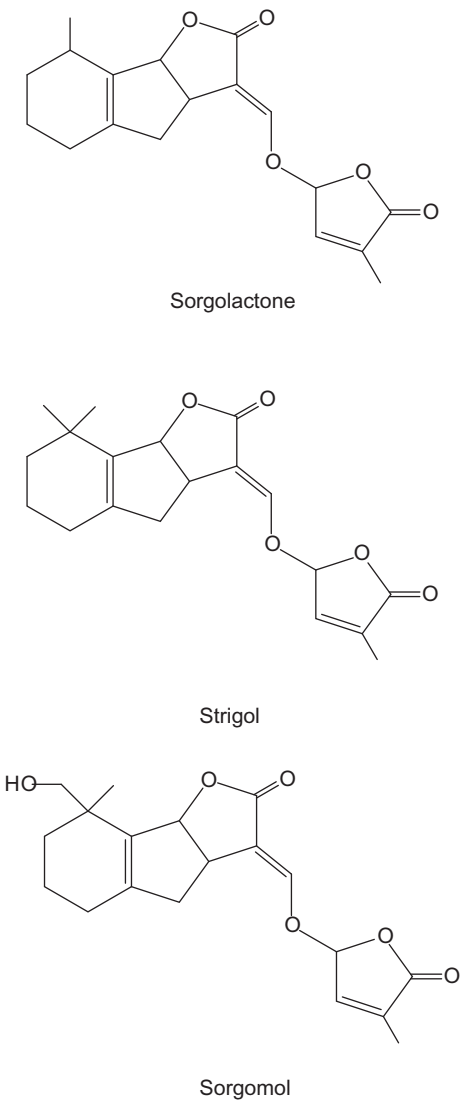

FIGURE 5.5 Depicting molecular diversity of strigolactones.

Gibberellins promote growth in different ways. These hormones are mainly involved in controlling and promoting stem elongation, flowering, and leaf expansion as well as seed germination. They are used in suspension cultures to enhance the growth of cells. These hormones also increase the activity of enzymes such as α-amylase, proteinase ribonuclease, β-glucanase, and pentosanases [125]. There is also some evidence which suggests that gibberellins influence synthesis of nucleic acids [125]. Commercial uses include the formulation of gibberellin containing preservatives for the prevention of postharvest leaf yellowing in monocotyledonous cut flowers.

Strigolactones are signaling compounds which serve as endogenous hormones involved in the control of plant development and as components of root exudates which promote symbiotic interactions between plants and soil microbes. In parasitic plants, these compounds also promote the germination of seeds once in close proximity to the roots of a suitable host plant. Strigolactones prevent the generation of secondary shoots and promote the formation of lateral roots and root hairs [126]. This enables the plant to effectively utilize the minerals available in the surrounding soil. This is especially important in situations of soil nutrient limitations (Fig. 5.5).

Brassinosteroids (BRs) are a group of naturally occurring polyhydroxy steroidal hormone isolated from plants. They have been identified in many plants including monocots, dicots, and gymnosperms [127]. Brassinolide is the most abundant and bioactive form of BRs. It regulates genes involved in the synthesis of bassinosteroids as well as cell wall loosening during brrassinolide-induced growth responses. BRs work in synergy with auxins and additively with gibberellins to promote growth in plants by causing cell elongation and division [128]. These plant hormones also stimulate ethylene

synthesis which appears to be the cause of root elongation inhibition [128]. Other physiological processes affected by these hormones include photosynthesis, stress response, and senescence.

5.6 SUMMARY

Plant constituents serve many functions, in both plants and animals. They play various structural and protective roles (cellulose and pectins in plants and chitins inarthropods) as well as serve as energy sources and metabolic reserves (polysaccharides, e.g., starch and cellulose). Plant hormones regulate various physiological processes of plants such as photosynthesis, growth, stress response, reproduction, longevity, and senescence. Constituents such as nonessential oils and resins play major roles in plant protection by serving as physical barriers against desiccation and in sealing wounds as a defense mechanism against diseases and attacks from insects and microorganisms. Medicinal uses of these plant biomolecules are extensive. They have been shown to display antimicrobial and anti-inflammatory properties as well asaiding in immune system functioningand cellular signaling and recognition. Plant constituents displaying evidence of cardioprotective properties include the pectins, nonessential, and essential oils. Overall, research points to the fact that these plant constituents can help to maintain organ integrity as well as good overall health. Commercial applications of these constituents include furniture production, flavoring, perfume fixative, rubber substitute, fuels, and in textile production.

5.7 QUESTIONS

1. How are carbohydrates classified and provide examples of carbohydrates that fall in each category?
2. Give examples of some important polysaccharides and highlight their uses.
3. List some economically important essential and nonessential oils.
4. How have essential and nonessential oils impacted overall health and well-being?
5. Highlight health benefits and economic benefits of resins and balsams.
6. Give an overview of the main classifications of plant hormones and highlight their uses.

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Chapter 6

Plant Crude Drugs

D.K. Daley[1,2]
[1]University of Technology, Jamaica, Kingston, Jamaica, [2]Biotech R&D Institute, Kingston, Jamaica

Chapter Outline

Teaching Objectives

- Define plant crude drugs and their different classifications.
- Medicinal plants and their therapeutic value.
- Production of plant crude drugs and herbarium specimen preparation.
- Extraction techniques used to obtain plant crude drugs and their active components.

6.1 BACKGROUND

Plant-based medicine and traditional healing has formed the basis for many drug developments today. Currently, approximately four billion people still practice the use of plant-based medicine for the healing of various ailments, especially in developing countries [1]. The use of plants as crude drugs plays a pivotal role in the discovery of new phytotherapeutic compounds. Plants have a universal role in the treatment of diseases in the major systems of medicine [2]. The folkloric use of plants can date back to prehistoric times, e.g., the Mesopotamian times, where the healing properties of plants were taught from generation to generation, along with the time of collection, preparation method, and the therapeutic property of the plant being used. This happened in tropical Africa, the Americas, and the Pacific nations by many indigenous groups. For example, a parent or grandparent would recommend a decoction of ginger for gastrointestinal disorders, or a garlic preparation to aid in lowering the blood pressure, while other cultures will use these same plants for different remedies. This folklore medicine information subsequently created the template for the development or isolation of over 12,000 bioactive phytochemicals, of which 122 are still being used in allopathic medicine today [3–6].

Plant crude drugs are unmodified preparations of plant material with medicinal properties.

As previously described in Chapter 1, Background to Pharmacognosy, the Egyptian Ebers Papyrus indicates several medicinal plants, their preparations, and therapeutic uses documented. The Assyrians and the Greeks also have ancient documentations of plant-based preparations used for medicine [7,8]. The use of botanical and traditional medicine is still being used today in third world countries and rural communities, as traditional herbal healing accounts for approximately 80% of the primary healthcare for many medical systems [9]. Plant-based medicines have been introduced into various alternative medical systems, such as the Traditional Chinese Medicine and the Ayurvedic Medicine by the Chinese and Indians, respectively [2,10]. These systems have been in existence for approximately 5000 years, and are still utilized as the main health care in many of these communities.

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Many ethnic groups and alternative medical systems, such as Traditional Chinese Medicine and Ayurveda, use herbalism and plant crude drugs in their medicinal remedies.

6.2 CLASSIFICATION OF CRUDE DRUGS

Crude drugs are unmodified natural preparations of plants, animals, fungi, bacteria, or minerals that are used for the prevention or treatment of an ailment or disease [11]. Within traditional medicine, the traditional pharmacopoeias list the officially used crude drugs, where approximately 85% are obtained from plants with 15% being shared in a 2:1 ratio for mineral and animal substances, respectively [12]. Crude drugs may be listed in the pharmacopoeia and categorized using different methods, such as morphological, taxonomical, alphabetical, therapeutic activity, or based on the active chemicals found within them. The alphabetical classification involves grouping the crude drug according to their alphabetical order, e.g., *Allium sativum* (garlic) would follow *Allium cepa* (onion) if categorized by their binominal Latin names, but would be the reverse based on their English names. Crude drugs and their active constituents may also be grouped based on their pharmaceutical names, such as paclitaxel for taxol (isolated compound), which will also have other generic names. The taxonomic classification is based on the Linnaean system developed by Carl Linnaeus, a Swedish botanist and zoologist [12]. He was instrumental in building the modern classification system as he traveled throughout Europe grouping plants and animals from the largest kingdom to the smallest species. These organisms were grouped based on similar physical characteristics and were ranked accordingly.

With the increased prevalence of technology in science, organisms, including plants, were then classified based on their phylogenetic profile as a method within plant systematics such as molecular phylogeny or deoxyribonucleic acid (DNA) barcoding [13,14]. The DNA is obtained from a crude extraction of the plant material and various polymerase chain reaction (PCR) amplification techniques are used to multiply the genome and identify the different codons (amino acid template from DNA). The sequenced DNA is represented as the respective nucleic acids which is printed as a barcode and placed within the Barcode of Life Data systems (BOLD) database and may be used to identify unknown species and subsequent relations as the arrangement of the DNA and subsequent proteins produced allows for the grouping of plants based on these similarities, which has been done for various animals thus far [10,15,16].

Therapeutic activity is also an effective way of classifying crude drugs. This involves the medicinal use of the plant crude drug based on its active component and its efficacy as a treatment for a particular ailment. Some examples include those used as purgatives, such as *Azadirachta indica* (neem), *Cassia acutifolia* (senna), *Cascara sagrada* (cascara), *Smilax sarsaparilla* (sarsaparilla root) [17], those used as analgesics (pain-killers), such as *Papaver somniferum* (opium poppy), *Salix* spp. (willow tree), *Ocimum suave* (ocimum), among others [18], and those with anticancer activities, such as *Catharanthus roseus*, commonly called periwinkle, for vinblastine and vincristine, or *Taxol* spp. for paclitaxel and other anticancer components [19,20] as shown in Fig. 6.1. This classification would also include plant crude drugs grouped based on the major component, such as glycosides (anthraquinone or cardiac), tannins, carbohydrates, alkaloids, phenolics, fixed fats, and proteins.

Plant crude drugs preparation should follow the guidelines of the pharmacopoeia, as it outlines all the information required for quality control and effective products.

Plant crude drugs may be organized or unorganized, and thus may be classified based on morphology. Those preparations made from the entire organ of the plant, where it contains a specific plant tissue, and used for treatment are referred to as organized crude drugs, e.g., leaves, roots, flowers, or seeds used to treat a particular ailment (Table 6.1). Unorganized crude drugs, as the name suggests, are those preparations from undifferentiated and differentiated plant parts, i.e., no specific organs were used in the preparation, e.g., balsams, resins, volatile oils, honey.

Plant crude drugs have various classes of active compounds, such as primary metabolites—proteins, carbohydrates and fats—and secondary metabolites—alkaloids, glycosides, terpenes—among others. Therefore, specific extraction methods may be employed to obtain the active constituents.

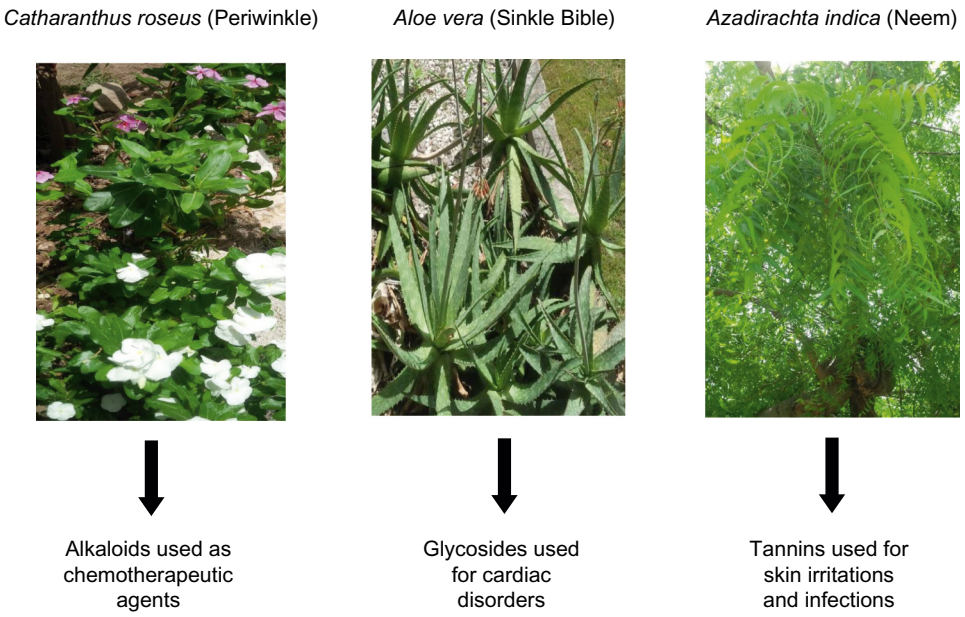

FIGURE 6.1 Different plant metabolites obtained from medicinal plants.

TABLE 6.1 Organized Plant Crude Drugs

Medicinal Plants	Plant Part (Organ) Used
Rhubarb, garlic, ginger, aconite, colchicum (corm), turmeric	Roots
Belladonna, peppermint, fever (lemon) grass, digitalis, and many others	Leaves
Cinchona, cassia, cascara	Bark/wood
Strychnos nux vomica, black and white mustard, colchicum	Seeds
Mangosteen, noni	Fruits
Clove, chamomile, saffron, yarrow, lavender	Flower
Cotton, silk, flax, hemp	Fiber

6.3 PLANT CULTIVATION AND COLLECTION

In the preparation of crude drugs, proper cultivation is imperative prior to collection of the raw material to be used, and is the most important step toward quality products. The cultivation and collection of the medicinal plant material is recorded in the various pharmacopoeias for crude drug preparations [21]. The wild species of medicinal plants have continuously been used as the source of crude drug production, as such cultivation systems are being used to maintain genetic diversity [22]. Cultivation technology incorporates the use of extensive methods of obtaining consistency in soil nutrient content, irrigation, gene modification, temperature control, fertilizer use, and other horticultural techniques to increase plant yield, phytochemical content, or therapeutic potency. Natural, artificial, and seed propagation are options used for the cultivation of desirable medicinal plants as vegetative organs (roots, stems, leaves, seeds) and are used to produce new offspring. Natural propagation involves the regeneration of new plantlets from runners, stolons, bulbs, or leaves. Cuttings, grafting, layering, and tissue culture are artificial propagation techniques and are often used to manage cultivation and growth of medicinal plants that are used for crude drugs [23]. These cultivation methods utilize good agricultural practices (GAP), this allows for the tracking of each plant from a seed or parent plant to the final product and ensures quality, quantity of the therapeutic phytochemicals, and reproducibility of the plant crude drug, unlike the use of the plants from the wild. The cultivation and usage of plants in phytotherapy within modern and alternative medicines leads to more ecoconservation strategies being employed. These strategies aim at ecosustainability in order to

prevent exploitation of a country's endemic and indigenous species. The implementation of *in vitro*, *in situ*, and *ex situ* plant protection strategies are amongst some of the methods used for conservation and sustainability [22,24–26]. These include:

- *In vitro* conservation has significantly reduced the risk of extinction for many species as germplasm or seeds are collected and stored. Tissue culture and other techniques are used to ensure continuity of the species, including medicinal plants.
- The protection and management of plants in their natural habitat that are considered high conservation locations, such as forests (*in situ*). This prevents unsupervised removal of medicinal plants for farming, housing, furniture, and other resources.
- *Ex situ* conservation involves plants being replanted in a controlled area (garden or greenhouse) for increased survival and sustainability of the species and is often done for those plants that are said to be endangered species.

There are several factors to consider before the plant material is collected. These include the weather conditions, the time of day, season, and the plant organ with the therapeutic value. These will evidently affect the active components present in the plant tissue as structural and chemical changes occur throughout the life of a plant. As a result, before a crude drug is prepared, these must be noted and then recorded for each plant batch (GAP) to ensure efficacy and quality control of the plant crude drug, also utilizing good manufacturing practices (GMP) for product tracking and reproducibility. There are other methods used in the assessment of plant-based products, such as microscopy and chemical analysis; they are also effective in the stabilization and standardization of plant crude drugs [27].

The time of day—some plants will produce compounds based on whether it is night, day or anywhere in between. The active components may be in higher proportions or present in their active form at certain times of the day. The *Ipomea* species (morning glory), for example *I. tricolor*, *I. purpurea*, and *I. violacea* which are best collected in the mornings or on a cloudy day [28]; these seeds are used as recreational drugs, as they contain an active tryptamine, lysergic acid amide (LSA/LSD), and other lysergamides [28]. Although the seeds have a psychoactive effect, the plant has significant medicinal properties, such as laxatives, diuretic, and as an expectorant for coughs. The ergot alkaloids, formed from the fungal endophyte, *Claviceps purpurea*, on rye wheat have been used in medicine and traditionally for uterine contractions, and also have a hallucinogenic effect due to their derivatives [29].

The time of year—the different seasons will affect the activity of the plant crude drug as the quantity and chemical structure of constituents may vary. For example, *Rheum rhabarbarum* (*Rheum officinale*), commonly called rhubarb, is harvested during the summer for its anthraquinone glycosides which are used as purgatives. Outside of this time period the plant's metabolome changes and thus different compounds may be extracted, e.g., during the winter, anthranol may be extracted and have an entirely different therapeutic effect, as anthranol is used to treat psoriasis and other dermatoses. Another medicinal plant, *Colchicum autumnale*, is used to treat gout and arthritis when the corm is collected during the spring when colchicine alkaloid may be collected.

Active plant parts—this refers to the morphology of the plant and the organ harvested for its medicinal properties. For example, *Zingiber officinale* (ginger), the rhizome is collected and used as a carminative, for gastrointestinal disorders, and high blood pressure. The hypercholesterolemia and hypoglycemic agent, 6-gingerol from ginger exhibits its effects at approximate concentrations of 60.44 ± 2.53 mg/g of the methanolic ginger extract [30,31]. *Allium sativum* (garlic) also has activity in its underground cloves. Underground plant parts are best collected when the aerial portions of the plant have fully matured and begin to dry and fall. Leaves are also used for their medicinal properties; the active constituents within leaves are best collected when the plant is flowering and as such, the leaves are fully mature with its full complement of phytochemicals [32,33]. For example, *Eucalyptus* species are used as an expectorant, hypoglycemic, and hypotensive agent, the medicinal properties are found in the slender blue-green leaves [34,35]. The leaves and fruit of *Morinda citrifolia* (noni) also contain many therapeutic compounds used as an antibiotic, antioxidant, anticancer, and for their wound-healing properties [36,37]. The metabolome of a plant will vary with the stage of development and thus, this will play a significant role when being harvested for medicine [32]. The pharmacopoeia, traditional medicine reviews and research papers, and farmers' agricultural guide books also have this information documented for many known medicinal plants and crops that are used as plant crude drugs.

Crude drugs made from medicinal fruits and seeds should be prepared from those collected when they are fully mature [33]. For example, *Garcinia mangostana* (mangosteen) is used to treat urinary tract infections, diarrhea, and a host of other ailments, due to its high antioxidant capacity [38,39]. Medicinal flowers should be collected during the dry times when it has fully bloomed in most cases. Some medicinal flowers include *Matricaria chamomilla* (chamomile) used to treat rheumatism, skin diseases, and to boost the immune system, gastrointestinal disorder, and inflammation [40]. *Calendula officinalis* (marigold) is commonly called poor man's saffron, as it is used to add color to rice or

curry dishes, but this flower also possesses antioxidant, antibacterial, anti-inflammatory properties, and aids in skin disorders [41–43]. Crude drugs obtained from the bark of deciduous trees are best collected during the spring or fall, when the plant sap is active [32]. However, there is no general rule *per se* as some trees may be harvested during other times of the year, as long as it is easily separated from the cambium. The alkaloid, quinine, used as an analgesic, antipyretic and as an antimalarial drug, may be extracted from *Cinchona officinalis* or *Cinchona ledgeriana* and more recently from the *Remijia peruviana* bark and these are best collected during the springtime when separation from the trunk is easiest [32,44]. The weather can also influence the collection time and type of drug preparation, in that a dry season is best for the collection of unorganized drugs, such as resins, balsams among others, as the quality would not be compromised as can happen with rainy weather. Once the plant material is collected, postharvest treatment will ensure quality products using GMP. The plant material is usually dried to remove excess water and prevent contamination by mold and bacteria, grinding is done before crude drugs are prepared as this increases the surface area for the release of the active component from the plant material. The plant material may then be processed or crude extractions may be done.

Good agricultural practices (GAP) and good manufacturing practices (GMP) will ensure consistency and quality control of the plant crude drug. Quality control refers to the procedures that ensure the standards of the product are maintained with each batch.

6.4 HERBARIUM SPECIMEN PREPARATION AND SIGNIFICANCE

Carbon dating has opened the gates to the history of plants on earth and their existence and has been used extensively in obtaining the age of many plants being preserved in a plant museum, also known as a herbarium. Preservation of plant material, especially medicinal plants with significant therapeutic properties, is important for the survival of species identity and thus methods can be put in place to preserve these species that may go extinct. Herbaria may also be used in biological and medicinal research, and knowledge of the flora that existed during a particular time. Herbaria are often used for plant sample observations after more than 10 years of preparation, from which valuable information can still be obtained [45]. This is due to the wealth of information that may be recorded when the plant material is collected, such as location, the habitat's biotic and abiotic factors, along with description of the plant at collection (flowers, bark, seeds, leaves, height). The scientific, family, and common names may also be included along with the date of collection and the name of the collector. Other optional information that may be added includes the classification of the specimen and with increasing use of technology, the direction to the location of the plant may be noted using the latitude and longitude recorded using global positioning system (GPS) technology. There are approximately 4000 herbaria covering 165 countries around the world and these include xylarium, fungarium, and hortorium for the collection of wood, fungi, and cultivated plants, respectively. The five largest herbaria are the National Museum of Natural History (housing 9.5 million species), New York Botanical Garden, (housing 7.2 million species), Komarov Botanical Institute (housing 7.2 million species), Royal Botanic Gardens (housing 7 million species), and the Conservatory and Botanical Garden City of Geneva (housing 6 million species) from France, USA, Russia, England, and Switzerland respectively. The oldest herbarium, developed in 1891, is the University of Florida Herbarium which houses roughly 470,000 specimens.

The materials needed to prepare a sample for preservation (herbarium) include: herbarium mounting paper, label, and tape. Prior to mounting the sample, the plant material needs to be flattened as best as possible, opening leaves to show the full lamina and blade, and dried using a plant press and paper to aid in stacking numerous plants and also in the removal of excess moisture. This may be oven dried at low temperature between 30°C and 40°C based on the thickness of the bark, or sun-dried based on delicacy of the plant material. The pressed-dried plant specimen can then be attached onto the herbarium mounting paper using herbarium tape/adhesive strips to ensure the specimen is securely fastened. The adhesive label with the plant names (scientific and common) and all the collection details should be placed at the bottom right of the specimen. The herbarium sample may then be filed in ranks according to the family, genus, species, variety, and cultivar, respectively, as is necessary comparing to existing samples; and stored in a dry area away from possible infestation damage.

6.5 PLANT CRUDE DRUG EXTRACTION AND PRODUCTION

After the plant is collected according to the guidelines outlined by the pharmacopoeia, the material would then be verified using key characteristics of the fruit, flower, leaves, stem, and growth habitat and a sample specimen placed in the herbarium within the identified classification family. The plant material may then be dried using either low temperature

oven at about 30−40°C, air dried for approximately 3−4 days or until dry, or via lyophilization. Over 50% of clinical drugs and pharmaceuticals being used today were first identified from traditional medicine and medicinal plants use [9]. The use of plants, microorganisms, and minerals have enhanced the pharmaceutical field as the isolation of novel compounds in the early 19th century has formed the basis for drug development. This is due to the overwhelming amount of scientific research that has been done to obtain pure compounds from plant crude drugs for various activities [7,18]. These isolated compounds are called derived drugs. They have formed the foundation for the discovery of novel structures due to increased technology for the identification and elucidation of isolated active components in plants. These novel structures and derivatives are also used as templates for the development of new drugs with improved efficacy or with new biological activities, thus the development of synthesized drugs [18]. The derived drug quinine was among the first crude drug isolations done and played a significant role in laboratory drug synthesis. Quinine was extracted from *Cinchona officinalis* (quinine or fever tree) bark after the extensive use of this plant crude drug in the 1600s for malaria and fever [46]. Later, Pelletier and Caventou in 1820 were the pioneers who isolated this white alkaloid as a pharmaceutical [6], other alkaloids have also been extracted, such as quinidine used for heart arrhythmias [47]. Plant crude drugs may be administered in the form of tinctures, tablets, capsules, salves, ointments, elixirs, creams, and various other forms that have been prepared traditionally in many different ways, some of which are outlined in Table 6.2.

Postharvest preparation of medicinal plants may produce two types of plant crude drugs—the use of the dried and milled plant material or the preparation of a plant crude extract. The plant crude extract is prepared via solid−liquid extraction, where a solvent may be used to obtain the soluble principles within the plant material. The solvent may serve as a part of the plant crude drug if it is edible, e.g., water (decoctions) or alcohol (tinctures), otherwise the solvent may be evaporated and the crude extract dried in vacuo.

Technology has broadened the extraction of plant crude drugs for the development of nutraceuticals and pharmaceuticals. Some of the techniques that are used in production and research include the use of noncarcinogenic solvents, aqueous extractions, and supercritical fluid and pressurized liquid extraction [21]. Other techniques used in purifying and isolating the active component include varying types of chromatography, such as thin layer chromatography (TLC), flash column chromatography (FCC), high-performance liquid chromatography (HPLC), ultrahigh performance liquid chromatography (UPLC), sonication (ultrasound extraction), and phytonics process extraction using fluorocarbon solvents. These chromatography technologies rely on the underlying principle of polarity with the mobile and stationary phases, in plant extracts the separation of the different proteins and pigments would be based on size [10]. The different components present with the desired activity may be isolated through bio-directed purification using in vitro or in vivo techniques for novel isolations or a review of the literature to aid in the isolation of the known active component [7,48]. Fig. 6.2 outlines the pathway needed in the production of plant crude drugs and pharmaceuticals, with further scientific evidence. The use of plants as medicine has led to the development of the nutraceutical and pharmaceutical framework. Ecoconservation and production cost are imperative and therefore synthesis is an option based on the type of drug once it is cost-effective [49].

Some important plant-derived drugs isolates include strychinine (from *Strychnos nux-vomica*), which was used as a stimulant for the central nervous system and to induce vomiting, emetine (*Cephaelis ipecacuanha*), which is used an antiprotozoal and also to induce vomiting, among many others for varying ailments including cancer [48,20]. These drugs have revolutionized the pharmaceutical industry as technology and organic chemistry reactions and reagents could now be used to obtain new compounds. The isolation of drugs from plants also creates the basis for the identification of new compounds and elucidation of their structure using various techniques such as proton and carbon nuclear magnetic resonance, Fourier transform infrared (FTIR) technology, gas chromatography−mass spectroscopy, and nowadays UPLC with mass spectrometry may be applied [14].

A synthesized drug is one that has been combined in the laboratory by complex chemical pathways and derivatization, without the use of plant material [20]. An extracted drug is one that has been isolated from the crude drug preparation of the plant and is further used for production. In new drug production, the most economical method will be utilized, as the pharmaceutical companies may spend up to $2.6 billion from the research phase through to drug development, which takes approximately 10 years, on resources needed to determine the potency, toxicity, and dosage prior to marketing [50]. This market resulted in sales of over $15 million for plant-derived drugs in the United States alone (1991), with similar trends observed in other regions [48,51]. Morphine was first extracted in 1804 by Friederich

TABLE 6.2 Traditional Preparation Methods of Plant Crude Drugs

Preparation Method	Description
Infusion	This method involves treatment of the dried plant material with cold or warm water which allows the readily soluble components to be removed from the plant material. Delicate fresh herbs may also be infused.
Decoction	Hot water is used to create a crude dried plant extract. The plant material (water soluble and heat stable) is boiled in water for a specific time, after which it is cooled and then filtered. Decoction may be used with wet plant material of tougher plants.
Percolation	The solvent is poured onto the ground, dried plant material within a percolator for approximately 24 h with agitation (maceration). This causes the soluble compounds to be extracted as it slowly flows down the percolator (extraction chamber). The sample is filtered, and is often used for tinctures and other fluid extracts.
Maceration	The fresh or dried plant material (ground or whole) is agitated with a solvent over a period of time (at least 72 h). Filtration follows and the marc (damped plant material) is pressed to remove any excess extract.
Soxhlet extraction	This is also referred to as hot concentrated extraction, and is very time, energy, solvent, and cost-effective. The Soxhlet apparatus is set up with the plant material in a porous bag. The solvent is heated and as the vapor condenses it causes the soluble components of the plant material to be removed on contact. The extract may be collected when no residue is observed from the vaporized solvent.
Digestion	This is similar to percolation and maceration; however, it involves the use of a slightly higher temperature above room temperature. This is often used for the extraction of tough plant material, such as wood and bark.
Fermentation	This is an aqueous alcoholic extraction as the plant material or prepared decoction is soaked in an earthen, metal, or porcelain vessel whereby alcohol is produced as the fermentation takes place over time. The compounds are extracted in the liquid, which is then filtered. Dried plant material is usually used.
Distillation	Distillation is a separation technique used to obtain pure samples due to selective evaporation (difference in boiling point). Steam distillation (hydrodistillation) is used to extract essential oils from plant material. As the essential oil reaches its boiling point, it then evaporates, condenses, and the pure sample collected. Fresh plant material is usually used.
Expression	This was one of the first methods used for obtaining essential oils. The pressing or squeezing of plant material under high mechanical pressure to extract the oils. This may be done hot (hot expression), cold (cold expression), or with chemicals.
Solvent extraction	A hydrophobic solvent, usually hexane or supercritical carbon dioxide is used to extract more sensitive oils that may be destroyed by the heat in distillation. This solvent is referred to as concretes and will contain other hydrophobic (oil-like) compounds such as resins and waxes. Ethanol may be added and chilled at −18°C for about 48 h, this will precipitate the other lipid compounds leaving the essential oils after evaporation of the ethanol.
Enfleurage	Another ancient method of obtaining essential oils for perfumery and is hardly used today. An odorless solid fat, usually lard or tallow, is placed on a glass cover (chassis) over the plant material (usually fresh) to be extracted, e.g., flower petals. The fragrance absorbs into the fat (pomade), is removed and purified using ethanol and subsequent evaporation to obtain the oils. This is called cold enfleurage. While with hot enfleurage the plant material is stirred in the melted fat.

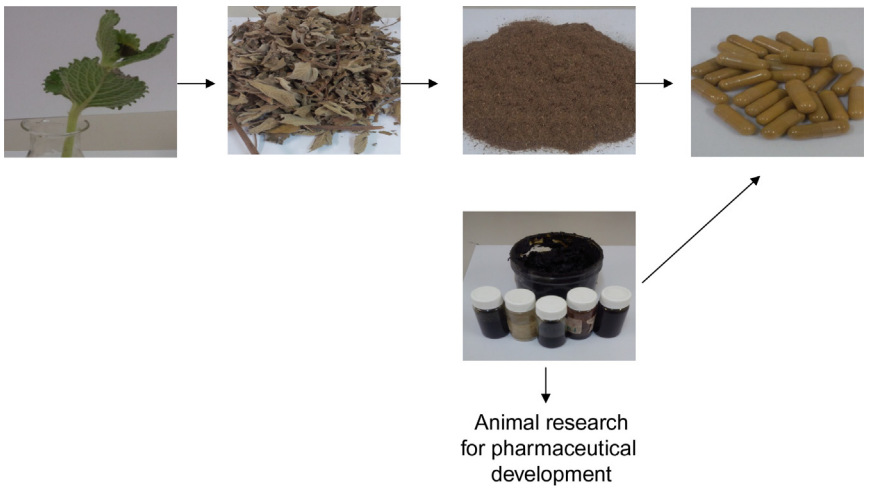

FIGURE 6.2 The processing of medicinal plant material for the production of plant crude drugs (nutraceuticals) or pharmaceuticals.

Serturner, as an effective analgesic; however, the drug was very addictive and as such attempts were made to modify the structure. However heroin, a diacetyl form of morphine, was produced with an enhanced addiction. Further isolations from the crude drug identified dihydromorphinone (codeine), which also had the pain-killing ability along with suppressing coughs and created the template for the synthesis of other morphinan class alkaloids, such as dextromethorphan, an antitussive agent, in cough medicines [52]. These are known as nonderived drugs as they were created from template compounds obtained from crude drugs.

6.6 CONCLUSION

Plants provide a remedy for many ailments. The crude preparation of plant-based crude drugs may lead to the development of natural products, such as nutraceuticals and cosmeceuticals, after the appropriate collection, preservation, and storage techniques are adhered to. Further extraction and isolation of the bioactive components within crude drugs are carried out, where efficiency parallels increased technology. These compounds in most cases increase the efficacy of treating various ailments and thus are useful in drug development. The purification of crude drugs thus gives rise to the formulation of pharmaceutical items. This aids in reducing production costs and prevents the continual removal of plant materials as sources of drugs.

6.7 SELF-EVALUATION QUESTIONS

- Describe a plant crude drug and its different types.
- Give examples of plant crude drugs and their use(s).
- How may plant crude drugs be classified?
- Discuss the importance of a herbarium and pharmacopoeia in plant crude drug production.
- Explain the difference between a synthetic and a derived drug.
- State the different stage requirements for producing a plant crude drug as a nutraceutical and as a pharmaceutical.

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Chapter 7

Evolutionary Perspectives on the Role of Plant Secondary Metabolites

R. Delgoda and J.E. Murray
University of the West Indies, Mona campus, Jamaica, West Indies

Chapter Outline

Learning Objectives

- Describe what secondary metabolites are.
- Describe how secondary metabolites have developed through plant phylogeny.
- Provide evidence that support the evolutionary theory.
- Appreciate the role of secondary metabolites for medicinal uses of man.
- Outline mass productions of secondary metabolites for man's use.

7.1 WHAT ARE SECONDARY METABOLITES?

A substantial portion of the human daily diet is formed of plants and plant constituents and their nutritional values have been extensively studied. These nutritional components of carbohydrates, lipids and amino acids are produced biosynthetically by photosynthetic green plants which form the root of almost all food chains on earth, and are known as primary metabolites.

In addition to the essential primary metabolites, plants are estimated to be able to biosynthesize at least a million [1] other diverse compounds as well, with related plant families and species expressing combinations of similar compounds. Selective expression of these compounds has in fact helped scientists classify plants into different chemotaxonomic groups. These compounds, typically of low molecular weight, appear not to contribute directly to the primary functions of the plant, and are therefore known as "secondary metabolites." The concept of secondary metabolites can be attributed to Kossel [2] who distinguished them from primary metabolites.

Although secondary metabolites were considered as waste products in the past, growing evidence has emerged displaying an intricate role in providing a distinct evolutionary advantage to the plants that express them, either directly or through indirect connections with others. Thus the definition of what a secondary metabolite is has changed somewhat over the years. The most accepted definition of secondary metabolites consider them to be naturally produced substances that do not play an explicit role in the internal economy of the organism that produces it [3,4] and stands in direct contrast to primary metabolites, which maintain fundamental cellular life processes. These secondary metabolites are argued to play an important role in the survival of the species that produces them via critical interactions with its environment.

Secondary metabolites are low molecular weight compounds that have no recognized role in the maintenance of fundamental life processes in the plants that synthesize them, but have an important role in the interaction of the plant with its environment [5].

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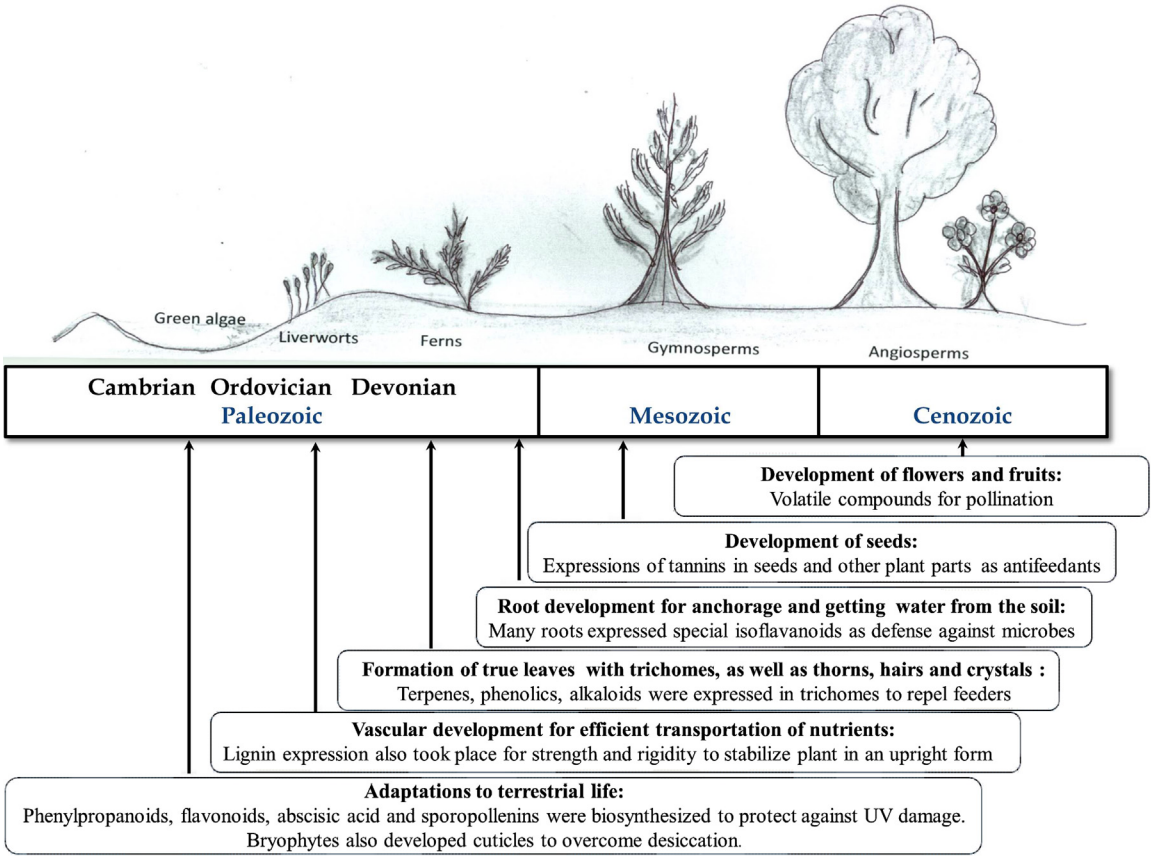

FIGURE 7.1 Expression of secondary metabolites at major branching points of the green plant phylogeny.

7.2 AT THE BEGINNING

Early plant forms, such as green filamentous algae surviving in aquatic pools in the early Ordovician age (~500 million years ago), were equipped to carry out the fundamental processes of photosynthesis, glycolysis, and Krebs cycle to produce primary metabolites. Specialized enzymes found in the green algae, *Chlamydomonas reinhardtii* [3], the closest living relative member of the Charophytes, can catalyze the biosynthesis of sterols, glucose, lipids, and nucleic acids and would have helped to maintain homeostasis within the aquatic pools of the early environment.

The first land plants, that looked like liverworts appeared around 450 million years ago, evidenced from the findings of their spores. Transitioning from the aquatic environment to land life, these early plant forms faced three huge challenges: large and rapid temperature changes, desiccation, and direct harsh ultraviolet light. Additional challenges included the need for anchorage on soil and rock, the combat of new forms of microorganic occupancies in the new soil environment, and eventually competition by other plants for space and resources as well as grazing predators. Adaptations that enable them to overcome these challenges would have been a necessity to thrive in the new environment and it appears that the generation of secondary metabolites has played a unique and significant role in meeting these biotic and abiotic challenges (Fig. 7.1). In this chapter, we examine how these secondary metabolites may have been developed (both in the plant and within microbes that they harbor), their role in the evolution of plants, along with purviews of evidence for such thought.

7.3 THE TRANSITIONS

The taxonomic distribution of secondary metabolites and the metabolic pathways that gave rise to them point to a gradual diversification to suit varying lifestyles during plant evolution, and are suggestive of a key role of secondary metabolites at major branchings of plant phylogeny. Fig. 7.1 depicts key nodal points of secondary metabolite expression, along with specialized tissue development.

A. The biosynthesis of the secondary metabolites, phenylpropanoids, flavonoids, abscisic acid, and sporopollenins, present in land plants provided protection against UV radiation, which played a significant role in the ability to survive in the harsh terrestrial landscape with direct exposure to sunlight. These metabolites have played a critical role in helping the early algal forms to transition into the mosses and liverworts found in terrestrial environments. The bryophytes also developed cuticles which aided the avoidance of some level of desiccation, although their approach was a rather rudimentary on and off method for metabolism, for wet and dry times respectively.

B. A more sophisticated method was needed to combat problems of desiccation and such was first seen in the Tracheophytes, nearly 40 million years later. The production of lignin, a phenolic, complex polymer, through the elaboration of the phenylpropanoid pathway provided strength and rigidity needed for vasculature, in addition to providing a protective layer against desiccation. The strength provided by lignin, which helped toughen cell walls, also stabilized the physical plant into an erect, upright life-form capable of standing up against the soil, independent of the amount of water available. The movement of plants from water environments to colonize drier environments was now possible, following vasculature and internal irrigation through xylem and phloem cell formations.

C. Late in the Paleozoic era, with the decline in atmospheric carbon dioxide, the extinct *Archaeopteris* and the ancestors of modern horsetails, ferns, and seed plants were thought to have evolved true leaves, containing trichomes, the hair-like appendages in the aerial epidermis. These trichomes (in addition to mechanical defenses such as thorns, hairs, and crystals) are widely believed to be a defense against insect feeders, and in many species there is a negative correlation between trichome density and insect feeding plus nutrition for their larvae. Different types of trichomes were also a repository for secondary metabolites exuding terpenes, phenolics, alkaloids, and others with repellent properties [6].

D. By the late Devonian era, following vasculature, plants were also seen to have a root system, critical for anchorage and tapping trapped water in the soil for larger trees. Many roots produced special isoflavonoids to face soil-borne microorganisms, which are factories of numerous secondary metabolites themselves [7].

E. The appearance of seed plants in gymnosperms (found today as conifers, cycads, gnetales, and the ginkgo tree) and beyond, blanketed with a seed coat containing tannins for further protection, was seen around 260 million years ago. The evolution of the angiosperms, which currently make up 85% of the 300,000 known plant species, appeared around 145 million years ago and within a 10 million-year period, scientists believe, arose the greatest speciation in the history of plant life [8]. With it came an explosion in secondary metabolites, yielding color, fragrance, and flavor central to the reproductive success of the flowering plants. Anthocyanins, anthocyanidins (phenylpropanoids C9−3C2) which provide color ranging from orange, red, purple to blue, carotenoids (terpenoids 4C2) that provide yellow, red, and orange colors, and flavones and flavonols, which contribute whites and yellows, are responsible for the rich color and fragrance of many plants, to attract the pollinators and symbionts for successful fertilization (Fig. 7.2). Terpenes (particularly monoterpenes), phenolics, benzoic acid derivatives, and aliphatics totaling to over 1700 volatiles have been identified as contributing to floral scent [8]. The nectar which rewards the visiting insect also contains volatile alcohols, aldehydes, and terpenes. Collectively these secondary metabolites also provide antioxidant, antifeedant and antimicrobial activities, thus acting as plant defenses. It is not coincidental that the

FIGURE 7.2 Expression of anthocyanins by plants for bright coloration, from left, *Allamanda cathartica*, *Rosa* sp., and *Hibiscus rosa sinensis*.

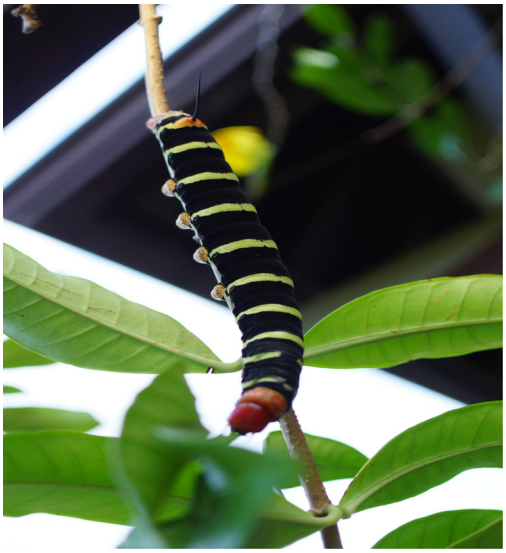

FIGURE 7.3 The Hawkmoth larvae feeding on *Allamanda cathartica*; the larvae have evolved antidotes for the poison expressed by the plant.

emergence of insects happened at the same time, with the first six-legged hexapod fossils found around 410 million years ago, and these two taxa (insects and plants) coexisted and coevolved largely alone on land for tens of millions of years (a good 50 million years before the first land vertebrates emerged) providing each other with key support for successful survival and reproduction [8]. An example of coevolution with insects is evident in orders of insects including *Coleoptera*, *Hymenoptera*, and *Lepidoptera* that feed on plants that synthesize defensive pyrrolizidine alkaloids, by evolving the ability to accumulate the toxic alkaloid in their external cuticles and wings as defense against predatory birds and mammals (see an example in Fig. 7.3).

It appears that as simple terrestrial plant forms evolved into more complex higher plants, secondary metabolites played a significant role in the key adaptations of traits and tissue formations.

7.4 EVIDENCE FOR EVOLUTIONARY THEORY

The premise that primary metabolites are the precursors for secondary metabolite biosynthesis provides the first point of evidence for evolution. As Fig. 7.4 depicts, few key primary metabolite building blocks lay the foundation for many known secondary metabolites. These are the acetate C2 unit (leading to polyketides and fatty acids), the phenylalanine/tyrosine derived C9 unit (leading to phenylpropanoids), the C5 isomeric units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), and some amino acids. In plants, the terpenoid and phenylpropanoid pathways are predominant in the production of secondary metabolites, while in microorganisms the polyketide pathways is particularly well developed. Besides its role in the biosynthesis of phenylalanine and tryptophan, both of which are also involved in secondary metabolism, the shikimate pathway is also directly involved in some secondary metabolite biosynthetic pathways, via its end product chorismate.

Although the numbers of building blocks are limited, it can be seen that the combinations of these to produce novel secondary conformations are nearly infinite. A plethora of different secondary metabolites have already been discovered, yet we still continue to uncover the breadth of nature's chemodiversity. For ease, secondary metabolites can be broadly classified into three main molecular families: the phenolics (including products from C2, C5, C9 + C2 pathways), terpenes (C10, C15, C20, C30, C40), and alkaloids, which are covered in detail in other chapters of this book, as well as other references [9].

There is a gradual evolutionary development of plant-specific metabolic routes for the production of certain classes of secondary metabolites, tissue types, organs, and lifestyles, with distinct taxonomic distributions. Key metabolic pathways and traits appear to be conserved (as seen in Fig. 7.5) with the propensity for new pathways to be continuously built upon existing pathways and this provides the second point of evidence for evolution. For example, the production of lignin appears to be conserved in all vascular plants, enabling the flow of water and nutrients efficiently, with varied other adaptations that have arisen in gymnosperms and angiosperms that have come millions of years after.

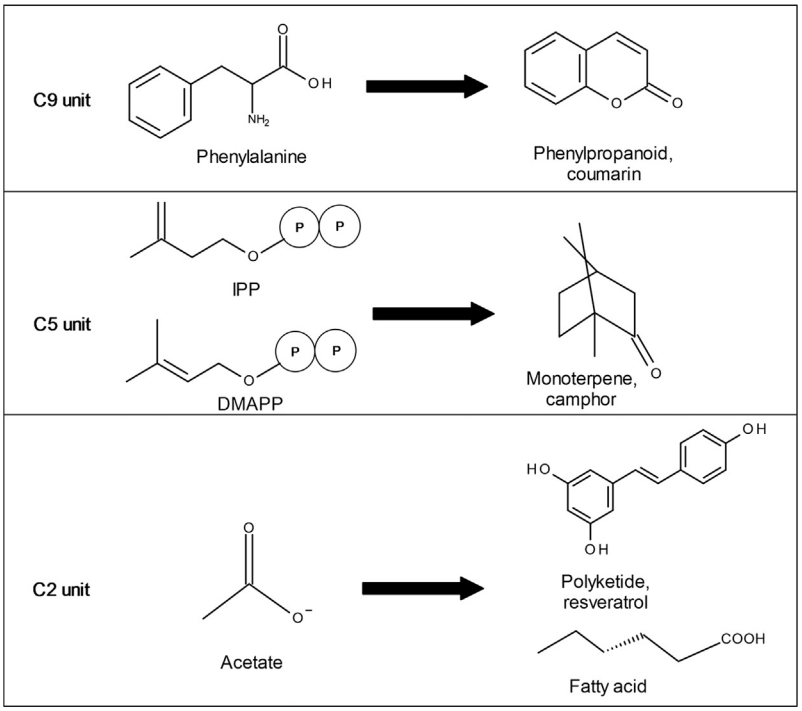

FIGURE 7.4 Building blocks commonly used in the biosynthesis of both primary and secondary metabolites.

FIGURE 7.5 *Couroupita guianensis*, commonly known as Canonball tree flowers evolving color and fragrance to attract pollinators.

The enzymes involved in the generation of secondary metabolites have also arisen as gradual adaptation of the primary enzymes themselves. Scaffolds of existing metabolic enzymes can be traced through to ancestral proteins, and as such this gives rise to the conclusion that indeed there was no emergence of new enzymes but rather the variations thrust upon existing structures by natural selection. Various examples of convergent, parallel, and divergent evolution can be seen in plant metabolic processes [10] and such examples include major enzymes such as cytochrome P450 (CYP) enzymes, which a play a role in the synthesis of divergent compounds including lignins, having relations to ancestral primary metabolic enzyme sterol 14-demethylase. Chalcone synthase, an enzyme involved in flavonoid synthesis in plants, is another great example as it shares the same protein folds as the fatty acid synthesis enzyme β ketoacyl ACP synthase III [3]. It is also very clear that catalytic promiscuity of plant enzymes, i.e., the ability to flexibly accommodate many substrates, drives the expansion of evolutionary diversity, and explains the ability to evolve new pathways as a response for environmental challenges.

It appears however that the fundamental evolutionary mechanism is gene duplication [8] whereby new copies of existing genes are created by a number of processes, allowing for variation and experimentation with the newly formed genes. Over 60 separate cannabinoids produced from *Cannabis sativa*, many of which have no known bioactivity, support the view that multiple variations of genes may give rise to many slightly modified chemicals with only a limited number of these needed to provide an advantageous outcome, which appears to be nature's synthetic experimentation method. Whole genome duplication can also be seen in the angiosperm, *Arabidopsis thaliana*.

7.5 THE EXPRESSION OF SECONDARY METABOLITES

Secondary metabolites are found expressed in various combinations in different parts of the plant (leaves, roots, shoots, bark), at different stages of growth (seedling, seed, plantlet, mature tree), under different environmental pressures (invasive microbes, herbivores), in numerous combinations of ways by different classes of plants. Although it is hard to draw general conclusions about secondary metabolite combinations with universal applicability, they have been classified into three categories [1] in Table 7.1, depending on how they are expressed, and this sheds light on the key roles they play.

7.6 SECONDARY METABOLITES, A WORTHY INVESTMENT: FURTHER SUPPORT

A glance at the metabolic routes of these secondary metabolites quite clearly points to complex and intricate systems of biosynthesis involving multiple pathways, enzymes, and genes, and thus supports the view that they must be expressed for an important adaptive, functional advantage for the plants that express them. The hypothesis that plants have evolved the ability to produce such secondary metabolites due to a selective advantage is based on the following tenets.

TABLE 7.1 Different Modes of Expression for Secondary Metabolites in Plants

Modes of Expression	Definition	Examples
Constitutive	Many secondary metabolites are expressed in plant tissue alone or often in many combinations of similar compounds to play a particular role.	A direct relationship has been found with the expression of high content of glucosinolates in young oilseed rape cotyledons and the deterred feeding of a slug species, *Deroceras reticulatum*.[a]
Constitutively expressed but require activation	Similar to prodrugs that require biochemical activation within the body, there are some metabolites which appear not to have a biological activity in themselves, but following activation do provide some value for the host plant.	Allicin in garlic (*Allium sativum* L.) is an antibacterial compound formed from its inactive form alliin subsequent to tissue and cellular damage caused by plant pathogens. Once cellular damage has occurred, the alliin lyase enzyme is able to mix with the alliin substrate and produce allicin as a line of defense against the pathogens.[b] This reaction gives rise to the characteristic aroma and flavor of garlic when it is crushed.[c]
Induced expression	Some secondary metabolites are expressed only following an environmental pressure that causes an induction of its production. These secondary metabolites are classified as phytoalexins, whereas those constitutively expressed as mentioned above are classified as phytoanticipins.[d]	The antimicrobial agents, anthraquinones, are not detected in healthy *Cinchona* plants, but are found in tissue cultured plants that are infected by fungi.[e]

[a] *Bennett RN, Wallsgrove RM. Secondary metabolites in plant defence mechanisms. New Phytol 1994;127(4):617–33.*
[b] *Curtis H, Noll U, Störmann J, Slusarenko AJ. Broad-spectrum activity of the volatile phytoanticipin allicin in extracts of garlic (*Allium sativum *L.) against plant pathogenic bacteria, fungi and oomycetes. Physiol Mol Plant Pathol. 2004;65(2):79–89.*
[c] *Whitaker JR. Flavor, odor and pungency in onion and garlic. Chichester CO, editor: Academic Press; 1976.*
[d] *VanEtten HD, Mansfield JW, Bailey JA, Farmer EE. Two classes of plant antibiotics: phytoalexins versus "phytoanticipins". Plant Cell. 1994;6(9):1191–2.*
[e] *Wijnsma R, Go JTKA, Weerden INv, Harkes PAA, Verpoorte R, Baerheim-Svendsen A. Anthraquinones as phytoalexins in cell and tissue cultures of Cinchona spec. Plant Cell Rep. 1985;4(5):241–4.*

FIGURE 7.6 *Pedilanthus tithymaloides*, commonly known as Monkey fiddle which expresses thick cuticles.

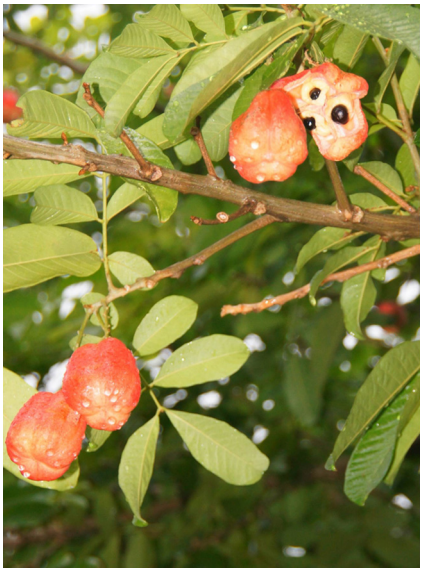

FIGURE 7.7 *Blighia sapida* that expresses the toxin, hypoglycin, to induce vomiting sickness in those who consume it at premature ripening.

FIGURE 7.8 *Hura crepitans* L. commonly known as the Sandbox tree with spines evolved to defend against predatory insects.

a. In general, plants (and other organisms that produce secondary metabolites) do not have an immune system, and are thus reliant on these compounds for defense. So these metabolites (phytoalexins and phytoanticipins) provide protection from pathogens due to their antibiotic, antifungal and antiviral capabilities, together with an arsenal of defense mechanisms from enzyme activities and potential resident endophytes.

b. There is an obvious adaptive advantage to those that produce it, e.g., to wade off competition; antigerminative or toxic for other plants (allelopathic); better adapted to harsh environments; and are able to combat feeding animals such as insects or cattle (antifeedant). Specific examples are the expression of estrogenic properties by clover or alfalfa, and the excretion of phenolic chemical juglone by walnut tree that creates bare patches of soil around it. Figs. 7.3, 7.6–7.8 display examples of occasions when secondary metabolites and tissues have been used to gain an adaptive advantage.

c. The biosynthesis of secondary metabolites requires sophisticated pathways that are energetically expensive, which are unlikely to have developed unless the products are advantageous.

d. The genes that produce these compounds reside right beside each other, demonstrating nature's selection for it.
e. Resistance genes are also organized beside it.
f. They are not merely artifacts of isolation procedures, and do in fact exist in nature.

7.7 CONCLUSIONS

It is estimated that there are over 170,000 known secondary metabolites [1] from a limited number of species studied so far, with a vast number of plants yet remaining to be fully examined. One of the noticeable features of expression is that plants express a multitude of compounds, not all of which are biologically active. It has been pondered upon therefore as to why such expressions are made, and one possible answer is based on the proposition that there might be a selective advantage to an organism if it can generate and retain chemical diversity at a low cost. Thus, the availability of many compounds perhaps allows the plant to do its own "screening" for optimal bioactivity subject to the environmental need [11].

There are 100 natural product secondary metabolites or natural product derived compounds being evaluated for clinical trials with 31 of them in phase III or in registration by the end of 2013 [12]. These figures clearly indicate that an important contribution is being made to modern pharmaceuticals by natural products, with the next few years likely to see natural product driven drugs continue to rise in the market.

Understandings gained from studying the evolutionary behaviors and triggers that incite the formation of secondary metabolites have provided useful means in the production of these natural products at a large scale. Due to their invaluable uses in multiple industries, including pharmaceutical, nutraceutical, cosmetics, and fine chemicals, large-scale production of secondary metabolites is desirous. Depending on the chemical compound and the plant that expresses them, the following options, apart from field cultivation, have been pursued to achieve that end: chemical synthesis, plant cell tissue and organ culture, and plant metabolic engineering.

Evidence gained over many species supports the view that secondary metabolites have arisen as a channel to meet a challenge, and that there is a selective advantage to the organism that produces it. These metabolites have been a useful source of medicine for man, and many methods of mass scale productions make use of the understanding gained about secondary metabolite production in plants.

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Chapter 8

Glycosides

M. Bartnik[1] and P.C. Facey[2]
[1]Medical University of Lublin, Lublin, Poland, [2]University of the West Indies, Kingston, Jamaica

Chapter Outline

Learning Objectives

- Plant glycosides and their structure–activity relationships and plant sources.
- Extraction methods of plant glycosides and chemical reactions.
- Therapeutic uses, mechanisms of action, possible adverse effects, and toxicity of natural plant glycosides as medicines and nutraceuticals.

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8.1 INTRODUCTION

Many plant secondary metabolites in nature occur as glycosides [1–4]. In plants, glycosides are derived mostly from postmodification of the secondary metabolites catalyzed by plant enzymes, glycosyltransferases. Further modifications of the glycosides, such as oxidation, acylation, and degradation, take place frequently [5–7]. While there remains much to uncover on the physiological roles of these water-soluble metabolites in plants, what is known is that they are stored and transported within the plant tissue and may play an important role in signaling, regulation of growth and development, and also in an allelopathy (biological phenomenon where one plant inhibits the growth of another). They are also important in the plant's defense system against pathogens and herbivores. In many instances, glycosides are produced as an answer to the environmental conditions, such as abiotic (humidity, soil composition, sunlight, temperature) or biotic factors (e.g., plant herbivores, coexisting plants) [3,4,8].

Glycosides are comprised of two chemically and functionally independent parts; the aglycone (genin) and the glycone (saccharide) parts. In a glycoside, the saccharide part (X) is linked to the aglycone (A) portion by a *glycosidic bond* as seen below:

$$\mathbf{A}-\mathrm{OH} + \mathbf{X}-\mathrm{OH} \leftrightarrow \mathbf{A}-\mathrm{O}-\mathbf{X} + \mathrm{H_2O}$$

The glycosidic bond is mostly unstable and susceptible to hydrolysis (by diluted acids or by enzymes, e.g., β-glucosidases). Accordingly, the types of glycosidic linkages are classified as:

- *O*-glycosides (if the glycosidic bond is via oxygen); the most abundant form in plants
- *C*-glycosides (linkage via a carbon); this type of linkage is resistant to hydrolysis
- *S*-glycosides (linkage via a sulfur; aglycone must have —SH group) present in glucosinolates (thioglycosides)
- *N*-glycosides (linkage via a nitrogen; aglycone must have —NH group) present in nucleosides.

The glycone is most frequently a monosaccharide, the most common being glucose (a glycoside yielding glucose is called a glucoside). Other frequently occurring glycones are L-rhamnose, L-fructose, L-arabinose, and D-xylose. The sugar unit can also be a di-, tri-, or tetrasaccharide (e.g., in cardiac glycosides). The configuration of the anomeric carbon of the glycone can exist as α or β diastereoisomer with the β-forms being most common and active. The number of saccharide units/chains attached to the algycone can be one (monodesmoside), two (bidesmoside), or three (tridesmoside), which are commonly seen in saponin glycosides. The main groups of glycosides highlighted are terpene, sterol, phenol, or phenylopropanoid glycosides on the basis of the aglycone structure. Glycosides also may be classified (due to the nature of the attached sugar) as galactosides, apiosides, rhamnosides, xylosides, rutinosides, etc.

Extraction of glycosides from plant material (previously dried and properly stored to avoid decomposition of glycosides by plant enzymes) is usually performed with the use of polar solvents (water, hydro-alcoholic solutions, and alcohols). The most used extraction methods are percolation or maceration, Soxhlet extraction, or special processes, e.g., pressurized liquid extraction (PLE/ASE), ultrasound-assisted or microwave-assisted extraction (UAE or MAE) [9,10]. Isolated glycoside fractions are subsequently purified by the use of solvent/solvent partitioning methods to remove chlorophylls or other pigments (water/hexane or water/chloroform are used), separated by precipitation or adsorption methods (column/planar chromatography) and then recrystallized.

The overall effect of glycosides is dependent on their two components; the aglycone portion primarily influences the therapeutic direction, while the saccharide part (sugar chain/s) increases water solubility, pharmacokinetics, and pharmacodynamics properties. The glycosidic linkage (normally β-linked in active plant glycosides) is resistant to human digestive enzymes and hence glycosides are often poorly absorbed from the digestive tract. They usually travel to the distal ileum or large bowel. Here, microbial activity forms the aglycone, which is less polar and can be absorbed into the bloodstream [11].

This chapter briefly discusses the various types of glycosides; their chemistry; occurrence in plants; extraction techniques, chemical detections; plant drugs containing glycosides and their bioactivity, including the therapeutic indications, metabolic profiles of those widely used, and also adverse effects and toxicities.

The main groups of plant glycosides are abbreviated in the text as follows: PHGs—phenolic glycosides; CMGs—coumarin glycosides; CHGs—chromone glycosides; FLGs—flavonoid glycosides; AQGs—anthraquinone glycosides; SPGs—saponosides; CRGs—cardiac glycosides; CNGs—cyanogenic glycosides; THGs—thioglycosides.

8.2 EXTRACTION OF GLYCOSIDES

The free phenols and phenolic acids are usually identified together during analysis of plant material. Phenols are soluble in polar organic solvents, in sodium hydroxide and carbonate solutions, and they can be extracted with organic solvents

under slightly acidic conditions [3]. Extraction of PHGs from fresh plant material is effected using alcohols or alcohol−water mixtures. Extraction from water with nonmiscible solvents with increasing polarity enables the isolation of aglycones, esters, and glycosides. Phenolic acids are normally detected after hydrolysis (acid or alkaline) of concentrated aqueous-alcoholic plant extract. Low temperature and neutral conditions are required to avoid decomposition or isomerization. Isomerization of cinnamic acid derivatives occurs during isolation when the extract is exposed to light. Cinnamic esters of caffeoylquinic acid isomerize in both acid and alkaline conditions and yield mixtures of positional isomers, e.g., chlorogenic acids (chlorogenic, cryptochlorogenic, or neochlorogenic acid) [3,12].

Simple coumarins are isolated by polar solvents such as simple phenols and phenolic acids. Furanocoumarins are generally lipid-soluble and can be isolated from dried plant material by petroleum ether, light petroleum, or dichloromethane. In many cases crude coumarin is obtained as a semicrystalline sediment when the concentrated petroleum ether extract is stored at low temperatures, and is next purified by use of column chromatographic techniques (LC), preparative TLC, or by crystallization from different solvents [13,14]. Advance chromatographic methods, such as centrifugal partition chromatography (CPC) or high-speed countercurrent chromatography (HSCCC), have been also developed and used for preparative isolation of coumarins from crude plant extracts [15,16]. The enzymatic cleavage of the glycosides is the mildest method of obtaining the aglycons, permitting the avoidance of the formation of by-products and artifacts.

FLGs can be degraded by enzymatic action when the plant material is fresh or not properly dried. It is thus advisable to use dry, lyophilized, or frozen samples. For flavonoid extraction the solvent is chosen as a function of the flavonoid polarity (which depends on chemical structure). Flavonoid glycosides are water-soluble and soluble in alcohols or alcohol−water mixtures. However, in some cases glycosides are sparingly soluble, e.g., rutoside or hesperidin. Certain flavanone and chalcone glycosides are difficult to dissolve in methanol, ethanol, or their water-containing mixtures. Flavanone solubility depends on the pH of water solutions. Anthocyanins are extracted with cold methanol acidified with acetic acid (c. 7%) or 3% trifluoroacetic acid. In general, powdered plant material can be extracted in a Soxhlet apparatus, first with hexane (or chloroform) to remove lipids and then with ethyl acetate or ethanol to obtain phenolics. If compounds are heat-sensitive, extraction must be performed at room temperature to avoid structure decomposition. Some lipophilic flavonoids are soluble in dichloromethane, although further steps for purification are required. The glycosides are extracted at high temperature with mixtures of acetone or alcohols; ethanol or methanol (80-50%) with water. Further steps are evaporation of the alcohol and partitioning of water residue with petroleum ether (removes lipids and chlorophyll), diethyl ether (extracts aglycones), and ethyl acetate (dissolves glycosides). The most polar glycosides are left in the aqueous phase together with sugars. Many applications of chromatographic separation of individual flavonoids are present in the literature, comprising TLC, LC, CPC, and HPLC techniques with structure elucidation by MS and NMR methods [3,12,17].

Free quinones are insoluble in water and can be extracted by common organic solvents (ether, benzene) and purified by use of chromatographic methods. Anthraquinone glycosides can be hydrolyzed by heating in aqueous acid. When recovering the reduced forms, it is required to use low temperatures, avoid light, and do the procedure under nitrogen (to avoid their spontaneous oxidation). Separation of anthraquinone mixtures is difficult, and special techniques were applied depending on the plant source [3,12,18].

Saponins form colloidal solution with water and the aqueous medium promotes the hydrolysis of the bi- or tridesmosides. In general saponins are extracted from plant material using polar solvents (alcohols or hydro-alcoholic solutions) after defatting the plant material with petroleum ether. The ethanol is next removed by evaporation and saponins are extracted from the water phase into *n*-butanol. Saponins precipitated from solutions by addition of sterol. After filtration we can separate sterol-saponin compound (precipitate) and next remove sterols after boiling precipitate with toluene (saponins are insoluble in toluene). Due to the ability of certain saponins to facilitate the formation of foam/emulsions, care must be taken to avoid this during extraction and preanalytical extract purification steps [19]. The choice of solvent for a particular application should be based on the effect of solvent on saponin yield and purity, and the chemical composition of the mixture of saponins (different selectivity of the solvents toward individual saponins and coexisting components). Recently there have been efforts to improve the methodology, mainly on the extraction of ginseng saponins, glycyrrhizic acid, and also aescin, and the use of supercritical CO_2 extraction in combination with modifiers such as methanol, ethanol, or aqueous methanol has proven successful [20]. Purification of the crude saponin extract usually requires a sequential approach; partitioning of saponins between aqueous extracts and a water immiscible solvent, precipitation, adsorption, ultrafiltration, and chromatography. For analytical scale purification of saponins an open column chromatography, thin layer chromatography, flash chromatography, liquid chromatography (low, medium, and high pressure), and countercurrent chromatography have been established and used [21,22]. Pure saponins are very hard to crystallize and they are hygroscopic and rarely give sharp melting points without decomposition.

The various CRGs show different solubility in aqueous and organic solvents. CRGs are usually soluble in water and alcohols (good solvents for both glycoside and aglycone form) and not soluble in nonpolar solvents (except chloroform and ethyl acetate), such as petroleum ether and diethyl ether. Primary glycosides are water-soluble, soluble in dioxane, and partially in chloroform. Specific plant enzymes split primary glycosides to secondary glycosides with fewer numbers of sugar chains, and acid hydrolysis cleaves CRGs to aglycones. The presence of the lactone ring which is easily opened in an alkaline medium renders the molecule unstable. During storage of plant material specified moisture content must be provided to avoid decomposition of compounds. Also inactivation of enzymes at higher temperatures (heat stabilizing) before storage of plant material containing CRGs is recommended [3,12].

Since they are β-glycosides, CNGs are hydrolyzed chemically in acidic solution and enzymatically by β-glucosidases. Products of those hydrolyses are sugar and unstable cyanohydrin (hydroxyl nitrile), which decomposes spontaneously depending on the pH (the more alkaline the faster) [23]. The instability of CNGs requires inactivation of enzymes present in plant tissues before extraction with alcohol or aqueous alcohol solutions (70%) [12].

The physico-chemical−biological properties of the glucosinolates are defined by the hydrophilic character of the thioglucopyranoside group and the strongly acid or anionic sulfate group, but these properties are more or less moderated by the properties of the side chain structure [24]. Glucosinolates are extracted from fresh plant tissue with boiling alcohol (under these conditions plant enzymes are destroyed). Due to their ionic nature they are purified on ion exchange resin columns (Amberlite), or by use of special PC, TLC, and HPLC methods.

For quantitative estimation of THGs two indirect methods are recommended. The first measured glucose (by standard glucose assay kit) released by action of enzyme myrosinase, which hydrolyze glucosinolates present in purified glucosinolate fraction. The second requires hydrolysis of the glucosinolate fraction by sulfatase for 30 min. and analysis of produced desulfoglucosinolates by HPLC (desulfoglucosinolates will be the hydrolytic products formed when glucosinolates without substituents on the thioglucose part react with active sulfatase) [3,12]. Carboxylic acids are the main product in acid catalyzed hydrolysis at elevated temperatures. In alkaline catalyzed transformations of glucosinolates at elevated temperatures, amino acids among other products are produced. In metal ion catalyzed reactions, glucosinolates can be transformed into nitriles [25,26]. Nonenzymatic glucosinolate transformations may also occur at temperatures of 20−40°C in reducing reaction media containing, e.g., Fe^{2+}, ascorbic acid, and thiol groups [24].

8.3 CHEMICAL TESTS

Visualization of PHGs components on TLC plates is possible by use of general reagents for phenols; ferric chloride, vanillin, and hydrochloric acid (give a range of pink colors with resorcinol or phloroglucinol derivatives), or using more specific tests with 2,4-dinitrophenylhydrazine (detects aldehydes). Folin−Ciocalteu's reagent is also useful for detecting phenols with catechol or hyroquinone nuclei (blue spots appear immediately after spraying of the TLC plate) or for other phenols, which show blue to gray spots when the plate is fumed with ammonia vapor. Gibbs reagent (2% 2,6-dichloroquinone chloroimide in chloroform) followed by fuming the plate with 2M NH_4OH gives variety of colors (e.g., it is able to distinguish vanillic acid—pink color—and isovanillic acid—blue). Cinnamic acid derivatives give characteristic light blue fluorescence when examined under UV (366 nm). *Cis* and *trans* isomers of cinnamic acids are presented on TLC plates when extract is chromatographed in aqueous solvents in two directions (2D-TLC). Spectrophotometric methods for the estimation of phenolic content are used frequently, e.g., method with Arnow's reagent or the previously mentioned Folin−Ciocalteu's reagent [3,12].

Some qualitative tests indicate coumarins in plant extracts, e.g., Lactone ring test (coumarins hydrolyzed by dilute alkali form yellow solution of O-coumaric acid salts, which can be reversed after acidification or saturation by CO_2); or Azo-coupling test (red color develops due to the reaction with diazotization sulfanilic acid in an alkaline solution). Coumarins are easily detected under UV light (365 nm), since they give characteristic color fluorescence (except simple unsubstituted coumarin which has no fluorescence). They are detected by their blue, violet, brown, green, or yellow colors. A 10% solution of KOH in methanol or 20% solution of antimony chloride in chloroform can intensify the color. Hydroxycoumarins do not exhibit bathochromic spectral shifts in alkaline solution [3,12]. In HPLC analysis with diode array detection various UV spectra of coumarins enable quick identification of compounds [27,28].

Chromones react in alkali solutions to give *O*-hydroxy-β-diketones without regeneration of γ-pyrone ring and also colored compounds with concentrated acid and concentrated alkali. Chromones are also visible in UV light (blue, yellow, greenish yellow, brown fluorescence at 365 nm).

The presence of the active phenyl ring (chromophore) in flavonoids makes them easy to detect under UV light. Their UV spectra are particularly informative, providing structural information that can distinguish the type of phenol and the oxidation pattern. Different chemical reactions by spraying TLC chromatograms are possible; e.g., visualization

in the presence of ammonia vapor (chalcones and aurones turn orange and red, respectively) or spraying with Naturstoff Reagenz A (1% solution of the ester of 2-aminoethanol and diphenylboric acid) and overspraying with 5% of methanolic solution of polyethylene glycol 4000 (PEG 4000), which enhances the sensitivity of this reaction. After derivatization, compounds could be observed in UV light (fluorescence from light yellow to green). Other tests include spraying with ferric chloride, diazotized sulfanilic acid (both are reactions for phenols), and the specific reaction; cyanidin with magnesium powder in the presence of hydrochloric acid (indicates presence of flavanones and dihydroflavanols) [3,12]. For quantitative colorimetric estimation of flavonoids, reaction with aluminum chloride ($AlCl_3$) is used (absorbance measured at 425 nm). Flavonoids dissolved in alkali solutions give intense yellow color, which decreases after the addition of acid. UV spectra of flavonoid compounds show two main bands (maxima of absorbance): band I at higher wavelength (attributed to the cinnamoyl part of flavonoid structure) and band II at lower wavelength (due to the benzoyl part). The band I is normally at 304−350 nm for flavones (H at C-3 in C ring), at 352−385 nm for flavonols (OH group at C-3), and at 328−357 nm for 3-substituted flavonols (O-substitution at C-3). The band II for most structures is at c. 250−280 nm. An additional phenol group (−OH) in ring A causes the bathochromic shift (shift to longer wavelengths) in band II, and an additional − OH group in ring B generates a similar effect in the band I [12].

Most anthraquinones or their glycosides form yellow or orange-red crystals and may show fluorescence dependent upon the pH [18]. The main color reaction is the Bornträger's test, which is effected by dissolving the quinone in alkaline aqueous medium. The color reaction ranges from orange-red to purplish-violet (depending on the structure and substituents of the quinone). Anthraquinones give a red color with this reaction. For 1,8-dihydroquinones, reaction with magnesium acetate is also used. Anthraquinones can be detected on TLC plates after spraying with 10% methanolic KOH solution. The original yellow or yellow-brown colors change to red, violet, green, or purple [3,12,18]. Powdered rhubarb could be examined in UV light to detect presence of raponthicin (stilbenoid glycoside, which is toxic). In original rhubarb (*Rheum palmatum*) a reddish-brown fluorescence appears, but no shining bluish-violet spots are seen (as in cause of rhapontic rhubarb—*Rheum rhaponticum*).

As was mentioned previously saponins are surface active compounds (modifying surface tension) and have emulsifying properties. For a quick qualitative check if a plant has SPGs, a foaming test by shaking plant material with water is commonly used. Saponins have membrane permeabilizing properties. Low concentrations of saponins are capable of destroying erythrocyte membranes (which can precipitate the cholesterol that exist in the membranes of the red blood cells), causing hemolysis of blood in vitro. This ability has led to the widespread use of hemolysis (hemolytic index—IH) as a method to determine biological activity. IH is defined as the amount of 2% isotonic suspension of bovine blood (in mL), which undergoes hemolysis when treated with 1 g of tested plant material (or tested extract). As a reference *Gypsophila paniculata* saponin mixture (IH = 30,000) or Saponin Album (Merck) (IH = 15,000) was used. As described by Hostettmann and Marston [22], hemolytic activity of saponosides varies considerably with the structure of the glycoside part. Monodesmosidic saponins (except acylglycosides and glycyrrhizin) are strongly hemolytic. No color reaction is particularly specific to SPGs. However, Lieberman reaction (with acetic anhydride in the presence of sulfuric acid) is available, where the colors differ depending on type of the aglycone (triterpene—pink to red —or steroid—blue-green) [3,12].

Chemical reactions to identify CRGs in plant material can be due to the aglycones or to the sugars. Liebermann's and Salkoviski's test indicates steroidal moiety, hence it is not specific for CRGs. Keller Kiliani's and Xanthidrole tests, both indicate deoxy-sugars (digitoxose or cymarose). The Kedde or Baljet reaction is more specific, because it is linked to the presence of α,β-unsaturated lactone ring. Fluorescent reaction of CRGs is also possible, e.g., digoxin hydroxyl group at C-14 and C-16 eliminates water with H_2SO_4 with formation of two additional double bonds. This results in a conjugated system (with a double bond in the lactone ring) that makes digoxin fluorescent in UV light. Jensen reaction (after spraying with trichloroacetic acid in ethanol) is used to visualize CRGs on TLC chromatograms [3].

Direct measurement of total HCN by acid hydrolysis of cyanogenic glycosides present in foods as well as decomposition of the intermediate cyanohydrins to HCN has the advantages of being applicable to all types of samples, although not all potential HCN of cyanogenic glycosides is likely to be available in vivo. Visualization of the presence of these compounds in plants is possible on filter paper impregnated with reagents, such as picric acid/sodium carbonate or benzidine/cupric acetate. These reagents are able to give color reactions with HCN released from crushed plant tissue. An impregnated paper is placed in a tube over the crushed plant material and left to incubate at 40°C for 2 h. A color change from yellow to reddish-brown indicates the enzymatic release of HCN. Picrate paper is not entirely specific to cyanogen (volatile isothiocyanates from *Brassica* species responds also to this reaction). Therefore another test is also used using a mixture (1:1) of freshly prepared solutions of 1% 4,4-tetramethyldiamine diphenylamine in chloroform (w/v) and 1% copper ethyl acetoacetate in chloroform (w/v). The color reaction turns from faint blue-green to a bright green. Another possible method requires distillation of the plant tissue in acidic water and titrating of the released HCN

with silver nitrate. Chromatographic methods such as HPLC/MS or GC/MS of trimethylsilyl derivatives are effective for analysis of individual cyanogenic glycosides or cyanohydrins, and provide chemical characterization as well as quantification of the compounds [3,12,29].

Because of the diversity of products formed in plants from glucosinolates (depending on its aglycone structure) estimation of isothiocyanates by the spectrophotometric method (where color products 1,3-benzodithiol-2-tiones are formed by isothiocyanate condensation with 1,2-benzene-dithiol) may be insufficient to determine the glucosinolate content in plants [12].

8.4 PHENOLIC GLYCOSIDES (PHG)

Phenols are defined as compounds that have at least one hydroxyl group attached to a benzene ring. Examples of naturally occurring plant phenols are catechol, guaiacol, hydroquinone, or phloroglucinol [3,4]. Phenolic glycosides (PHGs) are glycosides of phenols or compounds which have in their structure a C_6-C_1 or C_6-C_3 unit, one carboxyl group, and one (or more) hydroxyl group. These include phenolic acids such as hydroxyl derivatives of benzoic or cinnamic acids. (Fig. 8.1).

8.4.1 Plants Containing Phenolic Glycosides

Phenols and its glycosides are distributed widely in plant kingdom. Salicin and populin are present in the Salicaceae family; arbutin is distributed in Ericaceae and Rosaceae plants; coniferin in Coniferae; glucovanillin in *Vanilla* sp. and in some Graminae plants [4]. Among angiosperms, benzoic acid derivatives, phenolic acids, and their glycosides are universal. The most common are *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acid. Less common are salicylic and *o*-protocatechuic acid which are distributed mainly in the Ericaceae family. Gallic acid, which is very reactive, is found in woody plants (as galotannin) or as the dimer, elagic acid. Rosmarinic acid is distributed mainly in the Lamiaceae family. Free phenolic acids are relatively rare in plants as they are bounded as glycosides or esters, and also occur as condensation products [12]. The most important therapeutic plants containing PHGs are listed in the Table 8.1.

8.4.2 Bioactivity of Phenolic Glycosides

Well-documented and known bioactivities of plant-derived phenolic glycosides are summarized in Table 8.1. Here, some interesting and promising new activities are highlighted to illustrate the therapeutic potential of newly discovered

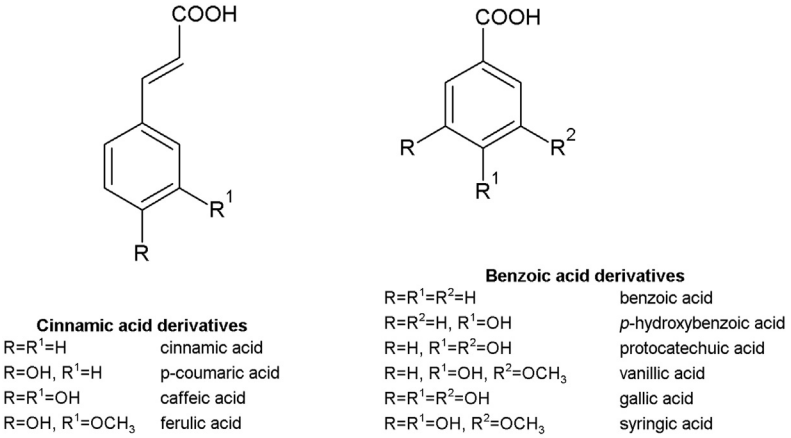
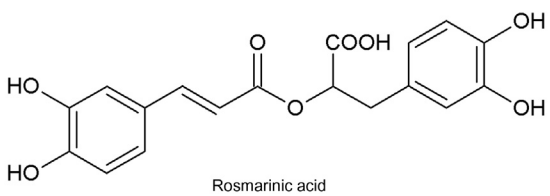


FIGURE 8.1 The phenolic acids examples of benzoic and cinnamic acid derivatives.



Rosmarinic acid

TABLE 8.1 The main plant drugs containing phenolic glycosides

Plant- botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose /undesirable effects /interactions	Clinical trials /preclinical studies /contra-indications
Phenolic glycosides				
*Salix alb*a L. *Salix purpurea* L *Salix daphnoides* Vill. *Salix fragilis* L. (Salicaceae)	***Salicis cortex*** **Willow Bark** phenol glycosides (10%) salicin (4-8% of total salicin in *Salix alba* bark) salicortin, tremulacin, salireposide fragilin, populin phenolic acids: salicylic, caffeic, ferulic [1]	Anti-inflammatory Antipyretic effect Anti-rheumatic Uses: relief of low back pain, symptomatic relief of mild osteoarthritic and rheumatic complains. Dried hydro-alcoholic or aqueous extract (120-240 mg of total salicin) = adult daily dose, for oral administration.	No toxic effects reported. Interactions: Salicin inhibits platelet aggregation.	In clinical studies (240 mg of salicin/for 2 weeks) moderate analgesic effect in osteoarthritis was observed. Also willow bark was useful and safe for treatment of low back pain (2x120 mg or 2x60 mg of salicin/4 weeks). Contra-indications: none known, but in case of sensitivity to salicylates the use should be avoided.
Filipendula ulmaria (L.) Maxim. = *Spiraea ulmaria* L. (Rosaceae)	***Filipendulae ulmariae herba (orflos)*** **Meadowsweet** phenol glycosides (up 0.5% of total); salicylaldehyde glycoside: monotropitin = gaultherin, methyl salicylate glycosides: spirein (1,2%) salicyl alcohol glycoside: isosalicyn phenolic acids: salicylic, caffeic, ferulic	Herbal preparations: powdered plant material, tincture (1:5 in 45% ethanol). Pharmaceutical preparations: tea, solid or liquid preparations for oral use. Used in supportive therapy of common cold, used to enhance the renal elimination of water (not supported by scientific evidence). Anti-inflammatory Anti-microbial Anti-rheumatic Antipyretic	Undesirable effects and interactions: none reported.	Contra-indications: none known, but in case of sensitivity to salicylates the use should be avoided.
Populus nigra L. *Populus tremula* L. (Salicaceae)	***Populi gemmae*** – ***Populi folium*** **Poplar** phenol glycosides; salicin, populin (6'-benzoilosalicin) phenolic acids: caffeic, chlorogenic, ferulic, salicylic [1,2]	Anti-microbial Anti-rheumatic Antipyretic Orally, popularly is used as an ingredient in herbal cough preparations. It is also used orally as a stimulant and expectorant. Topically, poplar is used for sores, bruises, cuts, pimples, external hemorrhoids, frostbite, and sunburn [3].	Undesirable effects and interactions: none reported.	Contra-indications: none known, but in case of sensitivity to salicylates the use should be avoided.
Arctostaphyllos uva-ursi (L.) Spreng. (Ericaceae)	***Uvae-ursi folium*** **Bearberry Leaf** arbutin (= hydroquinone β-D-glucopyranoside) (5-15%) methylarbutin (4%) gallic acid galloylarbutin hydroquinone methylhyroquinone	Infections of lower urinary tract e.g. cystitis. Adult: cold water infusions of the dried leaf (400-800 mg arbutin/day) divided into 2-3 doses. Not recommended for children. For oral treatment. Alkalinization (pH 8) of the urine may be beneficial and also consumption plenty of liquid.	Nausea and vomiting may occur due to stomach irritation from the high tannin content in leaf. Hydroquinone is topical irritant and hapatotoxic. Interactions: concomitant of acidification of the urine decreases efficacy of treatment. Patients should avoid highly acidic foods, such as acidic fruits or fruit juice, during treatment	Contra-indications: kidney disorders. Avoid during pregnancy.

(*Continued*)

TABLE 8.1 (Continued)

Plant- botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose /undesirable effects /interactions	Clinical trials /preclinical studies /contra-indications
Calluna vulgaris L. (Hull.) *Pyrus communis* L. (Rosaceae)	***Ericae flos*** Arbutin 0.5-1% ***Pyri folium*** **Pear Leaf** Arbutin 2-3%	Plant sources of arbutin. Arbutin as tyrosinase inhibitor is one of ingredients in cosmetic preparations applied topically to reduce undesirable skin pigments and for skin whitening [4].	No toxic effects and interactions reported. No toxic effects and interactions reported.	No clinical studies. No clinical studies.
Bergenia crassifolia (L.) Fritsch (Saxifragaceae)	***Bergeniae herba*** **Elephant-eared saxifrage** Arbutin 10-19% 2-O-caffeoylarbutin 6-O-galloylarbutin hydroquinone p-galloyloxyphenyl β-D-glucoside pyrogallol, Phenolic acids: acetylsalicylic, caffeoyl quinic, fumaric, furancarboxylic, gallic malic, protocatechuic quinic acid, ellagic acid, [5].	Used as infusions, decoction or hydro-alcoholic extract. Applied in ethnomedicine (TCM, Mongolian, Russian) anti-inflammatory antidiabetic adaptogenic diuretic skin whitening (arbutin) Infusions are recommended in gynecology for excessive menstruation, bleeding and cervical erosion treatment [6]. Also as a source of arbutin.	No toxic effects in rats and in humans. Humans are able to tolerate hydroquinone up to 450 mgdaily or 6.5 mg/kg for up to 5 months.	In animal *in vivo* studies; Anti-inflammatory and immunomodulating effect of dry extract of *Bergenia* leaves (min 18% arbutin) was observed in DBA/2 mice. Gastroprotective effect after pre-administration of arbutin (30, 60 mg/kg, per oral, 14 days) in aspirin induced ulcers in rats[5]. In the available literature no clinical data for *B. crassifolia* were found.
Phenolic acids and their glycosides				
Cynara scolymus L. (Asteraceae)	***Cynarae folium*** **Artichoke Leaf** Caffeoylquinic acids: chlorogenic, 1,5-dicaffeoylquinic acid cinarin (1,3-dicaffeoylquinic acid) only in traces in leaves after processing with hot water. caffeic acid [7]. Aliphatic acids: malic, lactic, hydroxymethylacrylic acid	Used in digestive complaints (stomach ache, nausea, vomiting, and feeling of fullness) and hepatobiliary disturbances. In low fat diet as adjuvant in the treatment of mild to moderate hyperlipidaemia. Adult daily dose: 5-10 g dried leaf as aqueous dry extract, infusion or other preparations. For oral administration. Choleretic, hepatoprotective, antioxidant, cholesterol-lowering and lipid-lowering effects (due to caffeoylquinic acids and flavonoids).	No toxic effects reported. Undesirable effects: mild gastrointestinal disturbances, allergic reactions in sensitive patients.	Contra-indications: obstruction of the bile tract, allergy to Asteraceae. Effects confirmed in clinical studies – see more in ESCOP 2009 monograph.
Echinacea purpurea (L.) Moench. (Asteraceae)	***Echinaceae purpureae herba*** **Purple Coneflower Herb** ***Echinaceae purpureae radix*** **Purple Coneflower Root** Caffeic acid derivatives: cichoric acid (2,3-O-dicaffeoyl-tartaric acid) 2-O-feruoyl-tartaric acid 2-O-caffeoyl-3-O-coumaroyl-tartaric acid	Internal use: as an adjuvant therapy and prophylaxis of infections of the upper respiratory tract (common colds). External use: in therapy of superficial wounds.	Toxicity – none reported. Interactions- none reported. Undesirable effects: hypersensitivity reactions e.g. skin reactions.	Hypersensitivity to plants of the Asteraceae. Not recommended in autoimmune diseases (tuberculosis, colagenoses, multiple sclerosis, AIDS and HIV infections).

Melissa officinalis L. (Lamiaceae)	***Melissae folium*** **Melissa Leaf** Phenolic acids (up to 4%); caffeic, chlorogenic and rosmarinic acid.	Internal use: Tenseness, restlessness, irritability, symptomatic treatment of digestive disorders e.g. minor spasms. External use (topical application): *Herpes labialis* (cold sores). Use: 2-3 g of the drug as an infusion 2-3times daily. Tincture (1:5, 45% ethanol) 2-6 ml/3 times/ day Antispasmodic, antiviral (anti-HIV-1 activity of aqueous extract), anti-inflammatory (rosmarinic acid), antimicrobial, antioxidant sedative effects	No toxic effects reported. Undesirable effects: none reported.	In clinical studies antiviral and sedative properties were confirmed. Contra-indications: none known.
Urtica dioica L. *Urtica urens* L. (Urticaceae)	***Urticae folium/herba*** **Nettle Leaf/Herb** Phenolic acids: caffeic acid esters (caffeoylmalic acid: up to 1.6%) chlorogenic acid, neochlorogenic acid.	Adjuvant in treatment of arthritis, arthroses and rheumatic conditions. Used as diuretic in e.g. urinary tract inflammatory complains. Adult dose: hydroalcoholic extract (= 8-12 g of nettle leaf daily/2-3 doses), Infusions (3-5 g drug/3 times/day), tincture 1:5 (25% ethanol) 2-6 mL/ 3 times/day. For oral or topical administration. Anti-inflammatory Diuretic Hypotensive Analgesic antirheumatic	Undesirable effects:none reported.	In clinical studies anti-arthretic and antirheumatic activities were confirmed. Preclinical safety data from in vivo studies in mice – positive. Contra-indications: none known.
Aldehyde glycosides				
Vanilla planifolia Jacks. ex Andrews (Orchidaceae)	**Vanilla Pods** **flat-leaved vanilla pods** glucovanillin and its' aglycone vanillin	Used as flavouring agent as whole pod, vanilla powder, extract (35% alcohol), vanilla sugar. Added to foods to reduce the amount of sugar needed for sweetening. Vanilla is used as a flavoring in syrups used in making medications and as a fragrance in perfumes.	Skin contact can cause irritation and swelling (inflammation). It might also cause headache and sleep problems (insomnia). No toxic effects reported.	No data available.

Data collected in the Table 8.1 were derived/summarized from EMA Assessment Reports, ESCOP and European Pharmacopoeia – Council of Europe monographs and from references below.

[1] Boeckler GA, Gershenzon J, Unsicker SB. Phenolic glycosides of the Salicaceae and their role as anti-herbivore defenses. Phytochemistry 2011;72:1497–1509.
[2] Si C-L, Wu L, Shu Z-Y. Phenolic glycosides from *Populus davidiana* bark. Biochem Syst Ecol 2009;37:221–224.
[3] http://naturaldatabase.therapeuticresearch.com/nd/PrintVersion.aspx?id = 245&AspxAutoDetectCookieSupport = 1
[4] Lim YJ, Lee EH, Kang TH, Ha SK, Oh MS, Kim SM, Yoon TJ, Kang C, Park JH, Kim SY. Inhibitory effects of arbutin on melanin biosynthesis of alpha-melanocyte stimulating hormone-induced hyperpigmentation in cultured brownish guinea pig skin tissues. Arch Pharm Res 2009;32:367–373.
[5] Shikov AN, Pozharitskaya ON, Makarov MN, Makarov VG, Wagner H. *Bergenia crassifolia* (L.) Fritsch – Pharmacology and phytochemistry. Phytomedicine 2014;21:1534–1542.
[6] Shikov AN, Pozharitskaya ON, Makarov VG, Wagner H, Verpoorte R, Heinrich M. Medicinal plants of the Russian Pharmacopoeia; their history and applications. J Ethnopharmacol 2014;154:481–536.
[7] IUPAC Commission on the Nomenclature of Organic Chemistry. CBN. Nomenclature of Cyclitols: Recommendations, 1973. Biochem J 1976;153:23–31.

and also new possibilities of well-known PHGs. The aqueous extract of the leaves of *Bergenia* sp., which contains arbutin, displayed antiviral activity in vitro against herpes simplex virus type 2, influenza virus A2 (Mannheim 57), and vaccinia virus at a concentration of 10% [30]. Arbutin a major phenolic of *Bergenia* sp. inhibits tyrosinase enzyme and has been employed as a cosmetic skin-whitening agent in humans. Arbutin also inhibits melanin production in B16 melanoma cells induced with α-melanocyte-stimulating hormone (α-MSH) and decreases tyrosinase activity in a cell-free system [31]. Two new phenolic glycosides, gnaphaffine A and B were isolated from *Gnaphalium affine* and anticomplementary activity of these components was confirmed as compared to heparin [32]. Three new phenolic glycosides, robustasides E, F, G, in addition to the known compound robustaside D, were isolated from the MeOH extract from the leaves and twigs of *Grevillea* "Poorinda Queen." In vitro and in vivo antimalarial activities as well as in vitro cytotoxic activities were analyzed and confirmed for robustasides E, G, and D [33]. Antioxidant activity of new hydroxy-3-methoxyphenyl-6-*O*-syringoyl-β-D-glucoside isolated from *Bridelia cambodiana* Gagnep. (Euphorbiaceae) was confirmed [34]. Through estrogen biosynthesis-guided fractionation in human ovarian granulosa-like KGN cells, five new PHGs, broussoside A−E were isolated from the leaves of *Broussonetia papyrifera* (L.) Vent. These compounds were found to potently inhibit estrogen biosynthesis in KGN cells [35]. Some new applications of modified PHGs in the cancer treatment are also promising, e.g., alkyl esters of gallic acid, which are more effective against tumor cell lines than gallic acid [36].

8.4.3 Nutraceutical Applications of Phenolic Glycosides

Bioaccessibility of nutraceuticals from willow bark (*Salix alba, S. daphnoides, S. purpurea*) was evaluated using in vivo studies. The results obtained indicate that high bioaccessibility and bioavailability of phytopharmaceuticals in extracts from bark of *Salix* genotypes can provide health promoting benefits to the consumers [37]. Phenolic acids and their glycosides are ubiquitous and can be found in many plant species consumed as a part of the human diet; in vegetables and fruits, and also in beverages. Health benefits from consumption of these dietary components (phenolic glycosides, especially phenolic acids, which contribute in biological activities) are obvious and well documented [3,4,11,38−40].

8.4.4 Pharmaceutical Applications of Phenolic Glycosides

Widely used plant drugs containing PHGs with their therapeutic use are summarized in the Table 8.1, where the division on plants containing PHGs and plants containing phenolic acids is presented. It must be mentioned that other different groups of compounds coexist in these plant drugs and have influence on their therapeutic potential.

8.4.5 Adverse Effects of Phenolic Glycosides

Adverse effects of PHGs are not significant and are listed in Table 8.1.

8.4.6 Metabolic Profile of Phenolic Glycosides

The most well-known PHGs used in treatment are salicin and arbutin, and their metabolic profiles are below described briefly. β-D-Salicin, upon oral administration, is metabolized (which involves glycon hydrolysis and oxidation of benzyl carbon) in the gastrointestinal tract and bloodstream into the pharmacological active form, salicylic acid. These compounds have been identified to exert a modulating role in inflammatory processes (inhibition of the activation of NF-κB and down-regulating COX-2 expression) [41]. Salicylic acid undergoes further metabolism in the liver and kidney. Cytochrome p-450 enzyme system in mitochondria is capable of catalyzing salicylate into different metabolites such as 2,3,5-trihydroxy benzoic acid, 2,5-dihydroxy benzoic acid, and catechol. Salicylic acid is readily available for the conjugation reaction with glycine to form salicyluric acid or D-glucuronic acid and form salicylacyl glucuronide or 1-salicylate glucuronide *via* the formation of ether or ester bonds [42]. Salicylic acid has recognized antipyretic and antiinflammatory properties that underlie the use of willow bark for fever and arthritis. Acetylsalicylic acid (aspirin) is a synthetic derivative of salicylic acid that in addition has pronounced antiplatelet properties due to the presence of the acetyl group [43].

After ingestion, arbutin is absorbed from the gastrointestinal tract. Intestinal flora hydrolyzes it to form the aglycone, the free hydroquinone. Hydroquinone glucuronate and sulfate esters are excreted in the urine in physiological pH. When the urine is alkaline (pH 8.0), active hydroquinone derivatives exert an antiseptic and astringent effect on the urinary mucous membranes, reaching a maximum of antibacterial action approximately 3−4 h after ingestion. Oral administration of 800 mg arbutin or an infusion of the leaves containing an equivalent amount of arbutin to healthy volunteers had strong antibacterial activity, as measured in urine samples after adjustment of the urine pH to 8.0 [44]. There is no direct

evidence, regarding human data, supporting the fact that free hydroquinone causes convulsion, hepatotoxicity, nephrotoxicity, or promotion of tumors in humans, and under the recommended use conditions *Uva-ursi folium* is safe for treating lower urinary tract infections [45].

8.5 COUMARIN GLYCOSIDES (CMG) AND CHROMONE GLYCOSIDES (CHG)

Coumarins owe their class name to "Coumarou," the name of the tonka bean (*Dipteryx odorata* Willd., Fabaceae), from which coumarin was isolated in 1820 [3]. The coumarin nucleus consists of a pyrone ring joined to a benzene ring. These structures are derived from the *trans*−*cis* isomerization of the side chain of *o*-coumaric acid followed by lactonization [3,4,46]. Coumarins are 1,2-benzopyrones (benzo-2-pyrones, chromene-2-ones) and are classified as: simple coumarins, furanocoumarins, pyranocoumarins, and the pyrone-substituted coumarins (benzocoumarins and furanobenzocoumarins with benzene or benzofuran ring respectively, condensed at C-3/C-4 to coumarin structure) (Fig. 8.2). Isomers of coumarins (isocoumarins) are also found in plants [3,4,46,47].

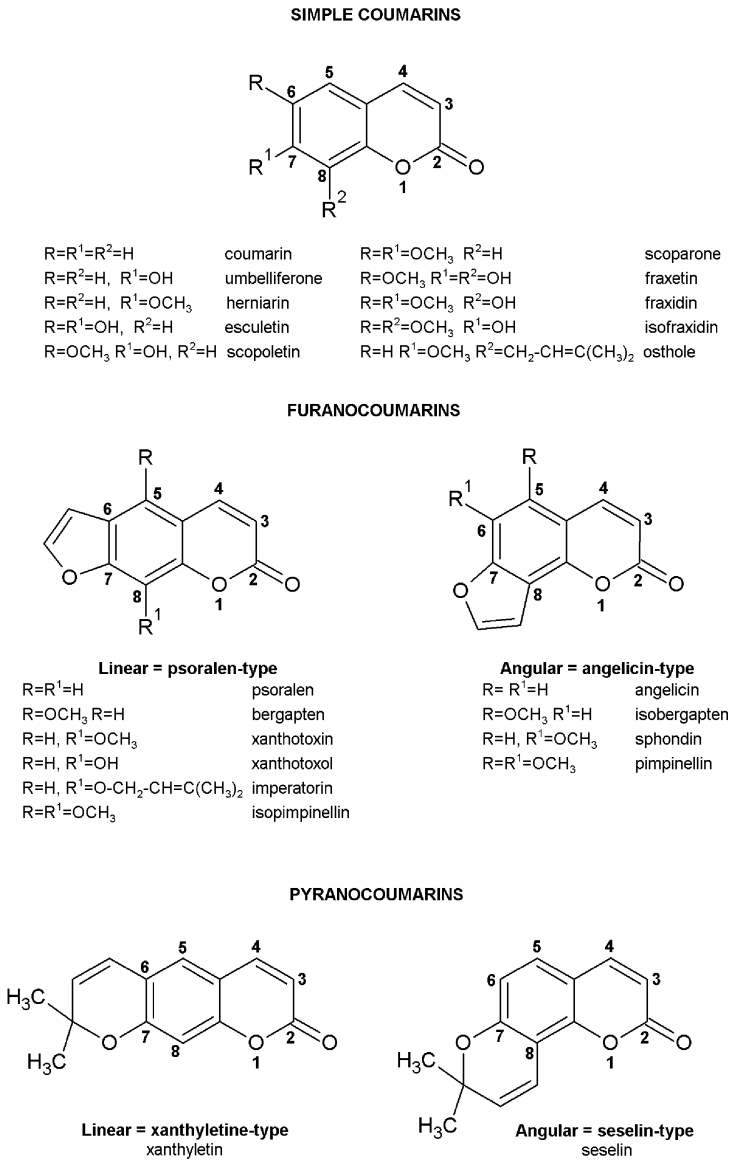


FIGURE 8.2 Examples of the main types of coumarin compounds: simple, furano-, and pyranocoumarins.

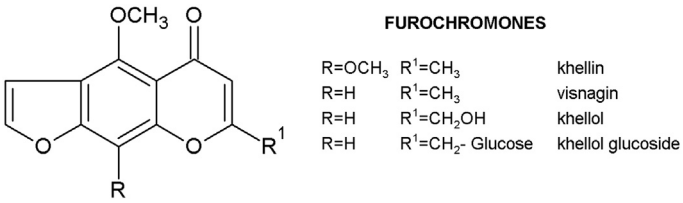


FIGURE 8.3 Furochromones from *Ammi visnaga* L. (Apiaeae).

In coumarin glycosides, the sugar moiety is most frequently attached to a hydroxyl group at position C-7 of the coumarin nucleus but it may also be located at position C-4, C-5, C-6, or C-8. All the coumarin glycosides isolated are *O*-glycosides, with the exception of dauroside D isolated from *Haplophyllum dauricum* (L.) G. Don., which is so far the only coumarin C-glycoside. The first isolated natural coumarins; daphnin (in 1812), esculin and fraxin proved to be glycosides. Most frequently attached sugars are D-glucose, D-galactose, L-rhamnose, and D-apiose, and more rarely D-xylose and L-arabinose. In coumarins from microbial sources, deoxysugar noviose is present [47]. Also disaccharides have been found as components of the coumarin glycosides, e.g., rutinose (6-*O*-α-L-rhamnopyranosyl-D-glucopyranose), gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucopyranose), primeverose (6-*O*-β-D-xylopyranosyl-D-glucopyranose), and 6-*O*-8-D-apiosyl-D-glucopyranose [48].

Chromones are chemically benzo-γ-pyrones (4*H*-1-benzopyran-4-ones) which constitute an important class of oxygen atom containing heterocyclic compounds which are ubiquitously found in the plant kingdom [46,49]. The word "chromone" is derived from the Greek word "*chroma*" (color), because many chromone derivatives exhibit a diversity of colors [46,50].

The structural diversity found in the chromone group led to their division into different categories: simple chromones and fused chromones (6,7-furanochromones, 6,7- or 7,8-pyranochromones, 6,7- or 7,8-benzochromones and hydroxypyranochromones) [51]. Furo(furano)chromones and pyranochromones are compounds in which the chromone structure is condensed at C-6/C-7 position with a furan or pyran ring respectively. The most well-known furochromones are khellin, wisnagin, khellol, and khellol glucoside (Fig. 8.3) found in *Ammi visnaga* L. fruit (Khella) from the Apiaceae family [52].

8.5.1 Plants Containing Coumarin and Chromone Glycosides

The most widespread plant coumarin is simple unsubstituted coumarin, which is common in many grasses and fodder crops (sweet smelling volatile, which is released during plant harvesting and drying). Substituted coumarins, e.g., hydroxy- or methoxy-coumarins such as umbelliferone (7-hydroxy), aesculetin (6,7-dihyroxy), herniarin (7-methoxy), scopoletin (6-hydroxy, 7-methoxycoumarin), are also common in Rutaceae, Rubiaceae, Apiaceae, Asteraceae, Oleaceae, and Thymelaeaceae plants. Furanocoumarins are typical for Rutaceae and Apiaceae while pyranocoumarins mostly occur in the Apiaceae family [53,54]. Regarded as part of the plant defense system, these compounds are released on the plant's surface under stress conditions and are mostly present in the young parts of the plant and in generative organs. The coumarins occur at the highest levels in the fruits, followed by the roots, stems, and leaves. Some members of coumarin group have been isolated from microbial sources, e.g., novobiocin and coumermycin from *Streptomyces*, and aflatoxins from *Aspergillus* species [47,55].

Chromones in plants have found to be active in a number of plant cycles, including growth regulation, indole acetic acid oxidation, and dormancy inhibition, as well as exhibitingcytokinin-type behavior and stimulating oxygen uptake in plant tissues [51]. Plants containing different types of coumarins and furochromones are listed in the Table 8.2.

8.5.2 Bioactivity of Coumarin and Chromone Glycosides

Coumarins and their derivatives are widely used as additives in food, perfumes, cosmetics, pharmaceuticals optical brighteners, dispersed fluorescent, and laser dyes [56]. These compounds have numerous therapeutic applications including photochemotherapy, antitumor, and anti-HIV therapy, and are known as lipid-lowering agents with moderate triglyceride-lowering activity. Antibacterial, anticoagulant, CNS stimulant, and antiinflammatory activities of coumarins are also known [57–59]. Other properties of simple coumarins include the gastroprotective activity of aesculin, antiasthmatic and antidiabetic activity of umbelliferone, and antithyroid activity of scopoletin [11,59].

TABLE 8.2 Plant drugs containing coumarins and furochromones

Plant- botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose /undesirable effects /interactions	Clinical trials /preclinical studies /contra-indications
Coumarins				
Simple Coumarins				
Aesculus hippocastanum L. (Hippocastanaceae)	***Hippocastani cortex*** **Horse-Chestnut Bark** Coumarin derivatives (up to 7%) esculin (esculetin-6-glucoside) fraxin (= fraxetin-8-glucoside), scopolin (= scopoletin 7-glucoside) esculetin, fraxetin, scopoletin	Used as herbal substance powdered or as preparation (capsules, dry extract), or topically as compresses. Venotonic, anti-platelet, anti-inflammatory activity. Anti-oxidant properties through inhibition of the peroxidation and some enzymes activity (β-glucuronidase, elastase, collagenase, hyaluronidase, 5-lipoxygenase) or a scavenging activity against ROS. Esculin acts as vitamin P and protects veins and is anti-haemorroidal, It absorbs UV light (370 nm) and protects skin against UV light damage.	It has been recommended that patients taking horse chestnut extracts concurrently with medications that have anticoagulant effects, such as warfarin, should be closely monitored for signs of symptoms of bleeding. /The acute toxicity of esculin and esculetin is low. Allergic reaction after the rectal administration of esculin (haemorrhoids treatment).	No data available.
Fraxinus excelsior L. *Fraxinus oxyphylla* M. Bieb. *Fraxinus angustifolia* Vahl. (Oleaceae)	***Fraxini cortex*** **Ash Bark** fraxetin, fraxin fraxidin, isofraxidin kelicanthoside (isofraxidin-7-glucoside) esculetin, esculin ***Fraxini folium*** **Ash Leaf** additionally; cichoriin, scopoletin	Anti-phlogistic Anti-rheumatic (enhances ureic acid excretion in urine) Vasoprotective	None reported for monopreparations.	*In vivo* study in mice revealed that esculetin and esculin have beneficial effects on hyperuricemia and renal dysfunction. Clinically effective in the treatment of gout, arthritis, diarrhea and bacillary dysentery in traditional Chinese medicine [1].
Melilotus officinalis L. *Melilotus altissimus* Thuill. (Fabaceae)	***Meliloti herba*** **Sweet Clover** **Melilot** Melilotoside (cis-O-coumaric acid β-glucoside) free coumarin melilotin (3,4-dihydrocoumarin) umbelliferone scopoletin From properly dried melilot dicoumarol is absent.	Treatment of symptoms of varicose veins; heavy legs, itching, and swelling. Internal use; drug or preparation corresponding to 3-30 mg of coumarin daily. External use; extracts in semi-solid preparations as topical application. Anti-edema Anti-coagulant (dicoumarol is vitamin K antagonist)	Undesirable effects: in rare cases headaches. Internal use may potentiate activity of anticoagulants.	In clinical studies administration of 200 mg of dry extract of melilot daily reduces symptoms of chronic venous insufficiency. Melilot extract significantly reduces lymphedema. Contra-indications none known.
Artemisia abrotanum L. (Asteraceae)	***Abrotani herba*** **Southern Wormwood** isofraxidin scopoletin umbelliferone	Use in dyspepsia and inadequate bile secretion, also as sedative in nervous intestinal disorders.	It causing miscarriages and have a stimulating effect on the uterus. Children under 12 should avoid internal consumption of the plant.May cause both skin rashes and allergic reactions.	It should not be used during pregnancy.

(*Continued*)

TABLE 8.2 (Continued)

Plant- botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose /undesirable effects /interactions	Clinical trials /preclinical studies /contra-indications
Furano- and Pyranocoumarins				
Archangelica officinalis Hoffm. (= *Angelica archangelica* L.) (Apiaceae)	***Archangelicae radix*** **Angelica Root** Furocoumarins (psoralen-type): bergapten, xanthotoxin, imperatorin oxypeucedanin, (angelicin-type): angelicin, archangelicin Simple coumarins: umbelliferone, prenylcoumarins: osthol and osthenol	Used in dyspeptic complains; spasm, sluggish digestion, flatulence, feeling of fullness, loss of appetite, bronchitis. Daily dose; 3-6 g of the drug, or as infusion (ethanol 25%, tincture 50% ethanol 1.5-6 mL divided into three doses). Coumarins are spasmolytic (Ca^{2+} channels blockers) Angelicin acts as sedative. Psoralen-type coumarins are isolated and used in vitiligo and psoriasis PUVA therapy. Root is used in the loss of appetite as bitter.	Can induce photo-dermatitis in case of prolonged use and exposition on to UV radiation (sunlight).	No safety data for monopreparations available. For mixture herbal preparations and liquid products over 40 years history of safe use in treatment of gastrointestinal complains exists.
Angelica sinensis (Oliv.) Diels (Apiaceae)	***Angelicae radix*** **Danggui** Simple coumarins: angelol G, angelicone, umbelliferone Furanocoumarins: bergapten, imperatorin, oxypeucedanin, psoralen, osthol	Used orally in water infusions. Effective in relieving menopausal symptoms. Anti-inflammatory Anti-platelet action. Anti-pyretic In Chinese tradition, Danggui is used mostly to enrich blood, activate blood circulation, regulate menstruation, relieve pain, and relax bowels.	There are no data on genotoxicity, carcinogenicity and reproductive and development toxicity. May induce phototoxicity.	The most important in exerting pharmacological effects (inflammatory, antiplatelet) in patients after taking oral forms are trans-ferulic acid and other hydrophilic compounds.
Pimpinella saxifraga L. *Pimpinella major* (L.) Huds. (Apiaceae)	***Pimpinellae saxifragae radix*** **Brunet Saxifrage** ***Pimpinellae majoris radix*** **Greater Brunet Saxifrage** Furanocoumarins: pimpinellin, isopimpinellin, bergapten, isobergapten, imperatorin	Spasmolytic in bile tract (cholagogue), in urine tract (diuretic) and in respiratory tract (bronchial tubes). In treatment of wounds. Internal use eases digestion, helps respiratory problems, used in treatments of kidney and urinary diseases. Anti-inflammatory. In symptoms of laryngitis and bronchitis (also expectorant). Used for kidney stones, fluid retention (edema).	May induce phototoxicity.	No data available.
Ruta graveolens L. (Rutaceae)	***Rutae herba*** **Herb of Grace** **Garden Rue** Psoralene-type furocoumarins:	Cholagogue as an antispasmodic to relieve cramps, as an emmenagogue to promote menstrual flow	Adverse effects; abortive, phototoxic, mutagenic, contact skin inflammatory, in case of long term use.	Avoid during pregnancy.

	psoralene, bergapten, xanthotoxin, isopimpinellin, isoimperatorin Pyranocoumarins: xanthyletin Simple coumarins: umbelliferone, herniarin	anti-bacterial anti-fungal Important veterinary medicine: used as an anthelmintic, antispasmodic, antiepileptic, rubefacient and an emmenagogue in herbal formulations for animals.	Large doses or prolonged use may cause headache.	
Pastinaca sativa L. (Apiaceae)	***Pastinacae sativae fructus/ radix*** **Parsnip Fruit/Root** Angelicin Psoralene-type furocoumarins: bergaptene, xanthotoxin, imperatorin, psoralen	The fruits are used in kidney and gastrointestinal complaints and for digestion problems.	Can induce photo-dermatitis in case of prolonged use and exposition on to UV radiation (sunlight).	
Ammi majus L. (Apiaceae)	***Ammi majoris fructus*** **Greater Ammi Fruit** Psoralene derivatives: xanthotoxin, imperatorin, bergapten	Used in photochemotherapy to treat a number of skin disorders, including mycosis fungoides, psoriasis and vitiligo.	Can induce photo-dermatitis in case of prolonged use and exposition on to UV radiation (sunlight).	
Furochromones				
Ammi visnaga L. (Apiaceae)	***Ammi visnagae fructus*** **Khella** Furochromones: khellin, visnagin, khellol, khellol glucoside, ammiol, khellinin Pyranocoumarins (seselin-type): visnadin, samidin, dihydrosamidin	Furochromones and visnadin have strongly antispasmodic action on the smaller bronchial muscles (in coronary veins, urinary tract, bronchial tubes). Dilate the bronchial, urinary and blood vessels without affecting blood pressure. Used in the treatment of asthma, angina, coronary arteriosclerosis, and kidney stones. Samidin and khellol glucoside induce positive inotropic effect on the heart [2]. Used also in treatment of vitiligo, antimicrobial and antioxidant.	Prolonged use may lead to: constipation, appetite loss, headaches, sleep disorders, vertigo, dizziness, nausea and vomiting. Photosensitivity.	Avoid during pregnancy and lactation. Avoid if on warfarin or other blood thinning medication. During therapy avoid exposition on sun light or other UV sources.

Data collected in the Table 8.2 were derived/summarized from EMA Assessment Reports, ESCOP and European Pharmacopoeia – Council of Europe monographs and from references below.

[1] Li J-M, Zhang X, Wang X, Xie Y-C, Kong L-D. Protective effects of cortex fraxini coumarines against oxonate-induced hyperuricemia and renal dysfunction in mice. Eur J Pharm 2011;666(1–3):196-204.
[2] Al-Snafi EA. Chemical constituents and pharmacological activities of *Ammi majus* and *Ammi visnaga*. A review. Int J Pharm Ind Res 2013;3(3):257–265.

The furanocoumarin xanthotoxin (and other psoralens) and UVA light were highly effective in the control of psoriasis and the malignant skin condition mycosis fungoid. Furanocoumarins in conjunction with UV light kill bacteria and inactivate viruses. They may be responsible for the enhanced bioavailability that grapefruit juice affords to several pharmaceuticals [11]. A number of coumarins have been tested for antifungal activity, and the most effective ones are osthole, psoralen, imperatorin, and ostruthin [57]. The biological activities of coumarins and furochromones are summarized in Table 8.2.

The simple coumarins such as scopoletin and umbelliferone are found to be active against *Mycobacterium tuberculosis* [57]. Osthole could be a potential therapeutic agent for the treatment of multiple sclerosis [46,60]. Two new natural CMGs, 6-methoxy-5,7-dihydroxy-3,4-dihydrocoumarin-8-*C*-glucopyranoside and 5-vinyl-6,7-dimethoxy-3,4-dihydrocoumarin-8-*C*-glucopyranoside, isolated from *Diceratella elliptica* (DC.) Jonsell. growing in Egypt, showed high activity against three human carcinoma cell lines: liver (HEPG2), cervix (HELA), and colon (HCT116) [61]. Coumarin-based selective estrogen receptor modulators (SERMs) and coumarin−estrogen conjugates have been described as potential antibreast-cancer agents [62]. *Exostema caribaeum*, containing glycosides of 4-phenylcoumarin, is used in Mexico as an antimalarial agent [63]. Antiplasmodial coumarin isolated from the roots of *Toddalia asiatica* supports the traditional use of this plant for the treatment of malaria [3]. Coumarins isolated from *Calophyllum lanigerum*, calanolide A and its two isomers costatolide and dihydrocostatolide, are highly effective inhibitors of clinical strains, including those representing various HIV-1 clades [64,65]. Coumarins from *Peucedanum ostruthium* [66], the dihydropyranocoumarin decursinol, the furanocoumarin isoimperatorin, and the dihydrofuranocoumarin nodakenin, isolated from underground parts of Chinese medicinal plant *Angelica gigas*, have shown acetylcholinesterase inhibitory activity [66,67]. The coumarins imperatorin and osthole have been found to possess anticonvulsant activities [68,69]. The anticonvulsant activity of xanthotoxin isolated from the fruits of *Pastinaca sativa*, was also found in in vivo experiments in mice [70]. Two new chromone glycosides, 5-hydroxy-2,6,8-trimethylchromone 7-*O*-b-D-glucopyranoside (uncinoside A) and 5-acetoxyl-2,6,8-trimethylchromone 7-*O*-b-D-glucopyranoside (uncinoside B) which were isolated from the Chinese herb *Selaginella uncinata* Spring., showed potent antiviral activities against respiratory syncytial virus and moderate antiviral activities against parainfluenza type 3 virus [71].

8.5.3 Nutraceutical Applications of Coumarin and Chromone Glycosides

Coumarins are found at high levels in some essential oils, e.g., in cinnamon bark oil and lavender oil [57], and also found in the fruits (e.g., bilberry, cloudberry), green tea, chicory, and in many cultivated plants from the Apiaceae family, such as parsnip, carrot, coriander, parsley, so they are common in a human diet [3,4].

8.5.4 Pharmaceutical Applications of Coumarin and Chromone Glycosides

For information about pharmaceutical application of plant drugs containing coumarins and furochromones see content of the Table 8.2.

8.5.5 Adverse Effects of Coumarin and Chromone Glycosides

Used as flavoring agents, coumarins show also their adverse effects, such as mild nausea, diarrhea, and hepatotoxicity, when used in certain amounts. The aflatoxins from *Aspergillus* sp. are a group of highly toxic fungal metabolites of coumarin origin [72]. Among them are aflatoxins B1, B2, G1, and G2, whereas aflatoxin B1 (AFB1) is the most commonly occurring member of the group, due to its extreme toxicity and worldwide occurrence in staple foods and feeds, such as peanuts and corn [73]. Toxic effects of AFB1 on humans are via its direct consumption in food products, or via the indirect consumption of products from animals taking AFB1-contaminated feeds. Aflatoxin B1 is a genotoxic hepatocarcinogen, and the liver is the organ most severely affected it. The primary effects include hemorrhagic necrosis, fatty acid infiltration, and bile duct proliferation, finally resulting in hepatotoxicity and carcinogenesis (hepatocellular carcinoma). AFB1 is metabolized at the liver and has several metabolites, such as aflatoxicol, aflatoxicol H1, and aflatoxin Q1, which are less toxic than AFB1, but aflatoxin B1-8-9-expoxide (AFBO) is the most toxic [73]. AFBO is a reactive intermediate which binds to liver cell DNA, resulting in DNA adducts, which interact with the guanine bases of liver cell DNA and cause a mutational effect [74,75]. Another possible cause of DNA damage is the aflatoxin B1-mediated stimulation of ROS formation, leading to the oxidation of DNA bases [76].

The furanocoumarins are potent photosensitizing agents, and exposure to these compounds can lead to severe photodermatitis and blistering. Only linear furanocoumarins (psoralens) are capable of binding to and cross-linking DNA,

which causes genetic damage and mutations. Nonlinear furanocoumarins (isopsoralens) can only bind to one base to form monoadducts. Use of PUVA therapy in psoriasis is associated with an increased risk of skin cancer, particularly squamous cell carcinoma; which is also associated with the number of treatments and the degree of exposure to UVA [77–79].

8.5.6 Metabolic Profile of Coumarin and Chromone Glycosides

Coumarins are competitive inhibitors of vitamin K in the biosynthesis of prothrombin. The minimal requirements for anticoagulant activity are 4-hydroxy group, a 3-substituent, and a bis-coumarin molecule [80,81], as in dicoumarol structure. Dicoumarol, found in sweet clover, exhibits anticoagulant activity as a vitamin K antagonist. This coumarin produces its anticoagulant effect by inhibiting vitamin K conversion cycle, thereby causing hepatic production of partially carboxylated and decarboxylated proteins with reduced procoagulant activity. The effect of coumarins can be counteracted by vitamin K1 (either ingested in food or administered therapeutically) because the second reductase step is relatively insensitive to vitamin K antagonists [81].

Some furanocoumarins are photosensitizers, therefore they are used in the therapy of psoriasis and vitiligo. Methoxsalen (=8-methoxypsoralen, or xanthotoxin) is used to facilitate repigmentation in idiopathic vitiligo (leukoderma) and for symptomatic control of severe, disabling psoriasis [77]. Linear psoralens can undergo cycloadditions at C-3, C-4, or C-4′, C-5′, or both to pyrimidine bases of DNA, and may each involved in one or two cycloadditions, and form cross-links between the base pairs of the nucleic acids. UVB, UVA, and psoralen plus UVA (PUVA; 320–380 nm) therapy exert a variety of immunomodulatory effects on human skin, and several studies have identified previously unrecognized immunosuppressive/antiinflammatory principles of photo(chemo)therapy [3,78]. Immunomodulation induced by UVB radiation appears to result from UVB radiation-induced generation of DNA photoproducts, in particular thymine dimers. In contrast to UVB radiation, UVA-1 radiation-induced immunomodulatory effects are thought to be based on oxidative mechanisms. Combinations of PUVA and UVB have shown increased efficacy in the treatment of psoriasis that suggests UVB and PUVA have different and perhaps complementary mechanisms of action [79].

Khellin, a furochromone found in the fruit of *Ammi visnaga Lam.* (Apiaceae), is a potent coronary vasodilator and bronchodilator in the treatment of coronary insufficiency, angina pectoris, and bronchial asthma. The mechanism of action involves a nonspecific inhibition of calcium flux, with no difference related to the specific calcium channels. Khellin has the capacity to interfere with the noradrenaline-sensitive mechanisms responsible to loading and releasing calcium stores. Khellin produces concentration-dependent inhibition of noradrenaline and it results in vasodilation [82].

8.6 FLAVONOID GLYCOSIDES (FLG)

Flavonoids are polyphenolic compounds synthesized in plants through the phenylpropanoid or acetate-malonate metabolic pathway [3]. Over ten thousand structural variants of flavonoids have been reported [56,83]. The term “flavonoid” generally describes a broad collection of natural products comprised of C6-C3-C6 structures forming a 2-phenyl-benzo-γ-pyrane (chromane) nucleus consisting of two benzene rings (A and B) linked through a heterocyclic pyran ring (C). These are a group of structurally related compounds divided by the degree of hydroxylation, methoxylation, prenylation, glycosylation, and attachment of the B-ring in C-2 (most frequently) or C-3 position [12,56,84,85]. The basic structural features of flavonoids are presented in Fig. 8.4.

Flavonoids are classified according to the oxidation level of the central pyran C-ring. In flavones and flavonols, hydroxyl groups are mainly present at the C-5, C-7 (ring A), and C-4′ (ring B) and sometimes one of those groups is absent. Other possible hydroxylation occurs at the C-3′ and C-5′ (ring B) and C-3 or C-2 (ring C). The difference also depends on the presence of C-2/C-3 double bond, which is absent in flavanones where asymmetric C-2 is usually in 2*S*-configuration. Isoflavones are characterized by the attachment of phenol B-ring at C-3 position. Chalcones and dihydrochalcones have the open-chain C_3 structure with a ketone functionality and α,β-unsaturation linking the A- and B-rings (in place of a heterocyclic C-ring), distinguishing them from other flavonoids. Chalcones are converted to the (2*S*)-flavanones in a stereospecific reaction catalyzed by the enzyme chalcone isomerase [86,87]. Aurones are 2-benzylidene coumaranones, and anthocyanidins have 2-phenylbenzopyrilium structure. Also anthocyanidins are frequently present as oligomeric and polymeric structures.

More complex structures are bioflavonoids, where two of the same type of structures or mixed structures (flavones, flavanones, and also aurones, chalcones, or isoflavones) are coupled via carbon atoms (C6 or C8) forming aryl linkage

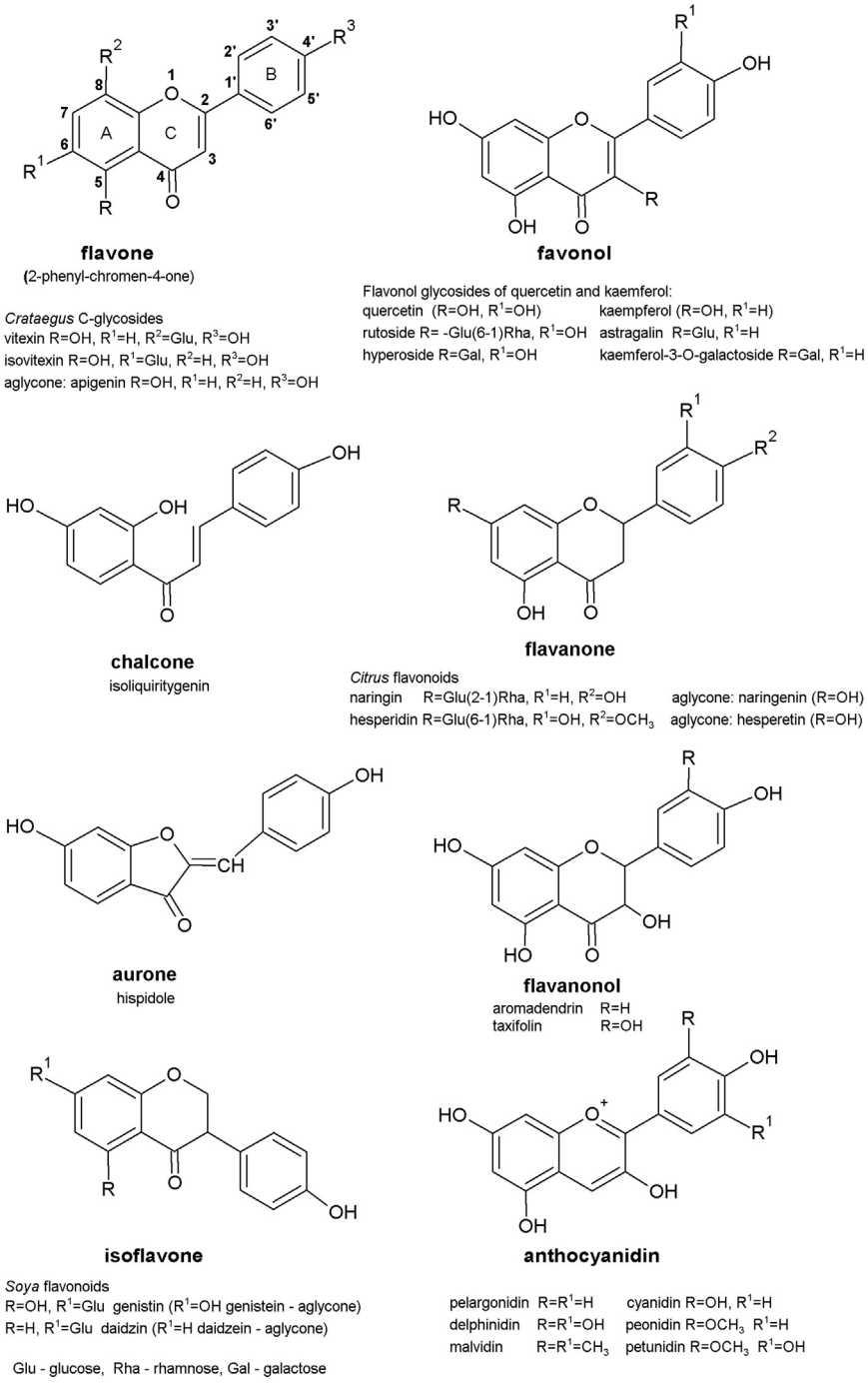
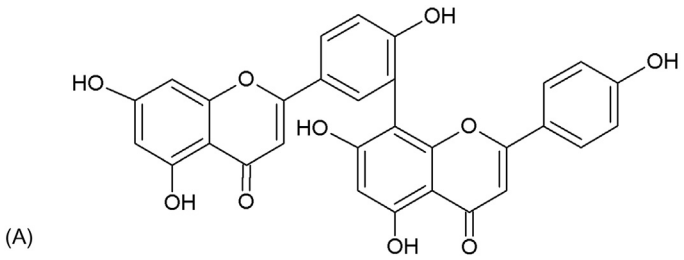


FIGURE 8.4 The structural diversity of flavonoids.

as in ginkgetin, bilobetin, and amentoflavone (Fig. 8.5A), or rarely, via carbon−oxygen forming aryl−ether linkage as in hinokiflavone (Fig. 8.5B).

In plants, tri-, tetra- penta- or hexa-flavonoids also exist, although are relatively rare [88]. Other types of flavonoids are flavonolignans, where a 3-OH-flavanone structure is connected to a coniferyl alcohol molecule. A well known example is silybin (Fig. 8.6).

In flavonoid *O*-glycosides, substitution with a sugar occurs mainly at C-3 (in C-ring), C-5, or C-7. The sugar moiety may be a mono-, bi-, or trisaccharide. The monosaccharides are usually D-glucose, D-galactose, D-glucuronic, or



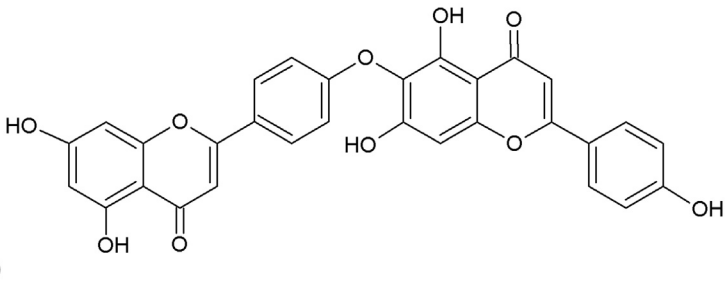


FIGURE 8.5 Examples of different types of biflavonoid structures: (A) amentoflavone (aryl carbon-carbon linkage) and (B) hinokiflavone (aryl−ether linkage *via* carbon−oxygen).

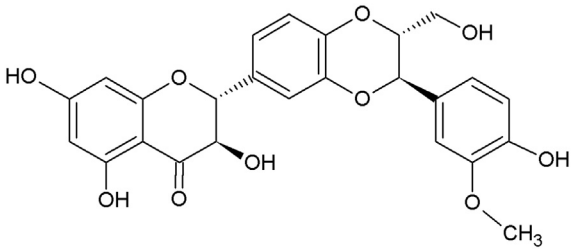


FIGURE 8.6 The chemical structure of silybin A.

D-galactouronic acid, or pentoses. D-Apiose, LLarabinose, D-xylose, or L-rhamnose are also frequently present. Disaccharides are mainly sambubiose, gentiobiose, rutinose, sophorose, or neohesperidose. In C-glycosides C-6 or C-8 in A-ring are linked with the asymmetric carbon atom of the monosaccharide (often glucose or galactose).

8.6.1 Plants Containing Flavonoid Glycosides

Flavonoids are widespread in higher plants. However they were found also in Bryophytes (mosses and hepaticas) as *O*- and *C*- glycosides and *O*-uronic derivatives, and in algae [88]. Various plants used as a food contain flavonols and flavones. Biflavonoids are relatively rare and are found mainly in gymnosperms particularly in the Cycadopsida and Coniferopsidaclasses, and are sporadic in angiosperms (*Hypericum* sp., *Garcinia* sp.) [3]. Chalcones are typical for the Asteraceae family while dihydrochalcones are found mainly in Ericaceae and Rosaceae [12]. FLGs, which are almost always water-soluble, are located in vacuoles, either concentrated in the epiderm or in mesophyll and in flowers in epidermal cells. Being plant pigments, they are responsible for the color of fruits, flowers, and in some cases leaves (yellow—chalcones, aurones, flavonols; red, blue, or purple—anthocyanidins). Flavonoids are also involved in electron transport during photosynthesis, and ensure tissue protection against UV radiation. Specifically released into the rhizosphere by roots these compounds are involved in plant/plant interactions, e.g., by the inhibition of seedling root growth. Flavonoids display various activities against microbes, between plants, and in animals [3,86,89]. Flavonoids (as well as enzymes of flavonoid biosynthesis) have been found in the nucleus (e.g., flavanols in gymnosperm species), and are capable of modulating the activity of enzymes and protein complexes involved in cell growth, including the regulation of gene expression and through chromatin remodeling [90−93]. The main medicinal plants containing flavonoids are summarized in the Table 8.3.

TABLE 8.3 Flavonoid-containing plant drugs

Plant-botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose /undesirable effects /interactions	Clinical trials /preclinical studies /contra-indications
Flavonols				
Betula pendula Roth (syn. *B. verrucosa* Ehrh. *B. pubescens* Ehrh. (Betulaceae)	***Betulae folium*** **Birch Leaf** Flavonols: quercetin-3-O-galactoside (hyperoside) quercetin-3-O-rhamnoside (quercitrin) quercetin-3-O-rutinoside (rutoside), miricetin-3-O-digalactoside kaemferol glycosides	diuretic, anti-inflammatory metabolic / Used as tincture, fresh juice, dried leaf infusion/adjuvant in the treatment of bacterial infections and in irrigation of the urinary tract	None reported.	In vivo studies in rats: excretion of urea and chloride increases, aquaretic effect was observed No toxicity and effectiveness of treatment of urinary infections was observed.
Vitis vinifera L. (Vitaceae)	***Vitis viniferae folium*** **Red Vine Leaf** Flavonols (up to 3.5%): quercetin-3-gucuronide isoquercitrin hyperoside kaempferol-3-glucoside Antocyanins: peonidin-3-glucoside malvidin-3-glucoside cyanidin-3-glucoside proantocyanidins flavan-3-ols hydroxycinnamic acids	Internal use: In treatment of chronic venous insufficiency. In painful and heavy legs. 360-720 mg of aqueous dry extract. 10 g of dried leaf in 250 mL of water as infusion (2-4 cups/day) or 10 mL of fluid extract daily. External use: Topical treatment of varicosis and couperosis. As bath or footbath – decoction of 60-80 g of dried leaf/1 liter of water.	No toxic effects reported. Undesirable effects: mild gastrointestinal complains. None interactions reported.	None infections was observed. No contra-indications.
Solidago virgaurea L. (Asteraceae) *Solidago canadensis* L. *Solidago giganthea* Ait	***Solidaginis virgaureae herba*** **European Golden Rod** *Solidaginis herba* (*S. giganteaand/or S. canadensis*) Flavonols: quercetin glycosides (quercitrin, isoquercitrin, hyperoside, rutoside, avicularin), kaemferol glycosides (astragalin, nicotiflorin), isorhamnetin glycosides	diuretic saluretic effect anti-inflammatory spasmolytic activity hypotensive effect. For oral administration as an adjuvant in the treatment of bacterial infections and in irrigation of the urinary tract.	No toxic effects reported. Minor adverse-effects were observed: contact dermatitis and heart burn (one case). No interactions reported.	Confirmed anti-inflammatory, spasmolytic, and diuretic effects in clinical studies. Contra-indications: do not use in patients with edema due to impairment heart or kidney function.
Equisetum arvense L. (Equisetaceae)	***Equiseti herba*** **Horse-tail Herb** Flavonols: kaemferol-7-glucoside (equisetrin),	diuretic anti-rheumatic rheumatoid diseases, Urinary tract inflammations.	Intoxications no reported. However, enzyme thiaminase can produce decrease of vitamin B1 (thiamine).	No sufficient data available. The efficacy of use was established on basis of long term traditional use.

	quercetin-3-glucoside (isoquercitrin) and its malonyl esters Flavones: luteolin-5-glucoside (galuteolin)	Herbal tea for oral use, expressed juice, dry or liquid extract, comminuted herbal substance in tablets	Mild gastrointestinal complaints and allergic reactions were reported rarely.	
Polygonum aviculare L. (Polygonaceae)	***Polygoni herba*** **Knotweed herb** Flavonols: hyperoside quercetin-3-arabinoside (avicularin) quercitrin Flavones: apigenin-C-glucosides (vitexin, isovitexin)	diuretic anti-rheumatic	The antioxidant effects of *Polygonum aviculare* L. on superoxide radical scavenging, lipid peroxidation, and DNA damage was studied and confirmed [1].	It has been reported that *P. aviculare* L. can be employed supportively in the therapy of gingivitis by oral rinse. It was suggested that this phenomenon was attributed to the flavonoid components that decrease capillary fragility and exert a cortisone-like effect on gingival tissues [2].
Fagopyrum aesculentum Moench. (Polygonaceae)	***Fagopyri herba*** **Buckwheat** Flavonols: Rutoside (1-10%) hyperoside quercitrin (quercetin-3-rhamnoside)	vasoprotectivum (vitamin-P –like activity) diuretic As a rutoside source. Evaluation of use in type II diabetes.	Buckwheat can be a potent allergen. In sensitive people, it provokes IgE-mediated anaphylaxis [3]. Rare: Light sensitivity, called "fagopyrism," can result from the fagopyrin in buckwheat.	In vivo studies in mice: antibiabetic in type II diabetes [4].
Sambucus nigra L (Caprifoliaceae)	***Sambuci flos*** **Elder Flower** Flavonols: quercetin glycosides (rutoside, hyperoside, isoquercitrin) kaemferol glycosides (astragalin, nicotiflorin) isorhamnetin glycosides	diuretic diaphoretic antipyretic anti-inflammatory Oral use as tea and as liquid preparations, tincture DER 1:5 in 25% ethanol Traditional medicinal use of elder flower used for relief of early symptoms of common cold.	Raw unripe fruits and other parts of *Sambucus nigra* that contains the cyanogenic glycoside sambunigrin can cause diarrhoea and/or vomiting [5].	Possible interaction with diuretic drugs. Possible interaction potential between the elder flower and the centrally acting drugs morphine and pentobarbitone. Data not sufficient to confirm [6].
Tilia cordata Miller *T. platyphyllos* Scop. *Tilia vulgaris* Hayne (Tiliaceae)	***Tiliae flos*** **Lime Flower** Flavonols; quercetin glycosides (rutoside, hyperoside,quercitrin, isoquercitrin) kaemferol glycosides (astragalin, tyliroside)	diuretic, diaphoretic, antipyretic anti-inflammatory anti-diabetic /Traditional use for the relief of symptoms of common cold and for the relief of mild symptoms of mental stress.	No cases of overdose have been recovered in the scientific literature. Possible allergy. It has been advised that lime flower should be avoided by individuals with an existing cardiac disorder, as excessive use may result in cardiac toxicity. However, the scientific basis for this statement, if any, is not known (EMA 2012).	No clinical data for single substance.
Polygonum hydropiper L. (Polygonaceae)	***Polygoni hydropiperis herba*** **Smartweed Herb** Flavonols; quercetin glycosides (rutoside, hyperoside,quercitrin) isorhamnetin 3-monosulfate (persicarin)	vasoprotective haemostatic, anti-haemorrhoid /Used traditionally for its astringent properties which makes it useful in treating bleeding, skin problems and diarrhea.	Skin irritant when used frequently.	In vitro mutagenic activity. In vivo experiments in rats: The aerial parts cause blister of the skin upon repeated handling that could be due to the skin irritant polygodial, and neurotoxic effects [7].

(*Continued*)

TABLE 8.3 (Continued)

Plant-botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose /undesirable effects /interactions	Clinical trials /preclinical studies /contra-indications
		The ethnomedicinal uses of the plant including antioxidant, antibacterial, antifungal, anthelminthic, antifeedant, cytotoxicity, anti-inflammatory, antinociceptive, oestrogenicity, anti-fertility, anti-adipogenicity, anticholinesterase activity, and neuroprotection [7].		
Lamium album L. (Lamiaceae)	***Lamii albi flos*** **White Dead Nettle Flower** Flavonols; quercetin glycosides (rutoside, isoquercitrin) kaemferol glycosides (tyliroside)	Vasoprotective haemostatic adstringent /An infusion is used traditionally in the treatment of kidney and bladder complaints, diarrhoea, menstrual problems, bleeding after childbirth, vaginal discharges and prostatitis.	None known.	For the flowers of *Lamium album* there are no known contra-indications, side effects or interactions with other drugs, and for the herb, there are no known risks.
Leonurus cardiaca L. (Lamiaceae)	***Leonuri herba*** **Motherwort Herb** Flavonols: hyperoside, rutoside, astragalin Flavones apigenin glycosides, genkwanin (7-methoxyapigenin glucoside)	cardiotonic hypotonic spasmolytic cholagogue	Overdose: A dose of 3000 mg of solid extract per day, taken in capsule or tablet form, is likely to cause diarrhoea, stomach irritation, or uterine bleeding. Undesirable effects: the bradycardic actions, renal and liver toxicity and the activity on the uterus.	Contraindication in pregnancy (toxicity of leonurin). No clinical studies with single ingredient products were found.
Calendula officinalis L. (Asteraceae)	***Calendulae flos*** **Calendula Flower** Flavonols: quercetin and isorhamnetin glycosides	anti-inflammatory antimicrobial cholagogue spasmolytic hepatoprotection antiulcerosum vasoprotectivum For topical application in the treatment of minor inflammation of skin and mucosa, healing of minor wounds.	Undesirable effects: weal skin sensation, but no cases of contact dermatitis. None interactions reported.	In-vivo studies in mice reported no toxicity. In pilot clinical study of hydroethanolic extract of calendula flower positive effects were observed in 30 patients. Contra-indications: sensitivity to members of Asteraceae family.
Arnica montana L. (Asteraceae)	***Arnicae flos*** **Arnica Flower** Flavonols: astragalin Flavonol glucuronides	anti-inflammatory anti-microbial activity anti-histamine effect	Undesirable effects: skin irritations, contact dermatitis.	Contra-indications: allergy to *Arnica* or members of Asteraceae.

	Flavones: luteolin-7-O-glucoside	/External use; topically as diluted tincture, ointments, creams, gels, compresses /symptoms of rheumatic complains, gingivitis and aphthae's ulcers, insect bites inflammatory		
Flavones				
Scutellaria baicalensis Georgi (Lamiaceae)	***Scutellariae baicalensis radix*** **Baikal Skullcap** baicalein, baicalin (baicalein-7-glucuronide), wogonin, wogonoside (wogonin-7-glucuronide) oroxylin A sculpcaflavone I and II chrysin scutelarein	antioxidant, free radicals scavenging activity, anti-inflammatory hepatic protection anxiolytic effect (binding with benzodiazepine receptor) /is used to treat respiratory infections, hay fever, and fever, gastrointestinal infections, as well as liver problems including viral hepatitis and jaundice, to treat attention deficit-hyperactivity disorder (ADHD), prostate cancer, alung condition called bronchiolitis, arthritis, and hemorrhoids.	Allergic reactions not excluded. Interactions with: alcohol, lithium, medications for diabetes (lowered glucose level), benzodiazepines and sedatives (caused sleepiness).	Inhibition of dengue virus was observed [8].
Passiflora incarnata L. Passifloraceae	***Passiflorae herba*** **Passion Flower** Flavones: C-glycosides of apigenin (vitexin, isovitexin, swertisin, schaftoside, isoschaftoside, vicenin-2) and luteolin (orientin, isoorientin)	cardiotonic antihypertensive sedative and anxiolytic effect / For oral administration in case of tenseness, restlessness, irritability with difficulty in falling asleep	No toxic effects reported. Hypersensitivity in very rare cases, once bradycardia and ventricular arrhythmia has been reported.	Contra-indications: none—known.
Crataegus monogyna Jacq. *C. oxyacantha* L. (*C. laevigata*) Rosaceae	***Crataegi folium cum florae*** **Hawthorn Leaf and Flower** ***Crataegi fructus*** **Hawthorn Berries** Flavones: C-glycosidesof apigenin (vitexin, isovitexin, swertisin, schaftoside, isoschaftoside) and luteolin (orientin, isoorientin) Flavonols: quercetin and its glycosides (hyperoside, rutoside) Di-and oligomeric procyanidins Flavanes - catechins In fruit additionally antocyanidins.	Increase in myocardial contractility (positive inotropic effect), Increase in coronary blood flow, antiarrhythmic effect (flavone-C-glycosides) Diuretic and hypotensive effect (anti-ACE activity of flavonols) sedative effect (procyjanidins). Hydro-alcoholic extracts in declining cardiac performance, herbal tea or other preparation in nervous heart complain and in support of cardiac and circulatory functions.	No toxic effect reported. Undesirable effects: none reported.	Several clinical trials confirmed effectiveness of hawthorn therapy. No mutagenicity and toxicity was observed. Contra-indications - none known.

(*Continued*)

TABLE 8.3 (Continued)

Plant-botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose /undesirable effects /interactions	Clinical trials /preclinical studies /contra-indications
Viola tricolor L. *Viola arvensis* Murray (Violaceae)	***Violae herba cum florae*** **Heartsease Herb and Flower** Flavones: luteolin-7-O-glucoside, apigenin-6-C-glucoside (violanthin), vicenin-2,violarvesin, vitexin, orientin Flavonols: rutoside Antocyjanidins: peonidin and delphinidin glycoside (violanin)	vasoprotective diuretic diaphoretic metabolic anti-inflammatory Oral administration and local application. In skin disorders: eczema, seborrhea, impetigo and acne.	No toxic effect reported. Undesirable effects: none reported.	Anti-inflammatory effects reported in animal model. No clinical data available. Contra-indications – none known.
Orthosifon stamineus Benth. *O. aristatus Miq.* *O. spicatus* Bak. *(Lamiaceae)*	***Orthosifonis folium*** **Java Tea** Flavones: sinensetin, isosynensetin, rhamnasin, salvigetin, eupatorin	diuretic, saluretic spasmolytic, efficient as scavenger of DPPH antibacterial hypoglycaemic effect For oral administration as an adjuvant in the treatment of bacterial infections and in irrigation of the urinary tract	No toxic effect reported. Undesirable effects: none reported.	Hypoglycemic effect, increases chleresis and cholekinesis, antibacterial effect, diuretic effect, and elimination of urea and chloride was confirmed in clinical studies. Contra-indications – none known.
Flavanones				
Drynaria fortunei (Kunze) J. Sm. (Polypodiaceae)	***Drynariae herba*** **Drynaria** **GuSuiBu (Chinese)** naringenin – naringin = naringenin7-rhamnoglucoside	↑ of Ca^{2+} bioavailability anti-osteoporotic vasoprotective anti-inflammatory antiatheromaticum anti-tumor activity (colon, breast, bone cancers)	Overdose of *Drynaria herb* might result in adverse reactions such as poisoning and stomachache.	*In vitro* studies and analyses in clinical studies revealed inhibiting osteoclast activity and increasing oosteoblast functions, stimulate osteogenesis, and is effective in treatment of osteoporosis [9–11]. No interactions found clinically.
Chalcones				
Helichrysum arenarium L. (Asteraceae)	***Helichrysi inflorescentia*** **Sandy Everlasting Flower** Chalcone: isosalipurposide Flavones (apigenin and luteolin glycosides) Flavonols (astragalin) Flavanones (naringenin glycosides) [12-14]	choleretic spasmolytic diuretic anti-oxidant anti-microbial as infusion At this time there is not enough scientific information to determine an appropriate range of doses.	Interactions: none known.	Contra-indications: Do not use when bile duct is blocked or obstructed, and in case of allergy to Asteraceae.
Carthamus tinctorius L. (Asteraceae)	***Carthami flos*** **Safflower**	blockers of Ca^{2+} channels and spasmolytic activity in veins (chalcones) anti-inflammatory,	Adverse effects: increased menstrual flow may occur. Dizziness, skin eruptions and transient urticaria have been reported.	Interactions: *Flos Carthami* inhibit platelet aggregation, it should therefore be used with caution in patients taking anticoagulants or antiplatelet drugs.

	Chalcone C-glycosides; hydroxysafflore A (HSYA), safflamin a and C, carthamin, carthamidin Flavonol glycosides: nicotiflorin, rutoside, hyperoside Flavone glycosides: luteolin and apigenin derivatives [15].	platelet aggregation inhibition /Used for the treatment of cardiovascular and hematological disease such as angina pectoris, cerebral hemorrhage, cerebral atherosclerosis, in menstrual disorders [16].	Contra-indications: in hemorrhagic diseases, peptic ulcers and in excessive menstruation.	
Isoflavones				
Glycine max (L.) Merr. syn. Soya hispida (Moenh.) Maxim. (Fabaceae)	***Soyae semen*** **Soybeen** Isoflavones: genistein – genistin daidzein – daidzin formononetin, ononin (formononetin -7 glucoside), biochanin A, sissotrin (biochanin A – 7-glucoside) Other important substances are; saponins, lectins, phytosterols, proteins, dietary fiber, vitamins and minerals.	estrogenic activity, decreasing effect on cholesterol and triglyceride level in plasma anti-oxidant Used against metabolic disorders (cardio-vascular diseases, diabetes and obesity) in chronic diseases as a cancer, osteoporosis, menopausal syndrome and anemia.	Adverse effects: Seems to be safe for most people when used short-term. It can cause some mild side effects such as constipation, bloating, and nausea. It can also cause allergic reactions involving rash and itching. Long term and excessive use might cause abnormal tissue growth in the uterus. Contra-indications: In case of cancer related to estrogen, in kidney diseases[17].	In clinical trials: effectiveness of prevention of atherosclerotic diseases. Epidemiological studies in Japanese women suggest that consumption of soy products has a protective effect against menopausal symptoms and osteoporosis. *In vitro* anticancer activity against prostate and colon cancer[18]. Interactions: fermented soy products (as tofu) that contain high amounts of tyramine may interact with MAO drugs (depression treatment) and caused of high blood pressure. May interfere with medications used as treatment of blood clotting disorders. Interactions: with contraceptive drugs (estrogens), anticoagulant / antiplatelet drugs (medications that slow blood clotting; aspirin, ibuprofen, naproxen, heparin, warfarin), methotrexate, tamoxifen (increases side effects of therapy), with medications of diabetes.
Pueraria lobata (Willd.) Ohwi. *Pueraria thomsoni* Benth. (Fabaceae)	***Puerariae lobatae radix*** ***Puerariae thomsonii radix*** **Kudzu Root** Isoflavones: daidzein – daidzin, puerarin (daidzein-8-C-glucoside)	spasmolytic hypertonic estrogenic activity Increasing activity of alcoholic dehydrogenase, enhances serotonin and dopamine level in CNS (applied in ethanol addiction). /used in high blood pressure, chest pain, irregular heartbeat, for upper respiratory problems.	Avoid use in case of hormone-sensitive condition such as breast cancer, uterine cancer, ovarian cancer, endometriosis, or uterine fibroids. Kudzu might affect low blood sugar (hypoglycemia).	
Biflavonoids				
Ginkgo biloba L. (Ginkgoaceae)	***Ginkgonis folium*** **Ginkgo Leaf** Biflavonoids: amentoflavone, bilobetin, ginkgetin) Flavonols (quercetin, kaemferol, isorhamnetin and their mono-, bi- and triglycosides) Flavones (luteolin, apigenin) Flavan-3-ols: catechins, Oligomeric and polymeric procyanidins.	antioxidant vasoregulatory, tissue-protective and cognition-enhancing effects, broncho-spasmolytic anti-coagulant and haemorheological effect of decreasing of blood viscosity anxiolytic (BZD receptors activity of amentoflavone) /For oral administration in cases of dementia and CNS disturbances (min. 12 weeks treatment and evaluation if patient response is observed). Adult daily dose: 120-240 mg of standard. Ginkgo extract divided into 2–3 doses.	Undesirable effects: in rare cases mild gastrointestinal disorders, headache, and allergic skin reactions. Interactions: cannot be excluded interactions with substances that inhibit blood coagulation not observed in controlled studies).	Effectiveness of dementia treatment and effect on blood viscosity confirmed in clinical studies. Contra-indications: hypersensitivity or intolerance to ginkgo leaf preparations.

(*Continued*)

TABLE 8.3 (Continued)

Plant-botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose /undesirable effects /interactions	Clinical trials /preclinical studies /contra-indications
Hypericum perforatum L. (Hypericaceae)	***Hyperici herba*** **St. John's Wort** Biflavonoids: amentoflavone, 13,II8-biapigenin Flavonols: quercetin -3-O-rutinoside (rutoside), -3-O-galactoside (hyperoside) and -3-O-glucoside (isoquercitrin) [anthracene derivatives e.g. hypericins (see anthraquinone part of the Chapter VIII) xanthones essential oil]	Activity of St. John's Wort is complex and depending on anthraquinones, xanthones, flavonoids, and phenylopropanoids. Water infusions: Spasmolytic cholekinetic effect. Hydro-alcoholic extracts (50-60% ethanol or 80% methanol) or tinctures (50% ethanol) in episodes of mild depressive disorders to moderate depressive disorders (treatment 3-4 weeks and evaluation of therapeutic effect). Daily adult (over 12 years) dose 450-1050 mg of hydro-alcoholic extract). anti-inflammatory anti-bacterial anti-viral anti-oxidant activity	Occasional mild gastrointestinal disturbance, nausea, restlessness, fatigue, headache, allergic reactions were reported. Overdose (extract c.a. 3600 mg) caused phototoxic reactions if exposition on direct sunlight. Interactions with antiretroviral protease and transcriptase inhibitors, anticoagulants (warfarin or phenprocoumon). theophylline and digoxin. Interactions with contraceptive drugs not confirmed.	Dopaminergic activity of the extract confirmed in in vivo studies in animal models. As mechanisms of action an induction of several subtypes of cytochrome P450 and increased expression of the P-glycoprotein drug transporter has been reported. Inhibition of serotonin, dopamine, noradrenaline and GABA uptake from synaptosomes was confirmed.
Flavonolignanes *Sylibum marianum* L. Gaertner (Asteraceae)	***Sylibi mariani fructus*** **Milk Thistle Fruit** Flavonolignanes: silymarin as a mixture of silybin A and B, isosilybin A and B, silydianin, silychrystin, Flavonols (quercetin, kaemferol), Flavones (apigenin and luteolin) Flavanones (naringenin) and their glycosides	Hepatoprotective among falloidine from *Ammanita falloides*, and intoxication of alcohol, paracetamol, CCl_4, toxins /in virus hepatitis A-C in alcoholism. antioxidant/free radical scavengeranti-fibrotic /standardized extract (70-80% silymarin) in encapsulated form, 100-300 mg three times daily being the typical adult dose.	Non-toxic in animals and humans. At high doses ($>$1500 mg per day) a laxative effect is possible due to increased bile secretion and flow. Mild allergic reactions have also been noted [19]. Interactions: Milk thistle might decrease how quickly the liver breaks down some medications. Interacts with medications used for lowering cholesterol (Statins). Milk Thistle might decrease the effectiveness of estrogen pills.	Silymarin is able to neutralize the hepatotoxicity of several agents, including *Amanita phalloides*, ethanol, paracetamol (acetaminophen) and carbon tetrachloride in animal models. Silymarin has been shown to increase hepatocyte protein synthesis, thereby promoting hepatic tissue regeneration [20].

Anthocyanidins				
Vaccinium myrtillus L. (Ericaceae)	***Myrtilli fructus recens*Bilberry**Anthocyjanidins3-O-galactosides,glucosides and arabinosides of cyanidin, delphinidin and malvidin	antioxidant/free radical scavenger vasoprotectivum anti-inflammatoryanti-edematic,rhodopsin synthesis enhancement,anti-retinopathy,anti-diarrhea/ Recommended daily dosages also vary greatly, for example, 20-60 g of dried berries and 160-480 mg of powdered extract [21]	Can be safely consumed when used appropriately.	In several studies it was revealed that anthocyanins in bilberry strengthen the walls of blood vessels, reduce inflammation and stabilize tissues containing collagen, such as cartilage, tendons and ligaments, have influence on vision improvement, and have anti-inflammatory effects [22].
Sambucus nigra L. (Caprifoliaceae)	***Sambuci fructus*** **Elder Berry** Antocyanidins: cyanidin-3-glucoside (chrysanthemin) cyanidin-3-sambubioside	vasoprotectivum ant-inflammatory	Abdominal pain, edema legs, After drinking fresh juice from elderberries mixed with leaves nausea and vomiting occur. Not recommended for children under 18 years due to insufficient data of safety and efficacy.	Elderberry preparations have been studied in clinical trials, but the published clinical studies cannot be considered to fulfill the criteria required for "well-established medicinal use".
Hibiscus sabdariffa L. (Malvaceae)	***Hibisci flos*** **Hibiscus** Antocyanidins: delphoinidin and delphinidin-3-glucoside, delphinidin-3-sambubioside (hibiscin) cyaniding-3-sambubioside Flavonoids – gossipetin derivatives	vasoprotectivum anti-inflammatory inhibition of ACE – anti-hypertonic antiatheromaticum (increase HDL level) diuretic antibacterial /used for treating loss of appetite, colds, heart and nerve diseases, upper respiratory tract inflammation, fluid retention, stomach irritation, for dissolving phlegm; as a gentle laxative; and as a diuretic.	Adverse effects and overdose effects not known. Hibiscus might decrease blood sugar levels and lower blood pressure.	In clinical studies in patients with diabetes II type hibiscus tea taken twice daily for a month increase in HDL ("good") fraction of cholesterol and a significant decrease in total cholesterol and LDL fraction.
Vaccinium oxycoccus L. *V. macrocarpon* Ait. Ericaceae	***Vaccinii oxycocci vel macrocarponis fructus*** **Cranberry** Proantocyanidins (PAC)- catechin polimers Antocyanidins: peonidin-3-arabinoside, peonidin-3—galactoside, cyanidin derivatives	urodesinficient anti-inflammatory PAC- anti-adherence activity to uroepithelial cells of various *E. coli* strains was confirmed. anti-inflammatory anti-hypertensive antioxidant /For oral administration in prevention of urinary tract infections.	No toxic effect reported. Undesirable effects: Recurrent stone development in patients with calcium oxalate nephrolithiasis. Cranberry should not be taken in pregnancy or lactation in amounts greatly exceeding those found in foods.	Patients taking warfarin (anticoagulant) should avoid cranberry preparations (increasing anticoagulant effect). Some risk of urinary stone formation. Clinical studies – see ESCOP 2nd edition 2009 supplement.

(*Continued*)

TABLE 8.3 (Continued)

Plant-botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose /undesirable effects /interactions	Clinical trials /preclinical studies /contra-indications
Centaurea cyanus L. (Asteraceae)	***Cyani flos*** **Cornflower** Antocyjanidins: cyjanin, pelargonin	antioxidant anti-inflammatory vasoprotective, diuretic /used for easing eye irritation and treating problems like sore eyes, conjunctivitis, and even skin irritation around the eye because of its effective anti-inflammatory and astringent properties.	Cornflower may cause an allergic reaction in people who are sensitive to the Asteraceae.	Interactions: insufficient evidence available.

Data collected in the Table 8.3 were derived/summarized from EMA Assessment Reports, ESCOP and European Pharmacopoeia – Council of Europe monographs and from references below.

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8.6.2 Bioactivity of Flavonoid Glycosides

Flavonoids have been reported to exert wide range of biological activities. These include antiinflammatory, antibacterial, antiviral, antiallergic, cytotoxic, antitumor and vasodilatory action [94,95]. Flavonoids are capable of affecting the behaviors of many cell systems and exerting beneficial effects on body [3,4,11,96]. These compounds are known for modulating the activity of enzymes e.g., inhibit lipid peroxidation, cyclooxygenase and lipoxygenase enzyme activities, contribute to inhibition of NADH oxidase and the balance of reactive oxygen species, platelet aggregation, capillary permeability and fragility [97,98]. The most known therapeutic activities concerning flavonoid-containing plant drugs are presented in the Table 8.2.

8.6.3 Nutraceutical Applications of Flavonoid Glycosides

Flavonoids are responsible for major organoleptic characteristics of plant-derived foods and beverages and contribute to the nutrition value of fruits and vegetables. Due to variety of pharmacological activities in the animal and/or human body, flavonoids could be referred to as nutraceuticals. Food-derived flavonoids, especially flavonols such as kaemferol, quercetin and their glycosides, are widely occurring flavonoids and are reported to exhibit various biological functions, e.g., antioxidant, antiinflammatory, cardioprotective, and vasodilatory effects. These compounds are present in vegetables such as carrot, spinach, cauliflower, onion, garlic, ginger, or cabbage and in fruits such as plum, apple, strawberry, and apricot and also in plant drugs (see Table 8.3). Concentrated extracts or pulverized or micronized flavonoid-rich plants, such as propolis, pine bark, soy isoflavones, green tea leaves, and grape seed, are marketed as nutraceuticals which are applied in the prevention of cardiovascular diseases, metabolic syndrome, and in neuroprotection [99–102]. A prospective study of 10-year duration has provided a strong indication that regular dietary flavonoid intake has influence on a better neurocognitive performance with aging [103]. Brain imaging studies in healthy young people have demonstrated that the consumption of flavonol-rich cocoa enhances cortical blood flow [104], so flavonoid contained nutraceuticals can produce cognition-enhancing effects. Flavonoids alleviate neuroinflammation by inhibiting NADPH oxidase activation and subsequent reactive oxygen species generation in astrocytes, inhibiting COX-2 expression, cytokine release, and NO production [105–107]. Intensive epidemiological studies have shown that regular consumption of fruits and vegetables is associated with reduced risk of chronic diseases, such as cancer, and cardiovascular disease; suggesting that natural phytochemicals in fresh fruits could be even more effective than a dietary supplement [95,108]. The reduction of plasma cholesterol levels, increased resistance of LDL against oxidation, and decreased atherosclerotic lesion area were observed in mice on a soy protein high-fat diet rich in soy isoflavones (21 g isoflavone in 100 g protein). Genistein and daidzein (isoflavones derived from soybeans) have been shown to inhibit the development of both hormone- and nonhormone-related cancers, including mouse models of breast, prostate, and skin cancer [95]. Chemopreventive studies have demonstrated that the mechanisms of action of phytochemicals and nutraceuticals in the prevention of cancer go beyond the antioxidant activity scavenging of free radicals. They also include regulation of gene expression (oncogenes, and tumor suppressor genes), induction of cell cycle arrest and apoptosis, modulation of detoxification enzyme activity, stimulation of the immune system, and regulation of hormone metabolism [109,110].

8.6.4 Pharmaceutical Applications of Flavonoid Glycosides

The most important plant drugs containing flavonoids and flavonoid glycosides as their compounds are briefly summarized in Table 8.3 (based on Pharmacopoeia Europaea, EMA and ESCOP Monographs, and literature data listed in the appendix of the Table). Many investigations on structure/activity relationships for flavonoids and influence of various modifications on their bioavailability and bioactivity have been conducted and recently reviewed [111–113].

8.6.5 Adverse Effects of Flavonoid Glycosides

High doses of flavonoids intake do not appear to cause serious side effects. Flavonoids are substrates for enzymes in small intestine, liver, and colon and are hydrolyzed or conjugated to *O*-glucuronides and sulfate esters. Excess intake of flavonoids is simply excreted in urine. Epidemiological studies have not found adverse effects from the dietary consumption of isoflavones or linseed lignans. The observation that phytoestrogens can stimulate the growth of estrogen-dependent tumors in some circumstances, suggest that intake of these phytochemicals should be limited to dietary levels in women with estrogen-sensitive breast cancer. The findings from 14 total clinical trials provide little evidence that soya foods or isoflavones adversely affect thyroid function in euthyroid iodine-replete individuals. In contrast, some

evidence suggests that soya, by inhibiting absorption, may increase the dose of thyroid hormone required by hypothyroid patients [11]. Some flavonoids such as myricetin or baicalein (both with a pyrogallol structure in the A-ring) have been reported to promote hydrogen peroxide radicals. There is also evidence that unsaturated 2,3-bond and 4-oxo arrangement of flavones may produce formation of ROS. However, those effects are dependent of some factors (enzyme activity as, e.g., COMT, and the presence of vitamin C) and need further investigations [113].

8.6.6 Metabolic Profile of Flavonoid Glycosides

After ingestion as a food, flavonoids are liberated and its absorption depends on its physicochemical properties such as size of the molecule, configuration, lipophilicity, or solubility. Whereas aglycons can be easily absorbed in the small intestine, flavonoid glycosides are mainly transported to the colon, and have to be converted to aglycons by intestinal flora. Oligomeric flavonoids may be hydrolyzed in the stomach under acidic conditions. Bioavailability of flavonoids differs in accordance to their structure. Their first sites of interaction after ingestion are digestive enzymes and transporters in the small intestine. Many flavonoids were found to be inhibitors of glycoprotein P (P-gp) and multidrug resistance-associated protein-2 (MRP-2) [114]. The most available are isoflavones, and in decreasing availability: flavanols, flavanones, flavonols, and galloylocatechins and anthocyanins [115]. Flavonoids can also cross the blood−brain barrier depending on their lipophilicity and interactions with P-gp (a transporter expressed in the blood−brain barrier). Some metabolic profile of members of flavonols (quercetin and rutoside), flavonolignans (silymarin), and isoflavones (daidzein and daidzin) are summarized below.

For quercetin (flavonol aglycone) we see that levels of a flavonoid aglycone in the bloodstream will vary according to whether it is administered as an aglycone or glycoside. Quercetin glycosides can deliver quercetin more effectively into the bloodstream, presumably via active uptake by enterocytes. For example rutoside (quercetin rhamnoglucoside), when administrated as a food (onion), is partially decomposed in a large intestine. The terminal sugar, rhamnose, is removed by bacteria. In this step, what is important is the nature of the individual bowel flora, which is partly dependent on the individual diet [116]. Exposition of glucose results in uptake of glycoside by enterocytes. Flavonoid-*O*-glycosides are converted into the aglycone by bowel flora and in the next step the aglycones undergo further breakdown by a process known as C-ring fission (the C-ring is the central ring in the flavonoid structure) to give two different phenolic products. These C-ring fission products are probably the main bioavailable and active forms of flavonoids [117]. Once absorbed, the flavonoid aglycones are subject to three main types of conjugation: methylation, sulfation, and glucuronidation [118]. Much of the flavonoid glycosides passes through unchanged to the colon and only a small percentage of free flavonoid aglycones is present in the plasma. Similar observations have been made for naringin (and aglycone naringenin) [117].

Silymarin, a mixture of structural flavonolignane components (silybin A and B, silydianine, and sylichristine) from *Sylibum marianum* fruits, was recently introduced as a hepatoprotective agent in numerous liver diseases characterized as degenerative necrosis and functional impairment [119,120]. Silymarin is marketed in many countries under the trademark Legalon™ or Hepatron™. Silymarin provides hepatoprotection against poisoning by phalloidin (*Amanita falloides* toxin), alcohol, and carbon tetrachloride and also from injury caused by ischemia, radiation, iron overload, and viral hepatitis [121−123]. After absorption by the oral route, it distributes into the alimentary tract (liver, stomach, intestine, and pancreas). It is mainly excreted as metabolites in the bile, and is subject to enterohepatic circulation. Hepatoprotective function, i.e., stimulation of liver regeneration by silymarin (and mostly silybin, which is the most active) is related to increasing of protein synthesis in hepatocytes. Histochemical and histoenzymological studies have shown that silymarin, administered 60 min before or no longer than 10 min after induction of acute intoxication with phalloidin, is able to neutralize the effects of the toxin [124]. Silymarin exerts other important effects, which include liver-specific actions: hepatocyte membrane stabilization and permeability regulation, stimulation of ribosomal RNA synthesis promoting liver regeneration, and the prevention of the transformation of stellate hepatocytes into myofibroblasts, which are responsible for the deposition of collagen fibers.

Isoflavones, proposed as natural selective estrogen receptor modulators [125] have both agonistic and antagonistic effects on estrogen receptors (strong ER-β agonists and weak ER-α agonists). The presence of a correctly positioned phenolic ring and also the distance between the two opposing phenolic oxygens in the isoflavones structure is similar to that of 17-β-oestradiol, and this similarity allows the isoflavones effectively displacing 17-β-estradiol from the ER binding site [125]. The in vitro and in vivo studies on phytoestrogens have shown that they prevent cancer or inhibit cancer cell lines in a variety of models and protect against breast cancer [126]. Isoflavones have been found to exert favorable effects in vivo and in vitro on parameters relevant to cardiovascular risk, including insulin resistance, lipid peroxidation, and endothelial function [127]. After ingestion, isoflavone glycoside, e.g., daidzin, is metabolized by gut microflora to

daidzein (its aglycone) and compared to equol is a more active estrogenic compound. Individual bowel flora and predispositions to equol production determines the bioavailability (from 15% to 35%) and hence pharmacodynamic activity of isoflavones [11]. Isoflavone glycosides are c.a. 60−80% more bioavailable than aglycones (genistin is about 60% more bioavailable than genistein, its aglycone, and daidzin 80% more than daidzein) [128]. Soya, which contains isoflavone glycosides, increased follicular phase length in women, because the glycosidic group delays the degradation of isoflavones, resulting in higher bioavailability of their aglycones or equol [129,130].

8.7 ANTHRAQUINONE GLYCOSIDES (AQG)

Anthraquinones are naturally occurring structures, which comprise a central 1,4-diketo-cyclohexa-2,5-diene (quinone) pattern (e.g., *p*-quinone, *o*-quinone, naphtoquinone, anthraquinone, anthracyclinone, and naphtodianthrone) fused to a phenyl ring on either side. The anthraquinone structure is based on anthracene, where three benzene rings are joined together (Fig. 8.7).

Anthrones (10-*H*-anthracen-9-ones) and their tautomeric form; anthranol, are reduced forms of anthracene derivatives (Fig. 8.7). Inmost anthraquinones hydroxyl groups are normally found at positions C-1 and C-8. Only C-3 and C-6 carbon may also be substituted: methyl, hydroxymethyl, or carboxyl group at C-3, and phenolic or methyl group at C-6. Not all anthraquinones are strictly quinones; e.g., the sennosides are dianthrones consisting of two anthrone units, each bearing only one carbonyl group [3,11]. Dianthrones built from the same anthrone units are called homodianthrones (e.g., sennidins A,B and sennosides A,B). In instances where the subunits are different, then they are classified as heterodianthrones (Fig. 8.8).

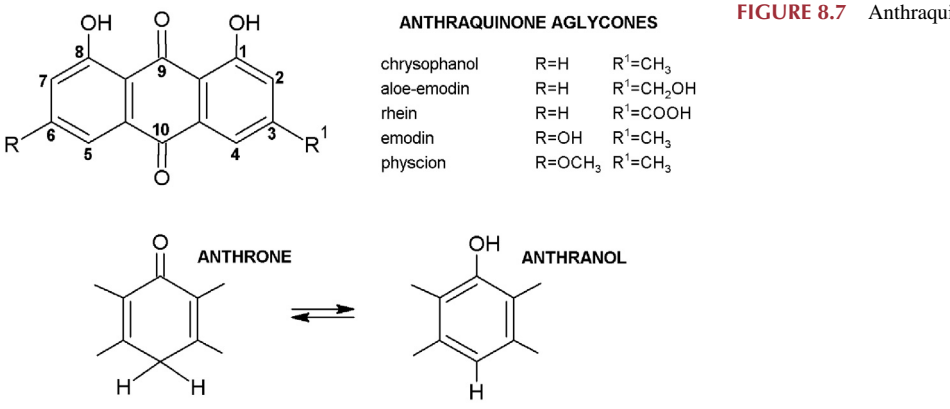

FIGURE 8.7 Anthraquinone aglycones.

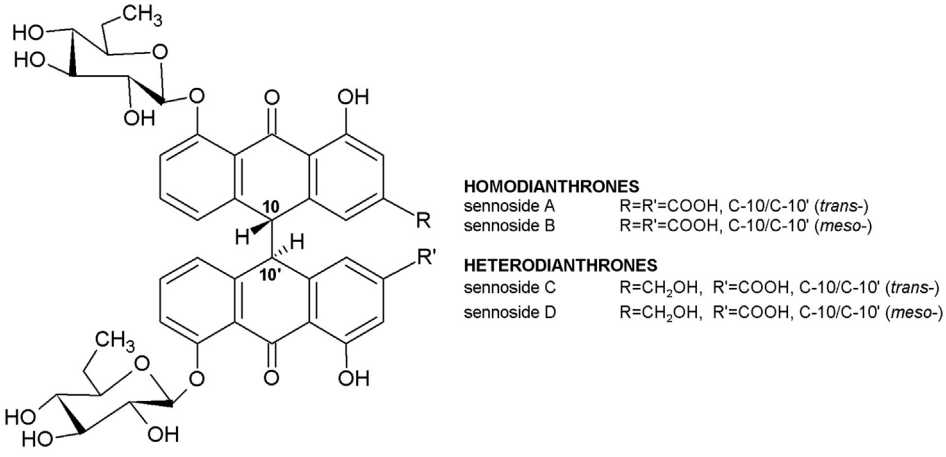

FIGURE 8.8 Sennosides as examples of homo- and heterodiantrones.

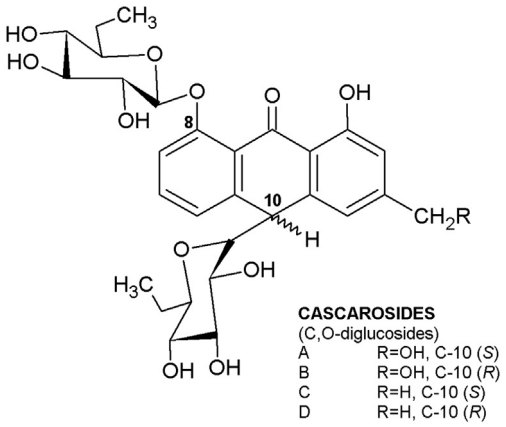

FIGURE 8.9 C,O-diglucosides—cascarosides from Cascara bark.

Anthrones are unstable and in plant drugs only anthraquinones occur as free aglycones. Anthrones and antraquinones are found in plants mainly as *O*-glycosides or rarely *C*-glycosides as aloins in *Aloe* species. *C*-glycosidic linkage introduces the chiral center into the molecule (as e.g., in aloin A [10*R*] and B [10*S*]). In some special cases *C*,*O*-diglycosides are found, as in the cascarosides from cascara bark (*Rhamnus purshiana*)—see Fig. 8.9.

In AQGs sugars are attached mainly at the C-8 or C-6 hydroxyl groups or as C-glycosides at the C-10 position (in case of cascarosides at C-8 and C-10 positions, respectively). Other examples of diglycosides occur when two different sugars are attached to the aglycone portion as in *Frangula*, e.g., in glucofrangulin, where at C-6 and C-8, rhamnose and glucose units are attached, respectively. After partial hydrolysis, glucose is removed giving frangulin, which contains only rhamnose. Differences in anthracene-type compounds exist in fresh plant (anthrone monomeric glycosides as the main form) and in dried plant material (oxidation during desiccation leads to formation of anthraquinones and the dimerization process forms dianthrone glycosides) [3,4,11].

8.7.1 Plants Containing Anthraquinone Glycosides

Anthraquinones are distributed in fungi, lichens, and spermatophyta. They are also found in limited groups of angiosperm families such as Fabaceae, Liliaceae, Polygonaceae, Rhamnaceae, Rubiaceae, and Scrophulariaceae [3]. These compounds are found in the rhubarb root, *Rhei radix* (the prominent Chinese laxative), senna leaf and pod, *Sennae folium et fructus* (a Middle Eastern laxative), cascara sagrada (most popular North American laxative), buckthorn, *Frangulae cortex* (also known as frangula; a European laxative), and aloe, *Aloe* (known worldwide). Anthraquinones are also found in other herbs, usually in small quantities, mainly in *Polgyonum* species, and also in vegetables, such as cabbage and lettuce, while being particularly high in beans (36 mg/kg fresh weight). Physcion is the dominant anthraquinone in foods. Plants containing AQGs are listed in the Table 8.4.

8.7.2 Bioactivity of Anthraquinone Glycosides

Plants like rhubarb, senna, and cascara have been used for their laxative effects since prehistory. The detailed description of anthraquinone plant drugs used as laxative is presented in Table 8.4. Structure−activity relationships in anthracene-derived laxative drugs and some other applications of anthracene derivatives are highlighted below. In anthracene laxatives hydroxylation of C-1 and C-8 is essential for activity. Glycosylation is also important (sugar moiety serves to transport the aglycone to the site of action in the large intestine). Aglycones are not active in animals. After ingestion they are absorbed in the stomach and never reach the colon to produce a local effect. In the case of *C*-*O*-glycosides (cascarosides), additional glycosidic linkage makes them more water-soluble and produces higher pharmacological effect. The oxidation level is also important. Glycosides of anthranol and anthrones are shown to have stronger laxative effect than anthraquinone glycosides. Some plant drugs such as cascara bark or frangula bark should be stored longer before use (for 1 year, or dried at 100°C for 2 h), because the fresh bark contains highly active anthrones that are converted by oxidation to the less active form (anthraquinone) and undesirable effects such as griping action can occur. Some structures are more stable such as C−C glycosides (less susceptible for hydrolysis) or structures with C-10/C-10' bridge, as in sennosides (C-10 position cannot be easily oxidized) [3,18]. Some natural anthraquinone

TABLE 8.4 Anthraquinone plant drugs

Plant-botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications [(a)]	Clinical trials /preclinical studies /contra-indications
Aloe ferox Mill. *Aloe barbadensis* Mill. (Liliaceae)	***Aloe capensis*-Cape Aloes** ***Aloe barbadensis*-Barbados Aloes** Concentrated and dried juice of the leaves (contain not less than 18% (in *Aloe capensis*) or not less than 28% (in *A. barbadensis*) hydroxyanthracene derivatives expressed as barbaloin) [PhEur.]. In *A. capensis*: Aloe-emodin anthrone-C-glycosides; aloin A[10 R] and aloin B[10 S]) 5-hydroxyaloin A Anthrone C- and O-glycosides: aloinosides A and B 1,8-dihydroxyanthraquinones: aloe-emodin In *A. barbadensis*: Aloe-emodin anthrone-C-glycosides; 20-45% barbaloin (mixture of aloin A[10 R] and aloin B[10 S]) 7-hydroxyaloins A, B and their 6-O-p-coumaroyl esters	Adults and children over 10 years; preparation equivalent of 10-30 mg of hydroxyanthracene derivatives, taken once daily at night. For oral administration. Do not use without medical advice longer than 2 weeks.	In clinical data no causal relationships between anthranoid laxative use and colorectal cancer could be detected. Contra-indications; intestinal obstruction and stenosis, atony, inflammatory colon diseases (Crohn's disease, ulcerative colitis), appendicitis, abdominal pain of unknown origin, dehydration states with electrolyte depletion. Avoid use in first trimester of pregnancy and use only under medical supervision. Do not use during lactation (excretion of metabolites in breast milk).
Rheum palmatum L. or *Rheum officinale* Baill. (Polygonaceae)	***Rhei radix* Rhubarb** Whole or cut dried underground parts (contains not less than 2.2% hydroxyanthracene derivatives expressed as rhein) [PhEur.]. Mono and diglucosides of rhein, chrysophanol, aloe-emodin, physcion and emodin Dianthrone glucosides (sennosides) Other compounds are gallotanins (5%), chromones, and phenylbutanones.	Adults and children over 10 years. Preparation equivalent of 15-50 mg of hydroxyanthracene derivatives (calc. as rhein) daily. For short-term treatment of occasional constipation. For oral administration. Not recommended during pregnancy. In low dose anti-diarrhoic activity due to activity of gallotanins is present.	In *in vitro* studies methanolic extract of rhubarb showed radical scavenging activity, water extract inhibited squalene epoxidase and strong inhibition of *Helicobacter pylori* growth was observed on agar plates [1]. An ethanolic extract shows strong antiviral activity against *Herpes simplex* virus. Rhein isolated from rhubarb shows strong activity against *Candida albicans*. [ESCOP 2009], [2] *In vivo* studies indicates anti-inflammatory activity of rhubarb. Contra-indications – see Aloes.
Rhamnus purshianus D.C. (Rhamnaceae)	***Rhamni purshiani cortex*-Cascara Bark** Dried whole or fragmented bark (contain not less than 8.0% of hyroxyanthracene glycosides of which 60% consist of cascarosides, both expressed as cascaroside A) PhEur.	Adults and children over 10 years. Dried bark: 0.3-1 g as single daily dose. Infusion: 1.5-2 g dried bark in 150 mL hot water.	In clinical data no causal relationships between anthranoid laxative use and colorectal cancer could be detected. In one case cascara was reported to be associated with development of cholestatic hepatitis after intake

(*Continued*)

TABLE 8.4 (Continued)

Plant-botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications [(a)]	Clinical trials /preclinical studies /contra-indications
	Cascarosides A-F (60-70% of the hydroxyanthracene complex); Cascarosides A and B (mixed C-O-glycosides of aloe-emodin anthrone) Cacarosides C and D are chrysophanol derivatives (= chrysaloins A and B) Cascarosides E and F (emodin derivatives) Aloins A and B Monoglucosides of aloe-emodin, chrysophanol, emodin, physcion.	Preparation equivalent of 20-30 mg of hydroxyanthracene derivatives. For oral administration. Do not use without medical advice longer than 2 weeks.	of 3x425 mg cascara (5% cascarosides) daily for 3 days. Contra-indications: In ileus of any origin and in inflammatory diseases of the colon, including ulcerative colitis, irritable bowel syndrome (IBS), and Crohn's disease.
Rhamnus frangula *(= Frangula alnus)* (Rhamnaceae)	***Frangulae cortex*-Frangula Bark** Dried whole or fragmented bark of the stems and branches (contain not less than 7% glucofrangulins expressed as glucofrangulin A). PhEur. Glucofrangulin A (emodin-6-O-α-L-ramnosyl-8-O-β-D-glucoside) Glucofrangulin B (emodin-6-O-β-D-apiosyl-8-O-β-D-glucoside) Frangulins A and B Frangulin C (emodin-6-O-β-D-xyloside), anthraquinone glycosides, dianthrones	Adults and children over 10 years; preparation equivalent of 20-30 mg of glucofrangulins daily. For short term treatment of occasional constipation. For oral administration. Do not use without medical advice longer than 2 weeks.	Epidemiological data suggests that it is no carcinogenic risk in humans from use of anthranoid laxatives. Contra-indications – see cascara.
Cassia senna L. *(= C. acutifolia* Delile*)* *Casia angustifolia* Vahl. (Cesalpiniaceae)	***Sennae folium*-Senna Leaf** The dried laflets (not less than 2.5% hydroxyanthracene derivatives calc. as sennoside B). ***Sennae fructus acutifoliae*** **Alexandrian Senna Pods** **(= Khartoum Senna Pods)** The dried fruit (not less than 3.4% hydroxyanthracene derivatives calc. as sennoside B) [Ph.Eur.]. ***Sennae fructus angustifoliae*** **Tinnevelly Senna Pods** The dried fruit (not less than 2.2% hydroxyanthracene derivatives calc. as sennoside B) [Ph. Eur.]. Rhein-dianthrone glucosides: Sennosides A and B Rhein-aloeemodin-dianthrone glucosides: Sennosides C and D Monoanthraquinone glycosides	Preparation equivalent of 15-30 mg of hydroxyanthracene derivatives (calculated as sennoside B).	Genotoxic risk detected for several anthranoids contraindicates their use in the first trimester of pregnancy. Epidemiological data suggests that it is no carcinogenic risk in humans from use of anthranoid laxatives. Contra-indications – see cascara.

Data collected in the Table 8.4 were derived/summarized from EMA Assessment Reports, ESCOP and European Pharmacopoeia – Council of Europe monographs and from references below. [(a)] Overdose reactions/adverse effects/interactions, which are similar for all members of this group of plant drugs - see text.

[1] Bae EA, Han MJ, Kim NJ, Kim DH. Anti-*Helicobacter pylori* activity of herbal medicines. Biol Pharm Bull 1998;21:990–992.
[2] Cyong J-C, Matsumoto T, Arakawa K, Yamada H, Otsuka Y. Anti *Bacterioideae fragilis* substances from rhubarb. J Ethnopharmacol 1987;19:279–283.

drugs have been used topically for psoriasis treatment, such as chrysarobin from a mixture of substances including chrysophanol, obtained from araroba (or Goa) powder. Araroba is extracted from cavities in the trunk of the Brazilian tree *Andira araroba* Aguiar. (Fabaceae). Chrysarobin irritates and stains the skin and there has been concern due to its tumor-promoting activity [131]. As a result this has reduced its use in therapy. Also some anthraquinone derivatives from Madder root (*Rubia tinctorum* L., Rubiaceae), such as alizarin, have been used as food coloring and also for chelating properties in the prevention of kidney stones. With oral doses of glycosides and aglycones, a pronounced calcium complexing effect and a significant reduction in the growth rate of kidney stones was observed in an animal model [132]. Madder root has been withdrawn from the market due to concerns over its mutagenicity and potential carcinogenicity [133]. Recently some studies indicating radical scavenging activity of alizarin were conducted [134]. Some dianthrones, e.g., hypericins from herb of St. Johns' Wort (*Hypericum perforatum* L., Hypericaceae), show no laxative activity (because of the absence of phenolic groups at C-1 and C-8). Hypericin and pseudohypericin are dianthrones with antiviral and antidepressant activity [135,136]. Several other anthraquinone aglycones including rhein, alizarin and emodin have also demonstrated antiviral activity against human cytomegalovirus [137]. This may explain the traditional use of applying the leaves of *Cassia* species to viral skin conditions [132]. Hypericin caused phototoxic reactions if exposure to direct sunlight and interacts with several drugs (see also *Hypericum perforatum* characterization in the Table 8.3).

8.7.3 Nutraceutical Applications of Anthraquinone Glycosides

Anthracene derived laxative drugs should not be recommended as nutraceuticals and for long-term use because of their undesirable effects.

8.7.4 Pharmaceutical Applications of Anthraquinone Glycosides

The list of the plant drug containing anthraquinone glycosides is presented in the Table 8.4. All these drugs are used as laxative. Some of these drugs are also used sometimes to aid in weight loss. However, most health authorities discourage the use of laxatives for weight loss, saying that they do not significantly reduce absorption of food calories [138].

8.7.5 Adverse Effects of Anthraquinone Glycosides

The adverse effects of laxative anthraquinone drugs are more likely to result from the excessive loss of fluid and electrolytes, particularly potassium, associated with the use of high doses. Habituation mechanism is a result of the fact that chronic abuse of laxatives raises aldosterone levels in response to the electrolyte loss diminishing their effectiveness. Higher doses also empty a larger portion of the colon and the resulting natural absence of defecation over the next day leads to anthraquinone reuse [11]. Long-term use of laxatives should be avoided, because of possible laxative dependence (stimulated peristalsis begins to replace natural peristalsis), and because it may produce a harmful effect on intestinal mucosa which leads to a condition known as melanosis coli (or pseudomelanosis). This is usually observed after a minimum of 9–12 months of regular stimulant laxative use [139]. The pigmentation of the intestine wall is due to staining by lipofuscin (peroxidized fatty acid). However, the intrinsic color of anthraquinones may play some part in the development of this pigment, and may be considered as a precursor to more serious intestinal problems, such as colon cancer [139,140]. However, senna is not carcinogenic in rats even after a 2-year daily dose of up to 300 mg/kg/day and the current evidence does not show that there is a genotoxic risk for patients who take laxatives containing senna extracts or sennosides [141]. Undesirable effects as abdominal spasms and pain, discoloration of urine by metabolites, and hemorrhoid congestion are frequent. In one study of colon submucosal nerves in patients with chronic abuse of laxatives, it appeared that nerve fiber damage was related to both dosage and duration of laxative use [142]. In a report from China, patients with addiction to senna leaf tea as a laxative were reported to suffer from symptoms of fidgetiness, sleeplessness, dilated pupils, and loss of appetite when consuming 5–9 grams of senna daily (10 times the range mentioned above that would be deemed safe) [143]. Cases of rare hepatic inflammation possibly induced by anthraquinone derivatives have been reported [144,145] and may be dose related. Hypokalemia, occurring as the effect of long-term use of laxative drugs, potentiates the action of cardiac glycosides and interacts with antiarrhythmic drugs. Use with other drugs inducing hypokalemia (e.g., diuretics, adrenocorticosteroids, and licorice root) may accelerate electrolyte imbalance. Contraindications for anthracene laxatives are intestinal obstruction, chronic intestinal inflammation, such as gastric or duodenal ulcers, or ulcerative colitis.

8.7.6 Metabolic Profile of Anthraquinone Glycosides

1,8-Hydroxyanthracenes possess a laxative effect. They act directly on the intestinal wall (in the colon region) to produce the desired result. The β-linked sennosides (and also glycosides from rhubarb or frangula) are not absorbed in the upper gut; they are converted by bacteria of the large intestine into the active metabolite (rhein anthrone). The mechanisms of action are firstly an influence on the motility of the large intestine (stimulation of peristaltic contractions and inhibition of local contractions) resulting in accelerated colonic transit, thus reducing fluid absorption [146]; secondly, there is an influence on secretion processes (change in absorption and secretion of water; retention of potassium, stimulation of active chlorine secretion) resulting in enhanced fluid secretion. The mechanism of action may be by a direct stimulation of peristaltic activity or possibly via an irritation of the intestinal mucosa and endothelial cells [11]. The bianthrones, especially sennosides, as found in rhubarb and senna, appear to be more active as laxatives than the simple anthraquinones. Defecation takes place after a delay of 8−10 h due to the time taken for transport to the colon and metabolization into the active compound. After oral administration, mainly anthraquinone aglycones are absorbed and were found in the blood as their corresponding glucuronide and sulfate derivatives. Emodin was found to be highly bound (99.6%) to serum protein [147]. Three to 6% of sennosides are excreted in urine and some in bile. Most sennosides are excreted in the feces as polyquinones together with unchanged sennosides.

8.8 SAPONIN GLYCOSIDES (SPG)

Saponins are generally known as nonvolatile compounds that are widely distributed in nature [9,21,148,149]. The name "saponin" is derived from the Latin word "*sapo*," (Eng. "soap"), reflecting their surfactant properties: ability to form stable soap-like foams in aqueous solutions. The amphiphilic nature of saponins; the combination of a hydrophobic aglycone backbone (sapogenin) linked to the hydrophilic sugar chain/s, distinguish these compounds from other glycosides [3,150]. The chemical structure of sapogenin defines the classification of saponins as steroidal or triterpenoid. Steroidal saponins consist mainly of a C_{27} spirostane skeleton, generally comprising of a six-ring structure (Fig. 8.10A), which occur as (25*S*)-spirostan derivatives (real saponins = neosaponins) or its (25*R*)-spirostan derivatives (isosaponins). In some cases the aglycone structure is referred to as a furostane skeleton, which is pentacyclic. It is seen mainly

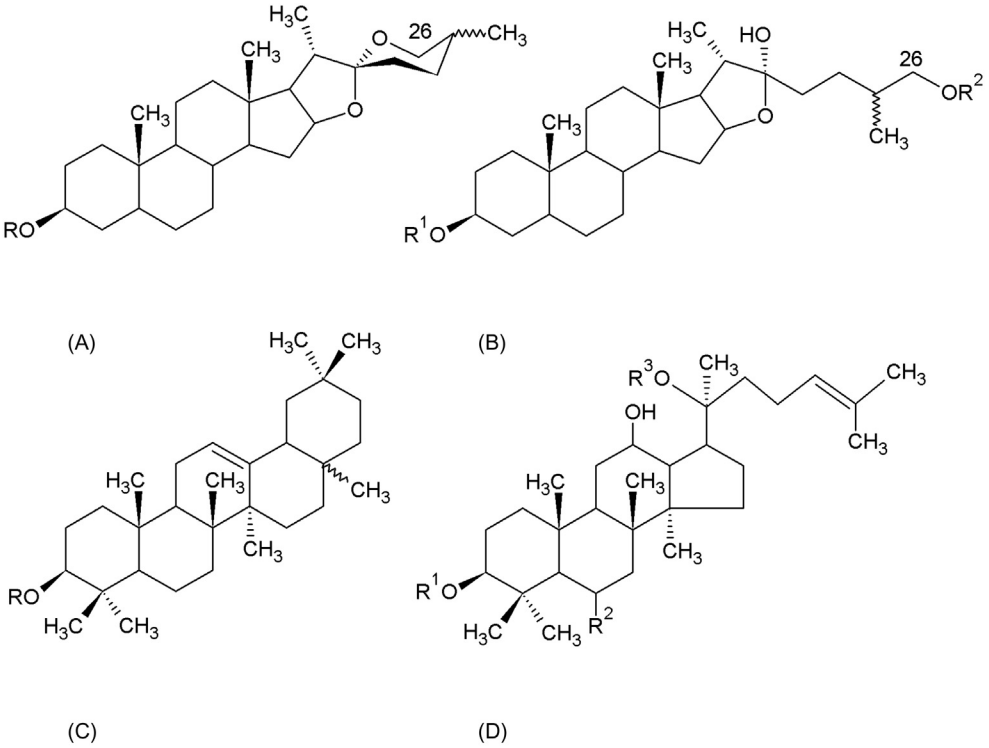

FIGURE 8.10 The main structural types of saponoside aglycones: steroidal spirostane (A) and furostane (B) skeletons, triterpenoid pentacyclic (C) and tetracyclic dammarane type structure (D).

as (25*S*)- or (25*R*)-furostan derivatives; in this case the C-26 OH group is engaged in a glycosidic linkage (Fig. 8.10B). Triterpenoid saponins aglycone consist of a pentacyclic C_{30} skeleton (Fig. 8.10C), and it is most commonly oleanane or ursane structures, or in a some cases a tetracyclic dammarane type structure as in ginsenosides (*Ginseng* saponosides) (Fig. 8.10D) [3,6].

Both classes of saponins are derived from the C_{30} carbon atoms precursor: 2,3-oxidosqualene. The starting point for cyclization reactions in triterpenoid biosynthesis is a result of linking two units of C_{15}-atom molecule farnesyl pyrophosphate in a head-to-tail manner [151,152]. Cyclization and rearrangement reactions of the 2,3-oxidosqualene molecule lead to the various triterpenoid and steroid structures [153] further classified, according to the sapogenin skeleton, into 11 main classes: oleananes, ursanes, dammaranes, lupanes, taraxasteranes, hopanes, cycloartanes, lanostanes, tirucallanes, cucurbitanes, and steroids [150]. In the oleanane type of saponins, a hydroxyl group at position C-3 is found in all structures. Very often hydroxyl groups are also reported at positions C-16, C-21, and C-22, and less often in positions C-2 and C-15. The methyl groups at positions C-23, C-24, C-28, C-29, and C-30 can be oxidized to CH_2OH- or COOH− moieties, and in some cases also to a CHO− group. The hydroxyl groups can be acylated, leading to the formation of ester saponins [154,155]. Monodesmosides are formed on the sugar chain, normally attached through an ether linkage at C-3. In bidesmosides the second sugar chain is attached through an ester linkage at C-28 (in triterpene saponins) or an ether linkage at C-26 (in furastanol saponins). The most common monosaccharides include: D-glucose, 3-methyl-D-glucose, D-galactose, L-rhamnose, D-fructose, L-arabinose, D-xylose, D-apiose, and D-chinovose, in addition to D-glucuronic acid and D-galacturonic acid [19,155].

8.8.1 Plants Containing Saponin Glycosides

Most known saponins are plant-derived secondary metabolites, found in more than 100 families of both wild and cultivated plants [155], although several saponins are also found in marine animals such as starfish (Asteroideae) [156] and sea cucumbers (Holothuroideae) [19,157,158]. The ability to synthesize saponins is widespread among plants belonging to the division of Magnoliophyta, in two major classes, Liliopsida (the class of monocotyledons) and the Magnoliopsida (the class of dicotyledons), where the majority of saponin-producing species was found [150,153,159]. Plant families accumulating triterpenoid saponins include the Amaranthaceae, Apiaceae, Aquifoliaceae, Araliaceae, Berberidaceae, Caryophyllaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Myrsinaceae, and Zygophyllaceae. The oleanane type of sapogenin is most common, followed by ursane type (which differs in the location of one methyl group). Triterpenoid saponins have been found in over 500 plant species such as alfalfa, licorice, horse chestnut, peas, soybean, quillaja, quinoa, tea, spinach, sugar beet, and sunflower. The steroidal saponins are typically found in members of the Agavaceae, Alliaceae, Amaryllidaceae, Asparagaceae, Bromeliaceae, Dioscoreaceae, Liliaceae, Palmae, and Scrophulariaceae families, and occur predominantly in the genera *Agave* (85 species), *Discorea*, and *Yucca*, and 56 other genera in plants, such as asparagus, ginseng, oats, and tomato. Two major commercial sources of these saponins are *Yucca* (*Yucca schidigera;* 8−12% of saponins) and *Quillaja* (*Quillaja saponaria;* 8−10% of saponins). Saponins have been isolated from different parts of the plants, which include the bark, leaves, stems, roots, seeds, and fruits. The relationship between the type of skeleton of saponins and the plant origin have been investigated and revealed that the same type of saponin skeleton can be obtained from various plant parts, and that the distribution of skeletons in the plant kingdom did not seem to be specific to order or subclass of plant [150]. The biological functions of saponins in plants are not completely clear. They are generally considered to be a part of a plant defense system due to their antimicrobial, fungicidal, allelopathic, insecticidal and molluscicidal, antiparasitic and antifeeding effects [159]. The concentrations of SPGs differ among plants and it is a function of plant species and variety, maturity, environmental conditions (sunlight, humidity, disease and insect attack, temperature), cultivation year, location grown, and season [19]. The most frequently used plants and plant drugs containing SPGs are listed in Table 8.5.

8.8.2 Bioactivity of Saponin Glycosides

SPGs have a diverse range of activities, which include the previously mentioned foaming and emulsifying properties [160,161], hemolytic properties [159,162], as well as sweetness (licorice saponins) and bitterness [163−165]. As surface active compounds in aqueous solutions, they form micelles as concentration reaches a critical level. They have solubilization properties for other compounds and can be used to enhance penetration of macromolecules such as proteins through cell membranes. Saponins have found wide applications in beverages and confectionery, as well as in cosmetics [161] and in pharmaceutical products [159], e.g., as adjuvants in vaccines. Steroidal saponins, e.g., diosgenin from fenugreek seeds or tubers of *Dioscoreavillosa* L.(Dioscoreaceae) are suitable precursors of partial synthesis of steroid

TABLE 8.5 Medicinal plants containing saponins

Plant- botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose/undesirable effects/ interactions	Clinical trials/preclinical studies/ contra-indications
Triterpenoid saponins				
Hedera helix L. (Araliaceae)	***Hederae helicis folium*** **Ivy Leaf** oleananes: hederasaponins: C,B,D F,G,E,H,I (= hederacosides) α-hederin	expectorant anti-inflammatory Adjuvant treatment of inflammatory bronchial diseases	Can provoke nausea, vomiting, diarrhea and excitation. /Fresh leaf can cause contact dermatitis.	Reduction in the frequency of coughs in children and adults suffering from various respiratory complains involving coughing.
Primula veris L. (*syn.P. officinalis* L.) *P. elatior* Hill. (Primulaceae)	***Primulae radix*** **Primula Root** Saponins - oleananes: prymverosaponin Phenolic glycosides; Primverin and primulaverin (2-primverosides of 4-methoxy- and 5-methoxy-salicilic acid)	expectorant productive cough, catarrh of the respiratory tract, chronic bronchitis Daily dose 0.5-1.5 g of the drug as decoction	Stomach upset, vomiting or diarrhea /in rare cases gastrointestinal disturbance	None reported. /low oral toxicity due poor absorption, favorable risk: benefit ratio
Glycyrrhiza glabra L. *G. uralensis* Fish. (Fabaceae)	***Liquiritiae radix*** **Licorice Root** oleananes; glycyrrhizic acid, glycyrrhizin – mixture of K and Ca salts of 3β-diglucuronide of glycyrrhetic acid	anti-inflammatory anti-allergic antiulcer hepatoprotective antimicrobial antiviral adjuvant therapy of gastric and duodenal ulcers and gastritis, also as expectorant in coughs and bronchial catarrh	Max daily dose 15 g of licorice root (or 600 mg of glycyrrhizin) should never be exceeded. Overdose (more 20 g/day) can cause hypermineralocorticoidism (hypertension, headache, lethargy, edema, muscle weakness to temporary paralysis, hypertensive encephalopathy and retinopathy and even heart failure). Do not use more 4-6 weeks without medical advice.	Contra-indications: cardiovascular-related disorders, renal disorders, hypokalemia, cholestatic or inflammatory liver disorders.
Panax ginseng C. A. Meyer (Araliaceae)	***Ginseng radix*** **Ginseng** dammaranes: protopanaxadiole derivatives: ginsenosides Ra_1, Rb_1, Rb_2, Rc, Rd; protopanaxatriole derivatives: ginsenosides Rg_1, Rg_2, Re, Rf oleananes: ginsenoside Ro	adaptogenic tonic effect on learning, memory and performance, immunomodulation hepatoprotective antioxidant. In weakness, exhaustion, tiredness, loss of concentration, during convalescence, also possible application in the Alzheimer disease treatment	Not associated with serious adverse effects if taken at the recommended dose level. Excessive use and uncontrolled ginseng products should be avoided. Interactions of ginseng with warfarin anticoagulant therapy was reported with unknown mechanism of action.	In clinical studies daily oral administration of 200 mg of standardized ginseng extract for 12 weeks did not cause any significant differences in blood levels of sex hormones. Contra-indications not known.
Eleuterococcus senticosus (Rupr. Et Maxim.) Maxim. *(syn. Acanthopanax senticosus* (Rupr. Et	***Eleuterococci radix*** **Eleutherococcus** oleananes: eleuterosides I,K,L,M acanthopanaxosides A-C [1] dammaranes protoprymulagenin A derivatives [2]	adaptogenic antiviral hepatoprotective cytostatic immunostimulant Use in weakness, exhaustion, tiredness, loss of	No toxic. Undesirable effects no reported. Dosage: Adults; 2-3 g of dried root/daily (65-195 mg of dry extract; 14-25:1 ethanol 40%)	In clinical studies (40 hospitalized patients with neurasthenic syndrome) performance improved after 3 weeks even after single dose/day of 120 mg dry extract. Other clinical studies concerning tumor patients, meningococcal or *Herpes*

Maxim.) Harms. (Araliaceae) Also known as Siberian ginseng		concentration, during convalescence.		*simplex* infections = in all cases lower ratio of post-operative complications and improvement of outbreaks was observed. Contra-indications not known
Polygala senega L. (Polygalaceae)	***Polygalae radix*** **Senega** Saponins based on aglycone presenegenin; senegins I-IV senegasaponins A-C Root	expectorant anti-inflammatory antiviral ↓blood triglyceride level hypoglycemic productive cough, chronic bronchitis, catarrh of the respiratory tract	Adult daily dose; 1.5-3 g (hydroethanolic prep.) or 2.5-5 g (aqueous prep.) In sensitive individuals gastrointestinal disturbance may occur. No interactions reported	Preclinical study: poor absorption from gut caused low toxicity. Contra-indications: gastric ulcer, gastritis
Aesculus hippocastanum L. (Hippocastanaceae)	***Hippocastani, semen*** **Horse-chestnut** Aescin (3 main fractions; α-,β- and cryptoaescin); mixture of more than 30 saponins based on barringtogenol C and protoaescigenin which is esterified with acids; angelic (21β position), tyglic or acetic (22α position), each saponin has trisaccharide group at C3 consisted from; glucuronic acid with substituent sugar: glucose, galactose and/or xylose (C2,C4)	vasoprotective anti-edema anti-inflammatory antithrombotic antihaemorrhoidal effect of venous tone Chronic venous insufficiency, varicosis	Adult daily dose: 50-150 mg (calculated as aescin) in divided doses. /gastric irritation or pruritus in rare cases, dizziness, headache or itching very rare	Standardized Horse-chestnut Dry Extract (SHDE) was recognized as safe and effective in short treatment of chronic venous insufficiency. In clinical safety studies excellent tolerability was observed. Contra-indications not known.
Astragalus mongolicus Bunge *var. mongolicus* or *var. dahuricus*	***Astragali mongolici radix*** **Milk vetch** <u>20,24-epoxycycloartanes:</u> astragalosides I-VII, agroastragalosides III, IV, isoastragalosides I and II, cycloartanes: agroastragalosides I and II oleananes: astragaloside VIII	adaptogenic antiviral hepatoprotective inotropic positive antidiabetic immunostimulant antirheumatic anti-inflammatory Psychotropic activity; anxiolytic (tranquilizer), antidepressant, antiamnesic, and antiaggressive effects [3].	Interactions: immunosuppressants may interact with Astragalus.	Contra-indications in autoimmune diseases. Astragalus might make the immune system more active. This could worsen the symptoms of auto-immune diseases.
Centella asiatica (L.) Urban (Apiaceae)	***Centellae asiaticae herba*** **Centella, Gotu kola** <u>ursanes:</u> asiaticoside, asiaticoside A madexaside, centelloside, bramoside, braminoside <u>oleananes:</u> asiaticoside B	adaptogenic antidepressive haemostatic antihaemorrhoidal ↑collagen production. Chronic venous insufficiency, varicosis, wound healing possible application in the Alzheimer disease treatment. External use in cosmetology.	No toxic effects reported. Contact allergy after topical application Gotu kola might cause too much sleepiness if combined with medications used during and after surgery. Interactions with sedative drugs and drugs which cause liver damage [4].	Effectiveness of modulation collagen production was confirmed. In the majority of randomized studies (60-180 mg centella triterpene fraction was taken orally/12 months) no side effects were reported. Contra-indications in case of hypersensitivity to Apiaceae plants, and in case of hepatitis.[4].

(*Continued*)

TABLE 8.5 (Continued)

Plant- botanical name (Family)	Plant drug /Compounds	Medicinal use /therapeutic indications	Overdose/undesirable effects/ interactions	Clinical trials/preclinical studies/ contra-indications
Steroidal saponins				
Ruscus aculeatus L. (Ruscaceae)	***Rusci rhizoma*** **Bucher's Broom** spirostanes: ruscogenin glycosides neoruscogenin glycosides: ruscin, deglucoruscin furostanes: ruscoside, deglucoruscoside	vasoprotective antihaemorrhoidal diuretic Supportive therapy; in chronic venous insufficiency (painful, tired and heavy legs, tingling and swelling), in symptoms of haemorrhoids (itching and burning) Applied in cosmetology	Adult daily dose: 7-11 mg of total ruscogenins. No toxic effects reported. May cause stomach upset and nausea. Interactions: with medications used for high blood pressure (Alpha-adrenergic antagonists) – when taken together may decrease effectiveness of the therapy. Also interactions with stimulant medications (Alpha-adrenergic agonists) – it taken together can speed up the nervous system, increase blood pressure, and make the heart beat to fast.	Very good efficacy 85-93% in treatment of haemorrhoids and anorectal complains was observed in clinical study (1800 patients). In clinical studies (166 women suffering from chronic venous insufficiency) effective reduction of lower leg volume was observed. In clinical safety data no toxicity was observed. Contra-indications: none known.
Trigonella foenum-graecum L. (Fabaceae)	***Trigonellae foenugraeci semen*** **Fenugreek** furostanol 3,26-diglycosides: trigofoenosides A-G after hydrolysis; spirostanes: diosgenin and its 25β-epimer yamogenin (3:2 ratio, total 95% of saponin fraction) tigogenin and fenugreekine (steroidal sapogenin-peptide ester)	antiatheromatic antimicrobial insulin-stimulating appetite stimulating hypoglycemic and hypocholesterolaemic: ↓ absorption of cholesterol, lipids and glucose form intestinal tract /adjuvant therapy in diabetes mellitus, anorexia and hypercholesterolaemia. External uses: ulcers, eczema, furunculosis	Large dose (100 g daily) cause minor gastrointestinal symptoms: diarrhea, flatulence. Allergic reactions in rare cases The absorption of the drugs taken concurrently (affected by high mucilaginous fiber content in fenugreek)	In clinical studies hypocholesterolaemic and hypoglycemic effects were observed. Fenugreek improved peripheral glucose utilization and may exert its effect acting at the insulin receptor as well as at the gastrointestinal level. In clinical safety studies no renal or hepatic and no hematological abnormalities were observed after ingestion of 25 g fenugreek powder for 24 weeks (in 60 non-insulin-dependent diabetic patients). Some patients complained of diarrhea and flatulence (subsided after 3-4 days).Contra-indications: none known.

Data collected in the Table 8.5 were derived/summarized from EMA Assessment Reports, ESCOP and European Pharmacopoeia – Council of Europe monographs and from references below.

[1] Jiang W, Li W, Han L, Liu L, Zhang Q, Zhang S, Nikaido T, Koike K.Biologically active triterpenoid saponins from *Acanthopanax senticosus*. J Nat Prod 2006;69(11):1577–81.
[2] Segiet-Kujawa E, Kaloga M. Triterpenoid saponins of *Eleuterocaccus senticosus* roots. J Nat Prod 1991;54(4):1044–48.
[3] Molodavkin GM, Voronina TA, Aldarmaa J. Psychotropic effect of the Astragalus mongolicus preparation. Eksperimental'naia i klinicheskaia farmakologiia (2000;63(6):12–14.
[4] Gohil KJ, Patel JA, Gajjar AK. Pharmacological Review on *Centella asiatica*: A Potential Herbal Cure-all. Indian J Pharm Sci 2010;72(5):546–556. doi: 10.4103/0250-474X. 78519.

hormones such as cortisone, progesterone, and pregnenolone [20,166]. Saponins are known to be major constituents of many traditional folk medicines (e.g., extracts of licorice, *Glycyrrhiza* sp., or ginseng, *Panax* sp.) [167–169], and are used to stimulate bronchial secretion and as an expectorant drugs (e.g., saponins from *Ipecacuanha*, *Senega*, *Glycyrrhiza*, *Verbascum*). Glycyrrhizin from licorice has well known antiinflammatory activity in gastric ulcers, and has cortisone-like properties in treatment of rheumatic arthritis. Saponins are poorly absorbed as glycosides in the intestine, therefore it is likely that any pharmacological activity observed would be as result of their aglycones. Well known and documented pharmacological and medicinal properties of natural drugs containing saponins (listed in European Pharmacopoeia, EMA and ESCOP monographs, and from the recent studies) are summarized in Table 8.6. Numerous reports highlighted their cytotoxic and antitumor properties [170], as well as antiproliferative activities [171,172]. Some antiviral properties of saponins were reported, e.g., triterpenoid saponins from the Fabaceae family have been reported to have antiherpes virus activity [173]. Triterpenoid saponins from the roots and flower buds of *Panax notoginseng* (Burk.) F.H.Chen (Araliaceae) showed potent hepatoprotective effects on liver injury induced by D-galactosamine and lipopolysaccharide [174]. In vitro studies revealed that ginsenosides Rb_1 and Rg_1, isolated from the roots of *Panaxginseng* act as neuroprotective agents [175]. Saponins from *Allium* sp. and *Acorus calamus* L. (Araceae) decreased the plasma total cholesterol levels [176], serum cholesterol, and triglyceride levels significantly [177]. Saponins isolated from *Polygala senega* L. (Polygalaceae), have potential vaccine adjuvant activity, increasing specific immune responses in mice immunized with rotavirus [178]. Triterpene saponins from *Silene fortunei* Vis. (Caryophyllaceae), enhanced the accumulation and cytotoxic activity of the anticancer agent cisplatin against human colon tumor cells [179]. The perennial creeping plant *Gynostemma pentaphyllum* Thunb. (Cucurbitaceae), known in China as "jiaogulan," is known as an adaptogen (which helps the body to maintain optimal homeostasis), and contains chemical compounds closely structurally related to the ginsenosides. These triterpenoid saponins called gypenosides reduced inflammation via COX-2 inhibition [180,181].

8.8.3 Nutraceutical Applications of Saponin Glycosides

Nutraceutical applications of saponosides are in many cases connected with plants containing these compounds as plant medicines, e.g., ginsenosides—see Table 8.5.

8.8.4 Pharmaceutical Applications of Saponin Glycosides

Plant drugs containing saponins are summarized in Table 8.5. Many new derivatives of plant saponins were synthesized and evaluated based on their different activities. For example, Kondratenko et al. [182], starting from penta-acetyl glycyrrhizin (GL) or penta-acetyl GL 30-methyl ester, synthesized new saponin compounds with monosaccharide residues of glucose and galactose through ester bonds. In an experimental model where rat stomach lesions were induced by acetylsalicylic acid, some derivatives expressed antiulcer effects at the dose of 25 mg/kg (administered orally), against 100 mg/kg of the standard drug Carbenxolone or 50 mg/kg of GL. For more interesting examples see the review where Graebinet et al. [183] focusing on a series of new derivatives synthesized using glycyrrhizin and its aglycon, glycyrrhetinic acid (GLA), as starting materials, reported on the pharmacological activities described for those compounds, as well as on the new activities observed for GL and GLA.

The second example is analysis of semisynthetic derivatives of oleanolic acid, bardoxolone (CDDO), and bardoxolone methyl (CDDO-Me), which were clinically evaluated as anticancer agents in patients with chronic kidney disease and type 2 diabetes, to improve kidney function. However, the worldwide phase III trial of the CDDO-Me drug was terminated following severe adverse effects and mortality in patients when the drug was administered (http://www.clinicaltrials.gov/show/NCT01351675) [184].

8.8.5 Adverse Effects of Saponin Glycosides

Many saponin glycosides exhibit toxic effects at high doses over an extended period, causing problems such as excessive salivation, diarrhea, vomiting, loss of appetite, and manifestations of paralysis (Table 8.5). Oral toxicity of saponins in warm-blooded animals is relatively low due to its low absorption from the intestinal tract (opposite to high toxicity for most cold-blooded animals, e.g., fish). However, saponins are gastrointestinal irritants and in milder examples, this can lead to esophageal reflux in sensitive or overweight patients. The low availability of saponins due to the intestinal tract, results in insignificant toxicity. However, they are highly toxic when given intravenously [11].

TABLE 8.6 Widely known plant species containing CRGs and the main compounds

Natural source	Compounds
Cardenolides	
Digitalis purpurea L. (Scrophulariaceae) *Digitalis purpureae folium* Foxglove leaf	Digitoxygenin: Purpureaglycoside A (Dt-Dt-Dt-Gl) Digitoxin (Dt-Dt-Dt) Gitoxigenin: purpureaglycoside B (Dt-Dt-Dt-Gl) gitoxin (Dt-Dt-Dt) *Digitalinum verum* (Dl-Gl) Gitaloxigenin: purpureaglikoside E (Dt-Dt-Dt-Gl) gitaloxin (Dt-Dt-Dt)
Digitalis lanata Ehrh. (Scrophulariaceae) *Digitalis lanatae folium* Foxglove leaf	Digitoxygenin: Lanatoside A (Dt-Dt-AcDt-Gl) Acetyldigitoxin (Dt-Dt-AcDt) Gitoxigenin: Lanatoside B (Dt-Dt-AcDt-Gl) Acetylgitoxin (Dt-Dt-AcDt) Digoxigenin: Lanatoside C(Dt-Dt-AcDt-Gl) Acetyldigoxin (Dt-Dt-AcDt) Digoxin (Dt-Dt-Dt) Diginatygenin: Lanatoside D(Dt-Dt-AcDt-Gl) Gitaloxigenin: Lanatoside E (Dt-Dt-Dt-Gl)
Convallaria majalis L. (Liliaceae) *Convallariae herba* Lilly of the Valley Herb	Glycosides derived from: aglycone with 5β-OH group and CHO at C-10 (strophantidin); convallatoxin (Rha) convalloside desglucocheirotoxin aglycone with 5β-OH group and CH_2OH at C-10 (strophantidol): convallatoxol (Rha) Aglycone with 5β-OH group, CH_3 at C-10 and OH at C-11: lokundjoside (Rha)
Adonidis vernalis L. (Ranunculaceae) *Adonidis herba* Spring Adonis Herb	Strophantidin: cymarin (Cy) β-strofantyna K (Cy-Gl) Adonitoxigenin adonitoxin (Rha)
Strophantus gratus Baill. *Strophantus combe* Olivier (Apocynaceae) *Strophanti semen* Strophant seed	strophantidin β-trophantin K strophantoside Ouabain (G-strophantin)
Nerium oleander L. (Apocynaceae) Oleander Rose laurel	oleandrin ouabain (G-strophantin)
Thevetia neriifolia Juss. = *T. peruviana* K.Schum. (Apocynaceae) Yellow oleander	thevetioside oleandrin peruvoside thevetin A
Bufadienolides	
Urginea maritima L. (Liliaceae) *Scillae bulus* Squill Squill bulb	Aglycone: scillarenin scillaridin (dehydratation product of scillarenin) glucoscillaren A (Rha-Gl-Gl) scillaren A (Rha-Gl) proscilaridin A (Gl)

Dt - digitoxose, Gl - glucose, AcDt - acetyldigitoxose, Cy - cymarose, Rha — rhamnose.

8.8.6 Metabolic Profile of Saponin Glycosides

The metabolic profiles of some active substances in saponin containing drugs (licorice, horse chestnut, and butcher's broom) are presented below. The antiinflammatory activity of glycyrrhetic acid has been well documented. Glycyrrhizin, one of the main bioactive constituents of licorice, was gradually metabolized to glycyrrhetinic acid monoglucuronide (GAMG) and glycyrrhetinic acid by human intestinal bacteria [185]. It is accepted that it acts indirectly by potentiating corticoids. Glycyrrhizin and its metabolite 18β-glycyrrhetinic acid, which is an inhibitor of cortisol metabolism (inhibits deactivation of cortisol by 11β-hydroxysteroid dehydrogenase), appear to behave like aldosterone without affinity for mineralocorticoid receptors [3,11,185]. The seeds of *A. hippocastanum* contain aescin, a natural mixture of triterpene saponins whose aglycons are derivatives of protoascigenin, acylated by acetic acid at C-22 and by either angelic or tiglic acids at C-21. Aescin exists in two forms, α and β, that can be distinguished by melting point, specific rotation, hemolytic index, and solubility in water and from which β-aescin appears to be the active component of the mixture and is the molecular form present in major available pharmaceutical products [186]. Horse chestnut extract containing saponins (and also flavonoids, procyanidins, and coumarins) acts on capillaries and veins and demonstrate antiedematous, antiinflammatory, and venotonic effects. Suggested mechanisms of action include a selective vascular permeabilization [187], allowing higher sensitivity of calcium channels to molecular ions, resulting in increased venous and arterial tone. Other mechanisms such as inhibition of the enzymes elastase and hyaluronidase, prevention of leukocyte activation, and influence on capillary filtration also occur [186,188–190].

Ruscus aculeatus saponins are efficiently absorbed when administrated orally and prevent the dilation of overloaded venous vessels. The activity of ruscogenins is linked to their stimulation of the postjunctional α1- and α2-adrenergic receptors of the smooth muscle cells of the vascular wall. Also their direct action on the venous wall fibers and stimulation of the release of norepinephrine from adrenergic nerve endings seems to be involved. Several clinical studies prove the vascular protective and venous tonic properties [3]. The butcher's broom extract is used for treatment of venous insufficiency such as fullness in the legs and the symptoms of hemorrhoids.

8.9 CARDIAC GLYCOSIDES (CRG)

Cardiac glycosides (CRGs) are natural metabolites in which small doses can affect the heart muscles in a specific way. Their effect (cardiotonic) was already known in ancient Egypt over 3000 years ago [115] and they have been used in the treatment of cardiac diseases for more than 200 years. CRGs form a well-defined group with homogenous structures. They comprise the 5β,14β-androstane-3β,14-diol (cyclopentane perhydro-phenanthrene) aglycone nucleus (Fig. 8.11A) and a sugar moiety (often an oligosaccharide) at a C-3β position. The first of two types of CRGs group are the cardenolide type (C_{23}), containing a five-membered unsaturated γ-butyrolactone (a butenolide) ring, and the bufadienolide type (C_{24}), characterized by a 2-pyrone (six-membered doubly unsaturated δ-valerolactone) ring system at C-17 (Fig. 8.12). The unsaturated C-17β-oriented lactone plays an important role in receptor binding and saturation of the ring significantly decreases the biological activity. Due to their stereochemistry, CRGs are structurally specific when compared to the classical steroidal carbon skeleton [191,192], because they have mainly a *cis*-conformation of A/B rings. Rings B/C are always *trans*-fused and rings C/D *cis*-fused (Fig. 8.11B). Opposite to steroidal sex hormones are mineralocorticoids and glycocorticoids, which are all *trans*-connected. CRGs rings have a characteristic "U" shape pharmacophore [193,194], which is very important for their activity. Structures with C/D *trans* fusion are inactive.

Members of CRGs family are additionally equipped with a β-configured hydroxyl group at C-14 (and frequently C-5). The OH group at position C-14β is not an essential feature for CRGs activity, because a skeleton without this

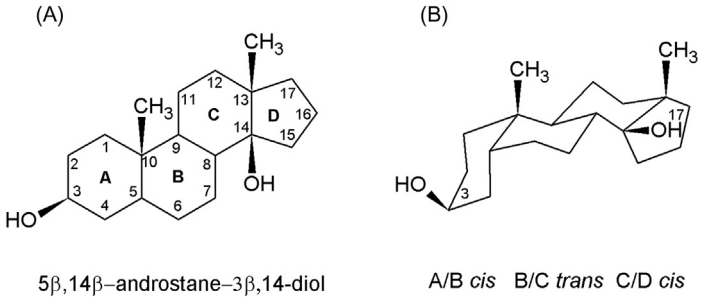

FIGURE 8.11 (A) 5β,14β-androstane-3β,14-diol aglycone nucleus and (B) *Cis-trans-cis* conformation (U-shape) of steroid skeleton of CRGs.

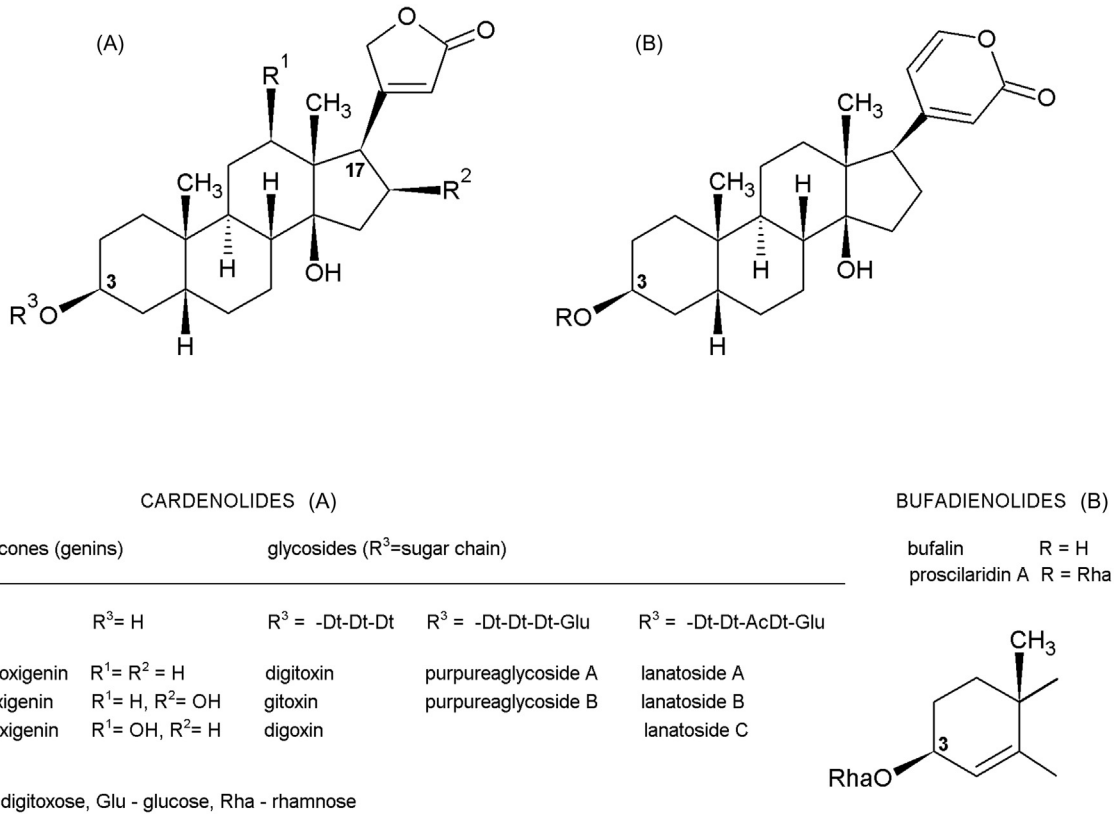

FIGURE 8.12 Cardenolide (A) and bufadienolide (B) structures with examples of both types of cardiac glycosides.

group but retaining the C/D *cis* ring fusion was still found active. When the C-14β-OH group is replaced by a hydrogen atom, the potency of the bioactivity decreased. A β − OH group at position C-16 reduces potency significantly, but if such an OH is esterified, potency increases. Attached to the 3β-OH group are normally one to four sugars units and predominantly in the *β*-conformation. The majority of saccharides are highly specific: 2,6-dideoxyhexoses (D-digitoxose) and 2,6-dideoxy-3-methylhexoses (L-oleandrose or D-diginose). D-Fucose (as an example of 6-deoxyhexoses) and 6-deoxy-3-methylhexoses; D-digitalose or L-thevetose also occur. D-Glucose is found mostly at the end of the oligosaccharide. The presence of an acetyl group on the sugar (as e.g., D-acetyldigitoxose) affects the lipophilic character and the kinetics of the entire glycoside. Sugar attachment modifies both pharmacokinetics and pharmacodynamics of CRGs. For example, free genins are absorbed faster and are metabolized more easily than their glycosylated counterparts. As a result the action of free aglycones is fast and short-lasting and in many cases creates toxic effects [193,195].

8.9.1 Plants Containing Cardiac Glycosides

Distribution of CRGs is limited to only some genera in families: e.g., Asclepiadaceae (*Asclepias*, *Calotropis*, *Cryptostegia*, *Periploca*, *Xysmalobium*), Apocynaceae (*Apocynum*, *Nerium*, *Strophantus*, *Thevetia*), Brassicaceae (*Erysimum*), Celastraceae (*Euonymus*), Crassulaceae (*Cotyledon*, *Kalanchoe*), Liliaceae (*Convallaria*, *Urginea*), Ranunculaceae (*Adonis*, *Helleborus*), Scrophulariaceae (*Digitalis*). All of the plant organs may contain CRGs. In plants CRGs are biosynthesized via the acetate mevalonate pathway (poliketide). The most important cardiac glycosides (e.g., digitoxin, digoxin, ouabain, and oleandrin) have been isolated from plants, including *Digitalis purpurea*, *Digitalis lanata*, *Strophanthus gratus*, and *Nerium oleander*. However, recognized as secondary plant metabolites, cardenolides and bufadienolides (e.g., digoxin, ouabain, bufalin, marinobufagenin, and telecinobufagin) may also be produced in animal tissues via the cholesterol pathway, and were found in amphibians and mammals (as was recently reported, probably also in a human body) [196–200]. Table 8.6 summarizes the most known plant species containing CRGs, focusing on investigated compounds.

8.9.2 Bioactivity of Cardiac Glycosides

Digitalis glycosides can be defined as allosteric inhibitors of Na^+-, K^+-ATPase, a protein cation pump, which uses the energy derived from the hydrolysis of ATP for active transport of potassium ions inside, and sodium ions outside cells (where K^+:Na^+ is as 2:3). The therapeutic concentrations of digitalis compounds produce a moderate enzyme inhibition (about 30%), which is enough for temporary increasing of the Na^+ level inside the cell. Thus (by inhibition of the Na^+/Ca^{2+} exchanger) the intracellular Ca^{2+} concentration rises and more calcium could be released by the sarcoplasmic reticulum, and is available to bind to the troponin-C (contractility of the heart muscle increases) [115,201–203]. However, recent results suggest that more than one mechanism or receptor is involved in effects of different CRGs [204]. The main effect of CRGs on the heart muscle is cardiotonic. It means that the force of systolic contractibility increases (positive inotropic effect) and the tone of cardiac muscle is improved (positive tonotropic effect). Therefore CRGs are used in the treatment of congestive heart failure and auricular fibrillation. *Digitalis* compounds also increase vagal efferent activity of the heart (para sympathomimetic action) and reduce sinoatrial firing rate (negative chronotropy). The conduction velocity of electrical impulses through atrioventricular node is also reduced (negative dromotropic effect). Some CRGs, mainly *Digitalis* glycosides (e.g., digitoxin), are cumulative and highly toxic (negative bathmotropic effect) and should be administered with a great care. In some countries, lily of the valley (*Convallaria majalis*), a herb containing cardiac glycosides is used, and it is combined with hawthorn (*Crataegus* sp.) in the management of milder forms of heart failure. Properties of *C. majalis* are similar to those of digitalis compounds, but much less cumulative. The main compound, convallatoxin, is poorly absorbed but the other components (saponosides) in the herb are said to aid its absorption [11]. CRGs increase in a renal blood flow and circulation and the additional effect of CRGs therapy is minor diuretic effect [205]. Recently some in vitro and ex vivo experiments have revealed that some cardiac glycosides (digitoxin, oleandrin) induce potent and selective anticancer effects [206–209]. Oleandrin, in Anvirzel has exhibited anticancer properties. Also a supercritical CO_2 extract of *Neriumoleander* L. (the botanical drug candidate PBI-05204), with oleandrin as the principal active constituent, exhibited potent anticancer activity. It was recently used in a phase I clinical trial as a treatment for patients with solid tumors [210]. Human melanoma and leukemia cells are more sensitive to oleandrin than murine tumor cells, normal human epithelial cells, peripheral blood mononuclear cells, and neutrophils. Therefore, the role of oleandrin as antiproliferative seems to be promising in a cancer therapy [211]. Recent screenings of drugs have identified several cardiac glycosides (e.g., ouabalin and bufalin) as potent inhibitors of cancer cell growth [212,213]. Found in mammalian tissues (also in human) and plasma endogenous ouabain (found in bovine renal gland and in hypothalamus), endogenous digoxin, proscilaridin, and telocinobufagin (found in uremic plasma) seems to be an important class of hormones synthesized in the adrenal cortex and similar to neurosteroids in brain (present in plasma in subnanomolar to nanomolar concentrations). An influence of endogenous (human tissue released) CRGs in a role in diabetes mellitus, sodium transport, blood pressure, central nervous function, ethanol addiction, cell growth and differentiation mechanisms was recently studied. It was proposed that dysregulation of these hormones seems to play an important role in a number of disease states, such as hypertension and cancer [199,200]. However, this promising idea needs to be further studied.

8.9.3 Nutraceutical Applications of Cardiac Glycosides

Because of toxicity of CRGs, no nutraceutical application has been reported.

8.9.4 Pharmaceutical Applications of Cardiac Glycosides

Plants containing CRGs are listed in Table 8.6. These plants are not used as crude drugs because of toxic effects and very close therapeutic and lethal doses. Also their galenicals, because of their irreproducible activity, have been abandoned. Pure isolated compounds or their derivatives are produced by the pharmaceutical industry. Digoxin and its derivatives (acetyl- and methyl-digoxin) are the cardiac glycosides most currently used in therapeutics [194]. Those drugs are administrated orally as tablets or intravenously as injections (e.g., Digoxin, Lanoxin). Ouabain from *Strophantus* sp. and proscilaridin from squill bulbs are also in use. Squill components are generally poorly absorbed from the gastrointestinal tract, and are less potent than digitalis. Meproscillaren, a semisynthetic derivative of proscillaridin, is absorbed orally and may be effective in some patients [214]. Preparations for oral administration are enteric-coated to prevent degradation by gastric acid.

8.9.5 Adverse Effects of Cardiac Glycosides

Most adverse effects of CRGs are dose-related. Binding cardenolides with plasma proteins may cause accumulation effect (toxicity). Toxic effects of digoxin (e.g., arrhythmias) occur when the cytoplasmic Ca^{2+} increases to concentrations exceeding the storage capacity of the sarcoplasmic reticulum. This gives rise to extrasystoles and sustained ventricular arrhythmias in vivo [201,215–217]. As more frequent adverse effects of CRGs: cardiac manifestations, (bradycardia, extrasystoles, tachycardia—generally linked to an overdose); digestive disorders (anorexia, nausea, vomiting, salivation, diarrhea, stomach pains); neurosensory disorders (headache, insomnia, and rarely: confusions, depression, visual disturbances of colors, dizziness, neuralgia of the trigeminal nerve), and endocrine adverse effects, such as gynecomastia in men (as result of an estrogen effect produced by CRGs because of their steroid structure) can be listed. A certain number of interactions between cardiac glycosides and other drugs were described, e.g., influence of potassium and calcium administration, which caused reinforcement of efficacy and toxicity of CRGs. Hypokalemia, as observed with certain diuretics, increases digitalis toxicity, hypercalcemia enhances digitalis-induced intracellular calcium level and arrhythmias. Quinidine competes with digoxin for binding sites and depresses renal clearance of digoxin (induces toxicity). Calcium-channel blockers and nonsteroidal antiinflammatory drugs also create a toxic effect.

8.9.6 Metabolic Profile of Cardiac Glycosides

The mechanism of action of several CRGs could be different; examples are digoxin and ouabain. Lipophilic digitalis glycosides increase the intracellular calcium concentration by entering the cell interior and acting on the ryanodine receptors (a class of intracellular calcium channels found in various forms of excitable animal tissue like muscles and neurons) and by forming transmembrane calcium channels. Ouabain, does not penetrate the plasma membrane but acts by activation of the $Na^{+}-K^{+}$-ATPase from the extracellular side, and via signal transduction pathways triggers release of calcium from intracellular stores and activates myocardial metabolism [180]. The pharmacokinetics of CRGs is closely dependent on the polarity of the molecule (e.g., on degree of hydroxylation of the aglycone; less hydroxylated are more lipophilic). Digitoxin is more lipophilic than digoxin and digoxin more than ouabain. Therefore the first is completely resorbed after oral administration, the second is less intense (80%), the third very poor. Ouabain may only be administrated intravenously and its renal excretion is very rapid. The half-life of digitoxin is about 6 days because it binds strongly to plasma proteins, while the half-life of digoxin is 36 h. Also nontoxic plasma concentrations are different for digitoxin (1 ng/mL), for digoxin (2 ng/mL), and for ouabain (10–30 ng/mL) [3].

8.10 CYANOGENIC GLYCOSIDES (CNG)

These are a specific group of glycosides comprised of compounds which can release hydrogen cyanide (historically called "Prussic acid") upon their hydrolysis. Chemically, these compounds are derived from aliphatic and aromatic L-amino acid precursors (Fig. 8.13), which are unstable but in plants can be stabilized by glycosylation [3,4]. CNGs are relatively stable when compared to their intermediate cyanohydrin. However in plants the β-glycosidic linkage can be hydrolyzed through the action of a β-glycosidase. To avoid autotoxicity, there is a separation of substrates and enzymes, which are stored in different compartments of the cell or in different tissues of plants which contain CNGs [218,219]; e.g., in sorghum leaves β-glucosidase (called dhurrinase) is located in the mesophyll cells whereas substrate (dhurrin) is found in the epidermal cells [220]. When the integrity of plant is disrupted (e.g., by herbivores chewing, crushing, or physical processes as maceration or freezing) those two components are in contact and a deglycosylation process takes place (Fig. 8.13). The released hydrogen cyanidehas a faint, bitter, almond-like odor which some people are unable to detect (20% of population is unable to detect HCN because of genetic trait).

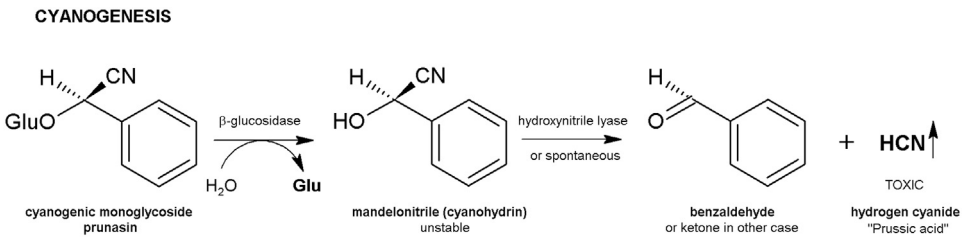

FIGURE 8.13 Cyanogenesis.

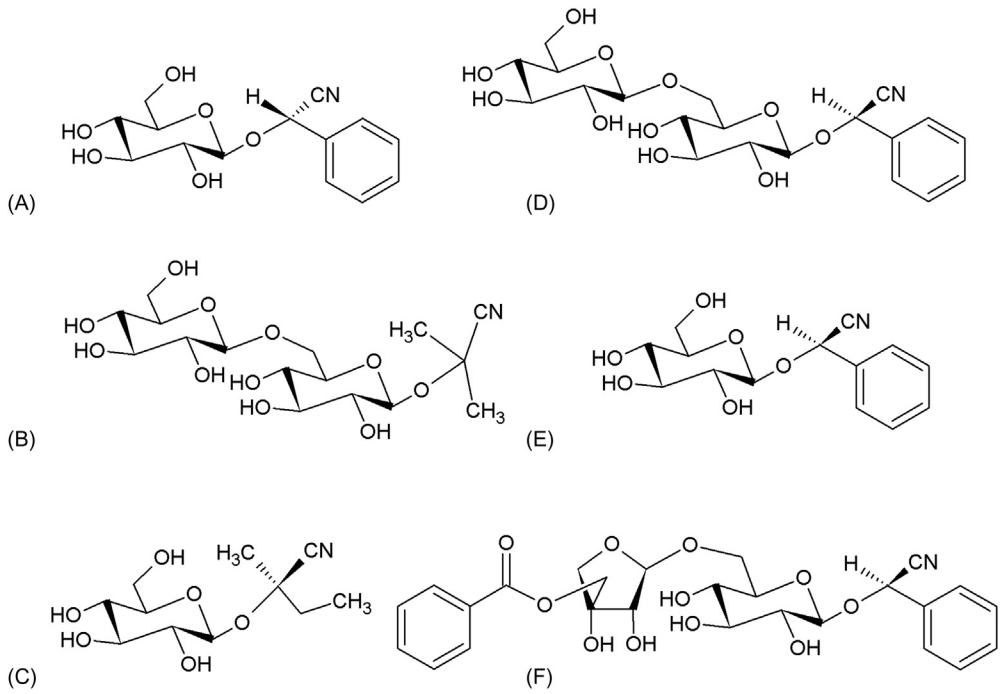

FIGURE 8.14 The most common cyanogenic glycosides of plant origin: (A) sambunigrin, (B) linustatin, (C) lotaustralin, (D) amygdalin, (E) prunasin, and (F) oxyanthin 5″- benzoate.

In cyanogenic glycosides, the most common sugar is glucose (most of CNGs are monoglucosides or diglucosides), although incorporation of other sugars such as apiose (together with glucose in oxyanthin-5″-benzoate) and arabinose or xylose are evident [23,221]. The most common examples are shown in Fig. 8.14. Some CNGs were isolated and their structures were elucidated years ago, e.g., amygdalin (in 1830, and in 1837 structure elucidation) and sambunigrin (in 1928) [23,222]. Recently, modern instrumental methods (MS, NMR) enable identification of new cyanogenic derivatives and hence there are still new findings in this expanding area [221].

8.10.1 Plants Containing Cyanogenic Glycosides

CNGs are hypothesized to be a part of an evolutionary defense mechanism developed in a large number of plant species—more than 3000 plant species are found to be cyanogenic [223,224]. The bitter taste and toxicity of cyanogenic compounds make plants containing them unattractive and not especially appetizing. Some animals (buds or the larvae of certain moths such as the six-spot burnet moth *Zygaena filipendulae*, family Zygaenidae) are able to sequester cyanogenic compounds (linamarin and lotaustralin) from a preferred feed plant or even to de novo biosynthesize these CNGs, and use them in their defense system against predators [219,225]. The defense role of CNGs in plants seems to be confirmed by the fact that most of the CNGs and their catabolic enzymes are located in periphery tissues and generative organs, and newly formed tissues seem to be always more cyanogenic than older tissues (e.g., bamboo immature shoot tip contains 8000 mg HCN/kg, sorghum whole immature plant 2500 mg HCN/kg) [226]. It is also believed that another important role for accumulated CNGs in plants is to provide a storage deposit of reduced nitrogen and sugar in angiosperm seeds for further development of seedlings [227,228]. This is confirmed by mechanisms of detoxification of CNGs, in which HCN released from cyanohydrin is bounded to cysteine by β-cyanoalanine synthase resulting in β-cyanoalanine synthesis (converted next by nitrilase to L-asparagine or L-aspartic acid and ammonia) [218]. Concentrations of cyanogenic glycosides in foods vary with climatic conditions and season. They are also highly variable depending on food preparation and processing. As we found in a WHO recent report concerning cyanogenic glycosides, their levels (expressed as "total HCN"—which is the total HCN content of all cyanogenic glycosides, cyanohydrins, and "free" HCN in a food) in plants used as food or for flavoring vary depending on both cultivar and environmental factors, e.g., in bamboo shoots (range 70−8000 mg/kg), bitter almonds (300−4700 mg/kg), lima beans

(to 3120 mg/kg), and bitter apricot kernels (90−4000 mg/kg) or cassava root (sweet 1−1064 mg/kg, and bitter 15−1120 mg/kg) [229]. Most commonly used plants containing CNGs are cultivated plants as listed below:

- The root of cassava (*Manihot esculenta* Crantz; cassava, manioc, tapioca, yucca) family Euphorbiaceae—contains linamarin and lotaustralin [230,231].
- The lima bean (*Phaseolus lunatus* L., family Fabaceae)—stores linamarin in seeds [232].
- Bamboo shoots (*Bambusa vulgaris* Schrad. ex J.C. Wendl. and *Phyllostachys edulis* (Carrière) J. Houz. = *Bambusa edulis* Carrière, family Poaceae)—both contain the cyanogenic glucoside taxiphyllin [233].
- Sorghum (*Sorghum bicolor* (L.) Moench., family Poaceae)—contains dhurrin [234].
- Flax seed (linen seed; *Linum usitatissimum* L., family Linaceae)—stores mainly the diglucoside linustatin and to a lesser extent neolinustatin [232,235,236].
- Stone fruits of *Prunus* sp. (Rosaceae); (e.g., *P. dulcis* var. *amara* = *Amygdalus dulcis* var. *amara*)—seeds are components in the production of beverages, pastry, or sweets/confectionary include almond—contains prunasin and amygdalin [3,232].
- Fruit of *Sorbus aucuparia* L. (Rosaceae)—has a small amount of amygdalin [3].

8.10.2 Bioactivity of Cyanogenic Glycosides

Many investigations concerning activity and mainly the toxicity of CNGs were conducted as in vitro and in vivo studies (see ATSDR reports [155] and WHO Food Additives Series: 65 [229]). Some CNGs were considered to be potential anticancer drugs, e.g., Laetrile also called amygdalin, although both are not exactly the same chemical structure. It was first used as a cancer treatment in Russia in 1845 with promising effects, and in the United States in the 1920s. Cyanide was thought to be the main anticancer component of this drug. To date, the effectiveness of this therapy remains controversial [237]. The cyanogenic plant drugs still used in pharmacies is Cherry Laurel (*Prunus laurocerasus* L., Rosaceae), which contains prunasin at the level from 1.2 to 1.8 g per 100 g. Crushed leaves of this plant release a characteristic bitter almond odor. Fresh cherry-laurel leaves are used to prepare cherry-laurel water (titrated to contain 100 mg/100 g in total HCN) used as an aromatizing agent, respiratory stimulant, and antispasmodic. *Prunus serotina* Ehrh. is also sometimes used to prepare a similar preparation, used for its expectorant and sedative properties [3].

8.10.3 Nutraceutical Applications of Cyanogenic Glycosides

Maximum levels for HCN in foodstuffs and beverages must be clearly stated, as cited from WHO Food Additives Series: 65 (2012): "Annex II of Council Directive 88/388/EEC on flavorings sets the following maximum levels for HCN in foodstuffs and beverages to which flavorings or other food ingredients with flavoring properties have been added: 1 mg/kg in foodstuffs and 1 mg/kg in beverages, with the exception of 50 mg/kg in nougat, marzipan or its substitutes or similar products, 1 mg per cent volume of alcohol in alcoholic beverages and 5 mg/kg in canned stone fruit. HCN should not be added as such to foodstuffs (EEC, 1988)" [229,238].

8.10.4 Pharmaceutical Applications of Cyanogenic Glycosides

As was mentioned previously the use of cyanogenic glycosides, e.g., amygdalin (named also vitamin B17), or products based on cyanogenic compounds (such as Laetrile) for the treatment or prevention of different forms of cancer has no satisfactory support in the scientific medicinal literature [222]. Laetrile has shown a little anticancer activity in animal studies and no anticancer activity in human clinical trials. The names laetrile, Laetrile, and amygdalin are not the same product. The US.-patented Laetrile is a semisynthetic derivative of amygdalin (mandelonitrile-β-glucuronide), which is different from the laetrile/amygdalin produced in Mexico (mandelonitrile β-D-gentiobioside), which is made from crushed apricot pits [239,240]. Several cases of cyanide poisoning have been linked to the use of Laetrile, and the US. Food and Drug Administration (FDA) has not approved it as a medical treatment in the United States. The use of Laetrile has been linked to cyanide toxicity and death in a few cases, especially when it was being taken by mouth. Although drug interactions are unknown, at least one case report suggests that vitamin C can increase the amount of cyanide released from Laetrile in the body (as reported on the website of American Cancer Society), and also ingestion of cyanide in Laetrile could be dangerous when combined with vegetarian diet with vitamin B12 deficiency [241−243]. Some recent systematic reviews which included all available up-to-date reports concerning cancer treatment with Laetrile, concluded that the claim that this drug has beneficial effects for cancer patients is not supported by clinical data [244,245].

8.10.5 Adverse Effects of Cyanogenic Glycosides

The toxic effects of cyanide ions in man and animals are generally similar and the chronic exposure to low concentrations of hydrogen cyanide affect the central nervous system of both animals and man [222]. Other primary targets of cyanide toxicity are the cardiovascular and the respiratory systems. Toxicity is due to inactivation of tissue cytochrome oxidase (cyanide combines with Fe^{3+}/Fe^{2+} contained in the enzyme) and inhibition of cellular respiration. Cyanide can inhibit several other metalloenzymes that contain iron, copper, or molybdenum. Cyanide causes an increase in blood glucose and lactic acid levels and a decrease in the ATP/ADP ratio indicating a shift from aerobic to anaerobic metabolism. Cyanide apparently activates glycogenolysis and shunts glucose to the pentose phosphate pathway decreasing the rate of glycolysis and inhibiting the tricarboxylic acid cycle [246]. Skin contact with hydrogen cyanide or cyanide salts can irritate and produce sores. Workers who breathed in amounts of hydrogen cyanide as low as 6−10 ppm over a period of time developed breathing difficulties, chest pain, vomiting, blood changes, headaches, and enlargement of the thyroid gland [247]. Acute toxicity and mortality induced by various cyanogenic glycosides in experimental animals are directly related to, and influenced by, factors associated with the release and detoxification of HCN [229]. An important way to avoid toxicity of CNGs is proper food processing, which eliminates most of CNGs from plant food (e.g., yucca, bamboo, sorgo, or flax seed in various forms of bread). As reported by WHO, the potential toxicity of HCN from ingested cyanogenic glycosides is dependent on a number of nutritional factors that are involved in detoxification mechanisms, including the availability of sulfur-containing amino acids and vitamin B12 [229]. Cyanide can react with hyroxycobalamine to form cyanocobalamine, and this reaction, with coexisting dietary deficit of sulfur amino acids, is partially involved in the degradation of peripheral nerves (*tropic ataxic neuropathy*,TAN—named *konzo*) [23,248]. Epidemic outbreaks of konzo have been reported in the Democratic Republic of the Congo, Mozambique, the United Republic of Tanzania, and the Central African Republic, with potential causal links with high consumption of inadequately processed cassava [229].

8.10.6 Metabolic Profile of Cyanogenic Glycosides

Following oral administration, ingested cyanogenic glycosides are absorbed and excreted intact in the urine. CNGs release HCN at slow rates as the activity of β-glucosidase is limited by the acidic pH of stomach. The unabsorbed fraction can be enzymatically converted to HCN by microorganisms in the gastrointestinal tract (GI). Only partial absorption of intact cyanogenic glycosides takes place from GI, where 10−20% of CNGs (e.g., linamarine) can be enzymatically converted to release cyanide [249]. The released HCN rapidly penetrate mucous and cell membranes (target tissues are; the liver, brain, spleen, blood, kidneys, and lungs). The cyanide is known to bind with iron in both methemoglobin and hemoglobin present in erythrocytes (approximately 99% of an absorbed dose) [229]. Because mechanisms of detoxification and excretion of HCN exists in plants, animals, and in fungi, poisoning occurs only when the rate of detoxification is slower than the rate of intake. Detoxification of HCN involves several different mechanisms. The first is common in plants and it is related to activity of β-cyanoalanine-synthase (mainly located in plant mitochondria). Its activity results in β-cyanoalanine synthesis in the presence of HCN and cysteine or serine. Next steps are catalyzed by nitrilases which convert β-cyanoalanine into asparagine or aspartate and ammonia. The second detoxification route proceeds by conversion of HCN (CN^-) in reaction with thiosulfate ($S_2O_2^{2-}$) into thiocyanate (rhodenide; SCN^-) and sulfite (SO_3^{2-}). This reaction is catalyzed by rhodanese enzyme (thiosulfate:cyanide sulfurtransferase) [250], which is present in humans, higher animals, plants, and insects [219]. Other mechanisms of HCN elimination are nonenzymatic such as excretion and exhalation (small amounts excreted through the lungs). Direct reaction of cyanide with cysteine gives 2-iminothiasolidine carboxylic acid, a product which is excreted with urine [247].

8.11 THIOGLYCOSIDES (THG)

Glucosinolates (thioglycosides; THGs) are the substituted esters of thio-amino acids; (*Z*)-(or *cis*)-*N*-hydroximinosulfate esters, known also as β-thioglucoside *N*-hydroxysulfates or as *S*-glucopyranosyl thiohydroximates [251,252], which contain a side chain (R) and a sulfur-linked β-D-glucopyranose moiety (Fig. 8.15). During biosynthesis of THGs, modification of the original amino acid occurs resulting in the formation of different (R) side chains. Methionine and cysteine are natural donors of the thio-group. Apart from alkylene glucosinolates, ω-methylthioalkyl, aromatic and heterocyclic are also present. Structural diversity of THGs reflects that of their amino acid precursor, which can be tyrosine (result benzylglucosinolate), phenylalanine (which gives benzylglucosinolateglucotropaeolin), tryptophan (3-indolylmethylglucosinolate), homomethionine (allylglucosinolate), or homophenylalanine (phenethylglucosinolate) [3,251,253]. Most of

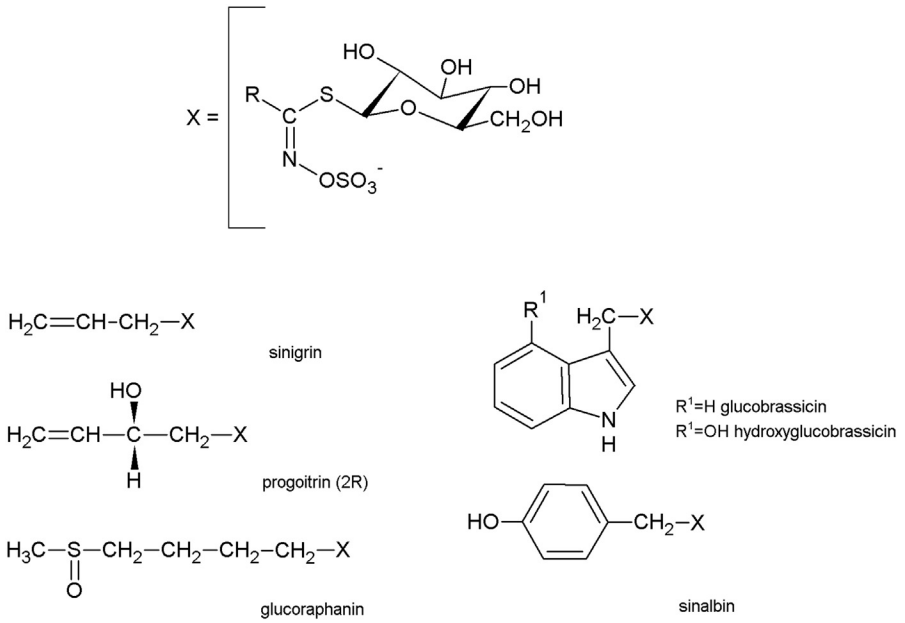

FIGURE 8.15 Chemical structures of widely known naturally occurring glucosinolates.

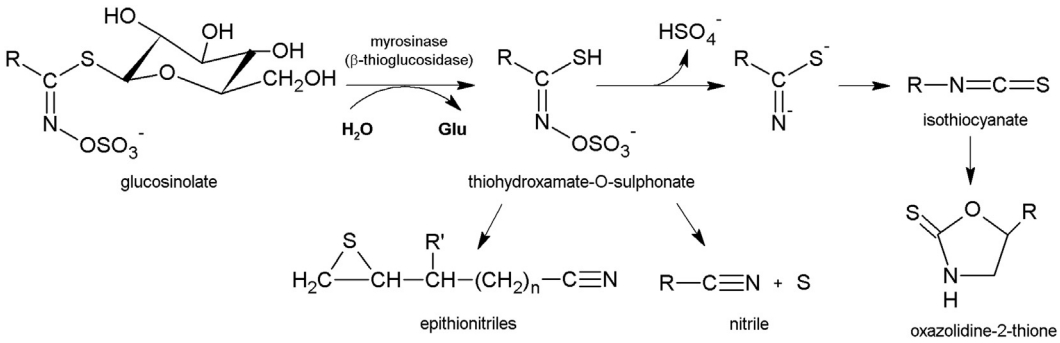

FIGURE 8.16 The enzymatic hydrolysis of glucosinolates and their main degradation products.

the known glucosinolates are presented in Fig. 8.15. Many of glucosinolates contain double bonds (olefins), hydroxyl or carboxyl groups, or sulfur linkages in various oxidation states (e.g., methyl-thioalkyl-, methylsulfinylalkyl-, or methylsulfonylalkyl). A small group of benzyl glucosinolates contain additional sugar moieties (rhamnose or arabinose) in glycosidic linkage to the aromatic ring [24,251,253]. Glucosinolates are strongly acidic and occur as salts (mainly potassium salts) in all physiological solutions. They are also hydrophilic compounds, because of conjunction with the thioglucoside group.

Glucosinolates co-occur in plant with myrosinase isoenzymes (β-thioglucosidases) in separated counterparts of the plant tissue. When contact with enzyme is provided hydrolysis take place. The unstable aglycone intermediate (the thiohydroxamate-*O*-sulfonate) undergoes spontaneous rearrangement into different possible products: isothiocyanates (ITCs), nitriles and elemental sulfur, thiocyanates, epithionitriles, oxazolidine-2-thiones, or indolyl compounds, depending on reaction conditions, myrosinase isoenzyme (multiple forms of the enzyme can exist even within the same plant), and type of glucosinolate (see Fig. 8.16) [24].

8.11.1 Plants Containing Thioglycosides

THGs have been found only in dicotyledonous plants in the families; Brassicaceae (Cruciferae)—350 genera with 3000 species—from which all are able to synthesize glucosinolates (approximately 20% of the known 140 glucosinolates are

accumulated in plants of the *Brassica* genus). Those found in *Brassica* plants are, e.g., sinigrin (in *Brassica nigra* L.—black mustard seeds) and sinalbin (from *Sinapis alba* L.—white mustard seeds). The most common are glucobrassicin, glucoraphanin, or progoitrin. THGs are also found in at least 500 species of noncruciferous plants within the families Capparaceae, Caricaceae, Resedaceae, and with sporadic occurrences in Euphorbiaceae, Tropaeolaceae, Tovariaceae, and Moringaceae [4,24,235,236,251].

Glucosinolate content in plants is highly variable and depends on various conditions, such as environmental factors, soil fertility, pathogenic challenge, and plant growth regulators. As was investigated in *B. napus* (rape), when soil is fertilized insufficiently by nitrogen, the glucosinolate content in seeds of rape increased. Distribution of the glucosinolates varies among plant organs with qualitative and quantitative differences between roots, leaves, stems, and seeds, and also depending on plants age and phenological stage, e.g., Broccoli sprouts contain about 15 times more glucoraphanin than the mature broccoli plant [254]. The role of glucosinolate defensive compounds against generalist herbivores is widely recognized (insecticide action). They are involved in host plant recognition by specialist predators, acting as an insect feeding attractant [251]. THGs containing planta are also attractive for humans, because of its characteristic organoleptic properties (characteristic smell and taste). Most known plants containing glucosinolates are cultivated plants being a part of the human diet, as listed below:

From the Brassicaceae family:

White cabbage—*Brassica oleracea* L. var. *capitata* L. (brassicin, glucobrassicin)
Field mustard—*B. campestris* L. = *B. rapa* L. (glucoraphanin)
Rape—*B. napus* (glucobrassicin, 4-hydroxyglucobrassicin)
Broccoli—*Brassica oleracea* var. *botrytis* (glucoraphanin)
Black mustard—*Brassica nigra* (L.) Koch. = *Sinapis nigra* L. (sinigrin)
White mustard—*Sinapis alba* L. (sinalbin)
Horseradish—*Armoracia rusticana* Gaertn. (sinigrin)
Black radish—*Raphanus sativus* L. var. *niger* (Mill.) Kerner (glucobrassicin, 4-methoxybrassicin, sinigrin, glucoraphanin).
From the family Tropaeolaceae: Nasturtium—*Tropaeolum majus* L. (glucotropaeoline).

8.11.2 Bioactivity of Thioglycosides

Zukalová and Vašák [253] present a division of glucosinolate physiological effects according to their decomposition products into three groups. The first group consists of aliphatic THGs (sinigrin, gluconapin, glucobrassicanapin), in which isothiocyanates are created by hydrolysis of THGs (the antinutritive effects, e.g., goitrogenic effect, antimicrobial, antifungicidal and thyroidal properties and a function of glucosinolates as biofumigants—substances excreted from plant into the soil). The second group are hydroxyl-aliphatic THGs, such as progoitrin and napoleiferin, where strong goitrogenic effect of hydroxyl-isothiocyanate products and their cyclic derivatives (2-oxazolidinethione = goitrin) is observed. The third group are cyclic (aromatic) THGs, e.g., sinalbin, glucobrassicin and neoglucobrassicin. Stereoisomeric glucosinolates, e.g., progoitrin and epiprogoitrin, exert highly diverse biological effects on organ and body weight in rats [24]. Sulforaphane, present in broccoli sprout and in the mature broccoli plant, has been shown to be protective against induced cancer at a variety of sites [13,255,256]. THGs when applied topically as "mustard oils" (products of distillation of THGs containing plant material, e.g., brassica seeds), act as rubefacients, causing local vasodilatation. Taken internally, they serve as effective stimulants of digestion, although large doses may be emetic. Fungistatic and antibiotic properties are also known [257]. The main component of nasturtium oil (*Tropaeolum majus*) is benzyl isothiocyanate, which has potent antibacterial and antifungal activity. In Europe, enteric-coated capsules of this oil are used to treat bronchial and urinary tract infections [52]. Indole-3-carbinol, the degradation product of glucobrassicin, may moderate estrogen metabolism and also have antioxidant and antiatherogenic activities [258].

8.11.3 Nutraceutical Applications of Thioglycosides

The anticarcinogenicity of THGs (specifically in *Brassica* vegetables) has been recently reviewed using in vitro assays, animal experiments, and various human studies [259,260]. Epidemiological evidence suggests that diets containing vegetables from the *Brassica* genus (including broccoli, cabbage, brussel sprouts, and cauliflower) are associated with a reduced cancer risk, and the association appears to be most consistent for lung, stomach, colon, and rectal cancer [11]. Phenethyl isothiocyanate has been shown to inhibit induction of lung esophageal cancer in rat and mouse tumor models

[251]. As was previously reported, postmenopausal women consuming one to two servings of Brassica vegetables daily had a 20−40% decreased risk of breast cancer [235]. Isothiocyanates as potent inducers of Phase II detoxification enzymes, increase the metabolism and detoxification of chemical carcinogens in vitro and in animal models, and also inhibit mitosis and stimulate apoptosis in tumor cells [255].

8.11.4 Pharmaceutical Applications of Thioglycosides

Glucosinolate-containing plant drugs can be used in the form of ointments, compresses, liniments, or plasters. The mustard compress or plaster is still used for bronchial infections and detoxification in chronic diseases. Mustard oil is highly corrosive and if applied for too long will cause blistering and may even permanently scar the skin. The plant drugs containing THGs are mainly black mustard seeds, white mustard seeds, and black radish. They are used mainly as rubefacient antirheumatic and antiarthritic drugs. Black mustard is a local irritant and an emetic. Nasturtium is used as medicinal plant known for its antibacterial and antifungal properties. Nasturtium-based preparations are used in dermatology and cosmetology to treat skin, hair (dandruff), and nail aliments [3].

8.11.5 Adverse Effects of Thioglycosides

The tyroid gland, pancreas, kidney, and liver are the main targets of the toxicity due to the hydrolysis products of THGs. Thyroid-toxicity in animal experiments shows that certain isothiocyanates interfere with the synthesis of thyroid hormones, whereas thiocyanates compete with iodine and inhibit iodine uptake by the thyroid gland (goitrin, which inhibits iodine incorporation and the formation of thyroxin) [261]. Oxazoline-thiones interfere with thyroxine synthesis. Goitrogenic as well as antigoitrogenic glucosinolates are present sometimes in the same plant material [11,24]. Damage to liver, kidney, and pancreas by some decomposition products has been also observed [261]. The unstable isothiocyanates formed from indole glucosinolates decompose to carbinols. Under the acid conditions of the stomach, the indole-3-carbinol may spontaneously condense to form compounds (e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin) which in structure, toxicity, and carcinogenicity closely resemble dioxin [251]. Caution should be exercised if recommending long-term intake of THGs containing plants at doses well in excess of optimum dietary levels because of their possible mutagenicity, tumor promotion, and carcinogenicity [262]. However, at normal dietary levels, they seem to be nontoxic and even beneficial (as discussed before).

8.11.6 Metabolic Profile of Thioglycosides

Preparation of food can make a large difference (e.g., in raw material) to both the intake of glucosinolates, and to the bioavailability of their breakdown products. Cooking reduces the concentration of glucosinolates in the plant tissue through thermal breakdown and leaching, but also inhibits the activity of myrosinase through denaturation of the enzyme. [263]. Lungs and intestines represent the entrance points where the uptake of bioactive glucosinolates and glucosinolate derived compounds occur. Upon ingestion by humans, β-thioglucosidase activity of the gut microflora is largely responsible for converting ingested glucosinolates to their cognate isothiocyanates [263,264], which is presumably required to generate biological activity [263]. Significant quantities of degradation products, such as isothiocyanate metabolites, nitrile, or thiocyanate, are excreted in the urine and partially in feces.

8.12 CONCLUSIONS

Natural plant glycosides and their aglycones are of great interest due to their widespread pharmacological properties, and this attracts many investigations for further screening them as novel therapeutic agents. Understanding of their activity, including adverse effects and toxicity, is important from both therapeutical and nutraceutical points of view.

8.13 SELF-EVALUATION QUESTIONS

GLYCOSIDES—General questions

1. Describe structure of typical glycoside. How we can classify glycosides in accordance to glycosidic linkage?
2. Classify glycosides on the basis of the aglycone structure.

3. Characterize briefly the extraction procedures used to obtain glycosides form plant material.
4. Describe part of the glycoside/activity relationships.

PHENOLIC GYCOSIDES—PHGs

1. Give some examples of simple phenols and phenolic acids from both benzoic and hydroxycinnamic groups.
2. Outline the methods of detection of PHGs.
3. Describe metabolic profile of salicin and arbutin respectively.

COUMARIN AND CHROMONE GLYCOSIDES—CMGs and CHGs

1. Explain the basic structure of coumarin and list basic types of coumarin structures with examples of main compounds.
2. Discuss the extraction methods for simple coumarins and furanocoumarins.
3. Outline the chemical reactions for the detection of coumarins in plant material.
4. What are the principles of PUVA therapy?
5. What are the biological activities of coumarins? List their benefits and adverse effects.
6. Give some examples of furochromones. What is the difference between furochromones and furanocoumarins main structure?

FLAVONOID GLYCOSIDES—FLGs

1. Give the main structure of flavonoids and their classification based on the oxidation of the C-ring.
2. What is a biological role of flavonoids in plants?
3. Give examples of the main compounds from an each subclass of flavonoids.
4. List examples of plant drugs containing isoflavonoids and outline their pharmacological activities.
5. Discuss the use of silymarin its plant source, chemistry, and pharmacological activity.
6. Describe the biological activity of flavonoids. Give examples of compounds and plant drugs.
7. How can we isolate FLGs from plant material?
8. What are the characteristic chemical reactions of flavonoids?

ANTHRAQUINONE GLYCOSIDES—AQGs

1. Give the chemical structures of anthracene derivatives; anthrones, anthranols, anthraquinones.
2. List AQGs containing plant drugs and the main compounds.
3. Discuss the structure−activity relationships of anthracene laxatives.
4. Describe AQG's mechanism of action.
5. Do you know the adverse effects, interactions, and contraindications for AQGs ?

SAPONIN GLYCOSIDES—SPGs

1. How can we group saponins based on their aglycone skeleton?
2. How many sugar chains may be connected with sapogenins and at which positions?
3. Summarize the most important pharmacological activities of saponins?
4. Describe the reactions to indicate saponin moiety of the plant material.
5. How we can explain the toxic effects of saponosides? Gave some examples of toxic saponosides?

CARDIAC GYLOSIDES—CRGs

1. Indicate differences between cardenolide and bufadienolide structure. Give examples of their main natural source (medicinal plants) and representative compounds.
2. Describe the role of aglycone (genin) stereochemistry, and glycoside chain in the pharmacological effects (and in pharmacokinetics) of cardiac glycosides.
3. What is the mechanism of action of cardiac glycosides, e.g., digitoxin?
4. Describe the contraindications and adverse effects of cardiac glycosides.
5. Explain term "endogenous cardiac glycosides." Give some examples.

CYANOGENIC GLYCOSIDES—CNGs

1. Describe the biosynthesis of CNGs and its occurrence in food plants?
2. What is cyanogenesis? Describe this process and its conditions.

3. Discuss the toxic effects of HCN in the human body and the detoxification mechanisms.
4. Describe the extraction of CNGs from plant tissues and possible chemical reactions for qualitative and quantitative estimation of HCN content in plants.

THIOGLYCOSIDES—THGs

1. Give some example of plants containing THGs and single compounds. Describe their degradation mechanism in the human body.
2. Discuss the pharmaceutical applications of plant drugs containing glucosinolates.
3. List the adverse effects and benefits of the intake of THGs containing plants?

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Chapter 9

Alkaloids

W.A. Kukula-Koch and J. Widelski
Medical University of Lublin, Lublin, Poland

Chapter Outline

Teaching Objectives

- Basic information on the classification, characteristics, biosynthetic pathways, and structures of alkaloids.
- An overview of medicinal applications, side effects, mechanisms of action, and toxicity of plants rich in alkaloids.
- A description of commonly used plant species containing these compounds.

9.1 INTRODUCTION

The first definition of alkaloids was introduced by W. Meissner in 1818 and was attributed to all organic compounds of plant origin characterized by basic character [1]. Subsequent studies indicated the presence of the nitrogen atom in a heterocyclic ring system within the structure of alkaloids. According to this definition, they were classified as primary, secondary, tertiary, or quaternary amines [2].

From then on, supported by several scientific achievements, the parameters which frame this group of secondary metabolites evolved, leading to an introduction of numerous chemical structures into this class of compounds. Alkaloids were established to be present in the plant kingdom, and in animals, including humans, marine organisms, fungi, and other microorganisms. Moreover, metabolites with a neutral character containing amide groups were also

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classified as alkaloids, as were compounds containing nitrogen atom(s) included in the side chain, a nitro group, or several other nitrogen-containing functional groups [3,4].

However, plants are still perceived as the main natural sources of these plentiful (ca. 27,000 alkaloids described so far) natural products [2]. They are widely distributed in the plant tissues of *inter alia* the Apocynaceae, Asteraceae, Berberidaceae, Boraginaceae, Buxaceae, Chenopodiaceae, Euphorbiaceae, Fabaceae, Fumariaceae, Lauraceae, Loganiaceae, Magnoliaceae, Menispermaceae, Papaveraceae, Rubiaceae, Rutaceae, and Solanaceae botanical families, and are rare in gymnosperms or monocots [5,6].

9.2 PHYSICOCHEMICAL PROPERTIES OF ALKALOIDS

Alkaloids are present in plant tissues as water-soluble salts of organic acids (tartaric, acetic, oxalic, citric, malic, and lactic acids), esters (e.g., atropine, scopolamine, cocaine, aconitine), or combined with tannins (*Cinchona* bark) or sugars (e.g., the glycoalkaloids of *Solanum* species) rather than as free bases [7,8].

Most alkaloids are isolated from plant matrices in the form of crystalline, amorphous, nonodorous, and nonvolatile compounds. However, low molecular weight alkaloids, such as arecoline and pilocarpine, and alkaloids with no oxygen atom in their structure (e.g., sparteine and nicotine) occur in the liquid form. Apart from the orange-yellow alkaloids berberine and colchicine, the red-colored betaine, the brick red sanguinarine, or the orange-colored canadine, the majority of alkaloids are colorless with a bitter taste. Indeed, quinine is still used as a bitter principle in tonic water.

Many alkaloids are optically active, with counterclockwise isomers (e.g., (−)-hyoscyamine) and are more pharmacologically active in contrast to the corresponding racemic mixtures which are characterized by lower activity, or a quite different activity, e.g., the enantiomers of morphine [3,7].

The free bases of alkaloids are soluble in nonpolar organic solvents (chloroform, methylene chloride, ether), while their solubility in water is low (exceptions include caffeine and ephedrine). In contrast, the salts of alkaloids are soluble in water or dilute acids, whereas they are insoluble or sparingly soluble in organic solvents. These differences in the solubility of alkaloids, depending on their form, are used in the pharmaceutical industry for their purification from complex plant matrices and for the production of pharmaceutically acceptable products [4].

9.3 TESTS FOR ALKALOIDS

The determination of alkaloids in a plant material may be performed either using the precipitation reactions with Mayer's (white precipitate with potassiomercuric iodide solution), Dragendorff (orange precipitate with potassium bismuth iodide), Wagner's (reddish-brown precipitate when mixed with iodine and potassium iodide), and Hager's (yellow precipitate with saturated aqueous solution of picric acid) reagents, with picric, picrolonic, phosphomolybdic, and silicotungstic acids, or in color reactions with sulfuric- or nitric acid-containing reagents (Marquis, Frőhde, and Erdmann reagents; e.g., yellow color for colchicine or bluish violet for tropane alkaloids), or with a Ce (IV) salt.

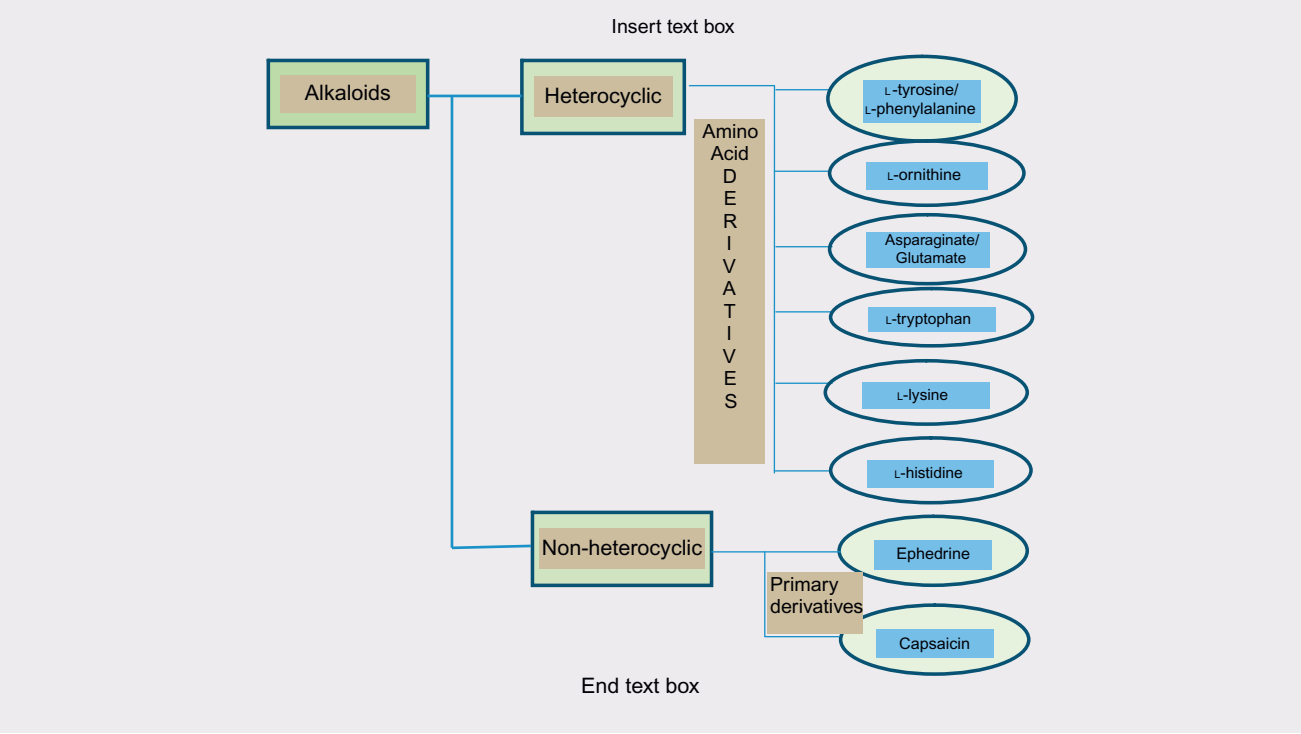
Positive precipitation reactions are observed also for proteins, so special attention should be paid to their removal from the tested sample.

Moreover, in order to determine the presence of certain groups of alkaloids, special reactions are performed individually for the group, e.g.:

1. For purine alkaloids: the murexide reaction resulting in purple color of a solution (see Section 9.7.1)
2. For tropane alkaloids:
   a. the Vitali-Morin color reaction (evaporated with fuming nitric acid with potassium hydroxide gives bright purple or reddish coloration);
   b. with Schaer's reagent (distinct green color occurs; reaction with perhydrol and sulfuric acid) [9].

9.4 CLASSIFICATION OF ALKALOIDS

Alkaloids can be classified in terms of their chemical structure, biological activity, the pathway of their biosynthesis, and occurrence into heterocyclic and nonheterocyclic alkaloids, sometimes called protoalkaloids or biological amines, see below.



9.5 HETEROCYCLIC ALKALOIDS

These compounds contain a nitrogen atom in a heterocyclic system (see Fig. 9.1). They are derived biosynthetically from the respective amino acids, and are often formed following a decarboxylation process. Six major groups of alkaloids have been recognized so far, depending on the amino acid of origin. These are the derivatives of: L-ornithine, L-lysine, L-tyrosine/L-phenylalanine, L-histidine, L-tryptophan, and glycine/aspartic acid [3].

This group contains the most diverse and pharmacologically active plant-derived alkaloids, which are known to exhibit very different biological activities even at very low doses. In the sections below, the detailed characteristics of alkaloids, together with their classification, based on their amino acid of origin, are presented. In addition, several lists of the most active representatives of these groups of alkaloids are discussed. Finally, a review of the alkaloid-containing plants and the most widely used purified alkaloids with their dosage, indications, and side effects is included.

9.6 L-TYROSINE DERIVATIVES

9.6.1 Isoquinoline Alkaloids

Isoquinoline alkaloids constitute one of the largest groups of natural substances. These compounds are biogenetically derived from phenylalanine and tyrosine, and include an isoquinoline or a tetrahydroisoquinoline ring as a basic structural feature in their skeleton (see Fig. 9.2) [5].

Isoquinoline alkaloids are not a structurally homogenous group. Based on different degrees of oxygenation, and intramolecular rearrangements, their distribution, and the presence of additional rings connected to the main system, they may be divided into eight subgroups [10]: benzylisoquinoline, aporphine, protoberberine, benzo[*c*]phenanthridine, protopine, phthalideisoquinoline, morphinan, and emetine alkaloids.

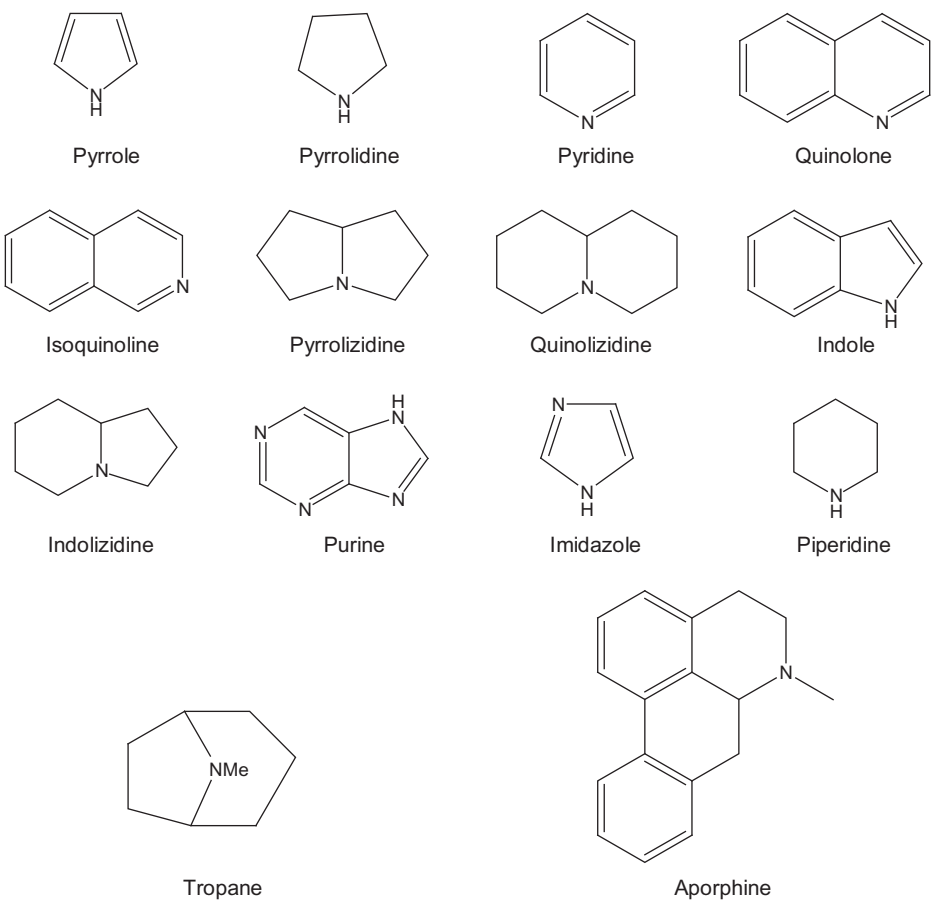


FIGURE 9.1 Basic alkaloid skeletal structures in natural products.

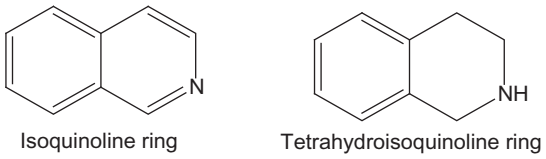


FIGURE 9.2 Basic elements of the isoquinoline alkaloid skeleton.

Among the listed subgroups, the protoberberines are the largest group—they constitute 25% of all elucidated structures of isoquinoline alkaloids, which makes them the most widespread secondary metabolites containing nitrogen among natural products [5].

Isoquinoline alkaloids are widely distributed among plants coming from the families Papaveraceae (where they are mostly present in the form of tetrahydro-bases), Berberidaceae, Fumariaceae, Menispermaceae, Ranunculaceae, Rutaceae, and Annonaceae (in the dehydro forms). A few plant species which belong to the Magnoliaceae and Convolvulaceae are also rich in these alkaloids [11,12].

Below, in Tables 9.1 and 9.2, the most widely used isoquinoline alkaloid-containing plants and their single alkaloids are reviewed, respectively.

9.7 L-ORNITHINE DERIVATIVES

Two amino acids, proline and L-ornithine (together with their decarboxylation product—putrescine), are found to be precursors of the tropane, necine, stachydrine, nicotine, and pyrrolizidine alkaloids (PAs).

TABLE 9.1 A Review of Plant Species Rich in Isoquinoline Alkaloids

Distribution	Examples	Major Pharmacological Activities	Pharmaceutical Applications	Dosage	Adverse Effects
Berberis vulgaris L.—European barberry, Berberidaceae, Root bark	Berberine, jatrorrhizine, palmatine, coptisine	Cholagogue, antileishmanial, antibacterial	– Gastrointestinal discomfort, digestion problems – Hypertension – Hepatic and rheumatic affections, particularly with urinary, hemorrhoidal, and menstrual complaints [1]	– Tincture (1:10): 20–40 drops a day – Dry extract: 250–500 mg 3 times a day – Tea: 1–2 teaspoons of berries or 2 g of bark	Gastrointestinal disorders, dyspnea, lowered blood pressure, flu-like symptoms and cardiac damage, when overdosed
Cephaëlis ipecacuanha Brot. (syn. *Psychotria ipecacuanha*, *Carapichea ipecacuanha*)—Ipecacuanha, Rubiaceae Root	Emetine, cephaeline, psychotrine	Emetic, expectorant, antiprotozoan	– Rarely used as total herbal drug – After suspected poisonings – In a severe amoebic dysentery	Tincture: – as expectorant: 30–50 mg twice a day – as emetic: 1 g once a day Herbal drug: 1–6 mg, 3 times a day	Safe when used for a short time; longer administration causes nausea, vomiting, ulcers, low blood pressure, tachycardia, blood in the urine, convulsions
Chelidonium maius L.—greater celandine, Papaveraceae herb	Chelidonine, coptisine, berberine, sanguinarine, chelerithrine	Cholagogue, analgesic, antiviral, antimicrobial, cytostatic, tranquilizing	– Diseases of bile ducts and gall stone complaints – Symptomatic relief of digestive disorders such as dyspepsia and flatulence (oral intake) – Treatment of warts, callus, and corns (cutaneous use)	No longer than 4 weeks; control of the transaminases required – tea infusion: 1.2–3.6 g – tincture (45% ethanol): 2–4 mL of a 1:5 preparation daily or 2–4 mL of a 1:10 preparation 3 times daily – liquid extract (25% ethanol): 1–2 mL of a 1:2 preparation daily or 1–2 mL of 1:1 preparation, 3 times daily – dry extract: 100–200 mg, 3 times daily External use: eyedrops: one drop, 3 times daily: grade of dilution: D4 to D30	Asthenia, nausea, abdominal pain, diarrhea, vomiting, nervousness, urine abnormal, jaundice, hepatocellular damage, hepatitis, cholestatic, SGOT and SGPT increased
Fumaria officinalis L.—common fumitory, Papaveraceae Herb	fumaricine, protopine, tetrahydroberberine, corydaline	digestive, spasmolytic in hepatobiliary tract (cholagogue), hypotensive	– Dyskinesia of the biliary duct, pain in cholelithiasis, cholecystitis and cholangitis, postcholecystectomia syndrome, posthepatic syndrome with cholestasis – pain in the biliary system in dyskinesia of the biliary duct, complaints after cholecystectomia, cholelithiasis when surgery is not possible	– tea infusions: 2–6 g per day – capsules/tablets: 960–1280 mg of powdered extract/day	High overdose may evoke trembling or convulsions

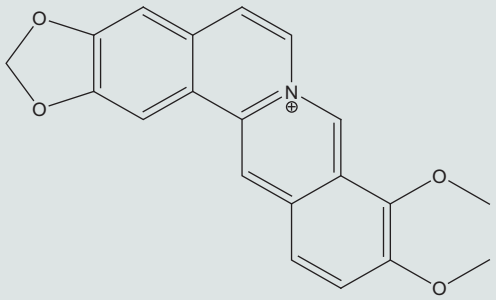
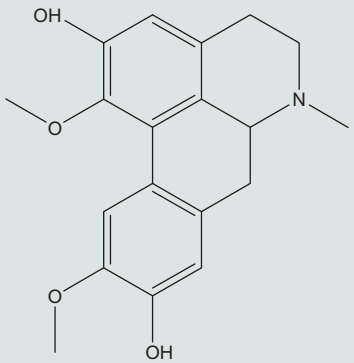
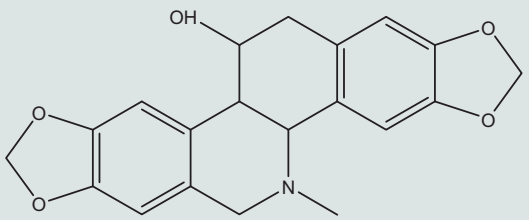
(*Continued*)

TABLE 9.1 (Continued)

Distribution	Examples	Major Pharmacological Activities	Pharmaceutical Applications	Dosage	Adverse Effects
			– supportive treatment of dyskinesias of the biliary duct in cases of an impairment of the liver		
Galanthus nivalis L.—snowdrop, Amaryllidaceae Tuber	Galanthamine, lycorine, phenethyliso-quinolines	Procognitive, anti-Alzheimer's	Dementia, intellectual deterioration, changes in personality and behavioral abnormalities	No registered herbal drugs from the crude tuber extracts; see Table 9.2 for galanthamine	Nausea, vomiting, diarrhea, heartburn, weight loss
Glaucium flavum Crantz—yellow horned poppy, Papaveraceae Herb	Glaucine, protopine, sanguinarine, chelidonine	Antitussive, bronchodilator, anti-inflammatory, calcium channel blocker, hypotensive, anticonvulsant, analgesic	– Reduction of a dry cough – traditional product for hypertensive patients	Oral administration, 40 mg per dose twice a day	Nausea, sedation, fatigue, hallucinogenic effects (colorful visual images)
Hydrastis canadensis L.—Goldenseal, Ranunculaceae, Rhizome	Hydrastine, berberine, canadine, jatrorrhizine	Digestive, cholagogue, emmenagogue, oxytocic, anti-inflammatory	– Gastrointestinal complaints, digestion problems – difficult menstruation – common cold and upper respiratory tract infections	0.5–1 g tablets or capsules 3 times a day, 0.5, 2–4 mL of a tincture (1:10, 60% ethanol), 0.3–1 mL of a liquid extract (1:1 in 60% ethanol), 0.5–1 g as a decoction	Nausea, vomiting, risk of bleeding; low white blood cell count, when overdosed; increased blood pressure (hydrastine)
Papaver somniferum L.—Opium poppy, Papaveraceae Opium	Morphine, codeine, papaverine tetrahydro-isoquinolines	Morphine: addictive, analgesic; codeine: addictive: antitussive; papaverine: nonaddictive: spasmolytic	As a source of alkaloids	See Table 9.2 for dosage (morphine, papaverine, codeine)	Addiction, constipation
Peumus boldus Molina—boldo, Monimiaceae Leaf	Boldine, isoboldine, aporphine	Cholagogue, choleretic, anticoagulant	– Traditional herbal medicinal product for symptomatic relief of dyspepsia and mild spasmodic disorders of the gastrointestinal tract	Up to 2 weeks; among adults; – tea preparations: 1–2 g of herbal substance, 2–3 times daily – dry extract (5:1, aqueous): up to 400 mg 2 times daily	Increase in the risk of bleeding, improvement of diuresis, bile duct obstruction due to overstimulation of its flow, concurrent use of boldo with alcohol may increase the risk of liver damage

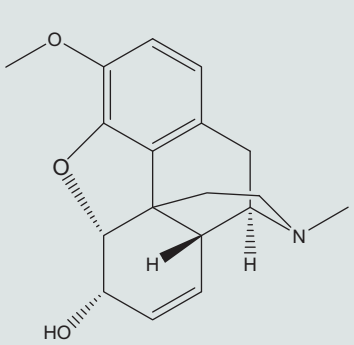
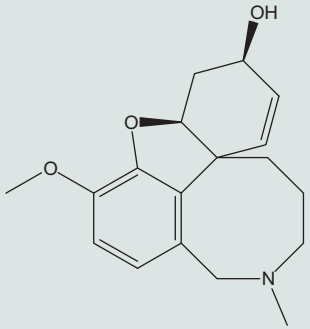
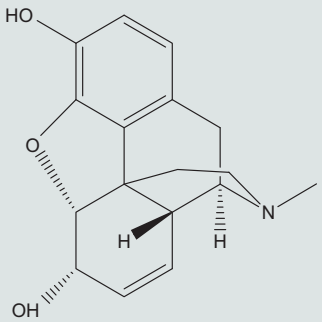
References Table 1: [13], Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP), European Pharmacopoeia 8.0, [1] Imanshahidi M, Hosseinzadeh H. Pharmacological and therapeutic effects of *Berberis vulgaris* and its active constituent, berberine. Phytother Res 2008;22:999–1012.

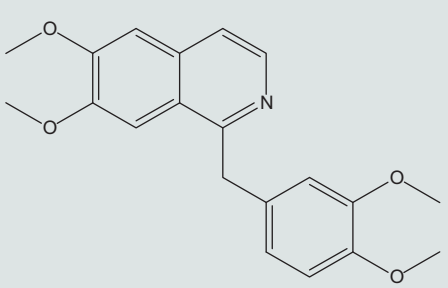
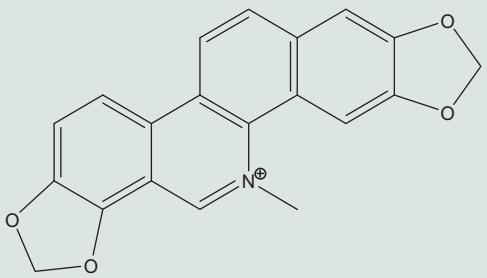
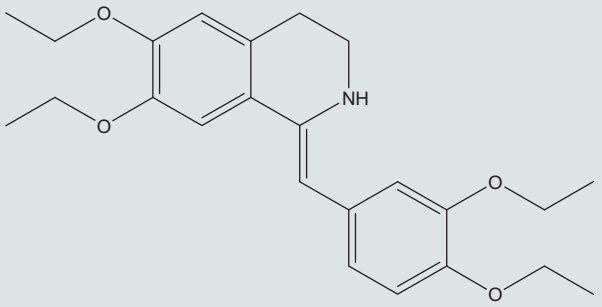
TABLE 9.2 The Most Commonly Used Alkaloids Among Isoquinolines

Alkaloid	Pharmacological Activity	Adverse Effects
Berberine 	– Improving the activity of the digestive system: cholagogue, choleretic, and laxative agent used in bowel movement disorders, hepatic malfunctions, intestinal ulcers and loss of appetite – Antibacterial, antifungal, antileishmanial, antimalarial drug – Anxiolytic, antidepressant, neuroprotective, antinociceptive – Vasorelaxant and hypotensive drug preventing tachyarrythmia Clinical trials confirmed its application in dyslipidemias, congestive heart failure, platelet aggregation, chloroquine-resistant malaria, and chlamydiosis.	Uterine contractions and miscarriages when overdosed
Boldine 	– Cholagogue and bile production increasing activity – liver damage prevention for CCl_4-induced hepatotoxicity in rats – anticancer properties in bladder carcinoma in vivo – anti-inflammatory activity (in vitro prostaglandin biosynthesis inhibition, prevention of edema, tissue damage and neutrophil infiltration, prevention of skin erythrema formation) – inhibition of the free-radical-mediated initiation and propagation of the peroxidative damage induced to various membrane types such as liver homogenates, hepatic microsomes and erythrocytes, – decrease in the free radical-dependent lysis of red blood-cells and intact hepatocytes [1]	– Hypersensitivity – Anatomical alterations in the fetus and a few cases of abortion at high doses
Chelidonine 	– Antiviral against *Herpes simplex*, polio virus, adenoviruses and HIV-1 virus in in vitro tests – antispasmotic activity, resulting in the cholagogic and hypotensive properties – analgesic properties	Dosage exceeding 0.79 mg daily for 4 weeks induces hepatotoxic and cytotoxic effects; forbidden during pregnancy

(*Continued*)

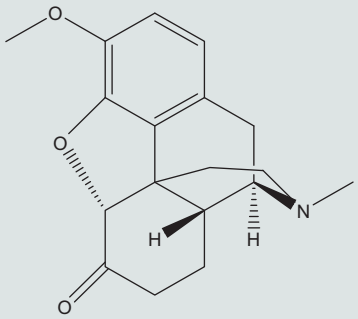
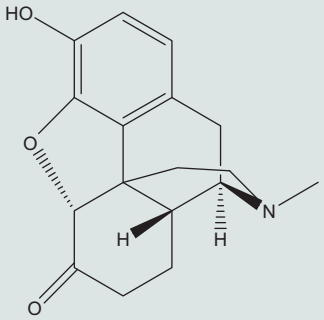
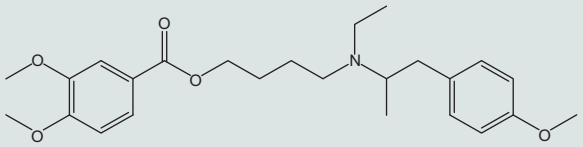
TABLE 9.2 (Continued)

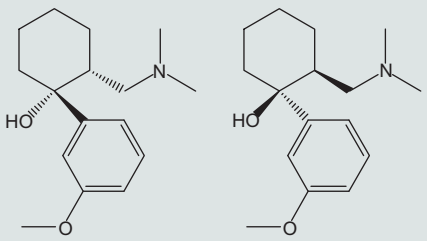
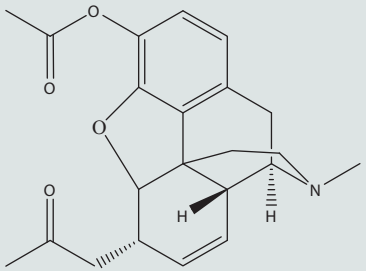
Alkaloid	Pharmacological Activity	Adverse Effects
Codeine 	– Pain relief drug due to its conversion into morphine in human body by the enzyme called CYP2D6; often combined with other analgesics—acetaminophen or acetylsalicylic acid – antitussive by the agonist activity toward opiate receptors in cough center – euphoric effects result from its conversion to morphine when metabolized – maximum oral daily dose: 60 mg/day for children, 120 mg/day for adults for codeine in the form of phosphate salts	Should not be used as a pain relief drug among children under 18 years of age due to respiratory depression (obstructive sleep apnea); addictive potential; numerous drug interactions (alcohol, antiviral, antihistamines, antidepressant, travel sickness, antifungal, anticonvulsant drugs); apathy, drowsiness, obstipation, euphoria, or sedation, tolerance
Galanthamine 	– A selective, competitive and reversible acetylcholinesterase (AChE) inhibitor which augments central cholinergic transmission; inhibiting the decomposition of acetylcholine in the synaptic cleft, it enhances neurotransmission and guarantees a protective function against dementia [2] – used in mild and moderately severe dementia of the Alzheimer's type and schizophrenic patients [3] – registered as 8, 16, and 24 mg tablets	Nausea, vomiting, loss of appetite, heartburn, dizziness, headache, depression, runny nose, difficulty falling asleep, difficulty urinating, slowed heartbeat
Morphine 	– An opioid analgesic, antitussive, and antidiarrheal drug, the major constituent of opium poppy—dried latex obtained from *Papaver somniferum*, used for chronic pain in terminal cancer treatment; causes addiction, tolerance, dependence, and withdrawal – maximum daily doses: morphine sulfate: i.v.—20 mg, per os (p.o.)—100 mg; morphine hydrochloride: p.o.—100 mg, per rectum (p.r.)—30 mg, s.c.—60 mg	Constipation, dysphoria, depression, fetal poisoning, blood changes, increase in breathing frequency, tachycardia, hypertension, gastrointestinal afflictions, addiction

Papaverine 	– Antispasmotic drug obtained from *Papaver somniferum*, which does not belong to the opiate-like compounds and does not induce addiction or tolerance – used to treat spasms in the digestive system (tablets or suppositories) – coronary vasodilator and prophylactic in migraine headaches. It is a selective phosphodiesterase inhibitor – papaverine hydrochloride dosage: ischemia: p.o. 100–300 mg, 3–5 times daily, constipation/spasms: intramuscular (i.m.), s.c., p.r. 40–120 mg	Tachycardia, constipation, increased transaminase and phosphatase levels, hyperhydrosis, tachycardia, allergic reactions.
Sanguinarine 	– Effective in the control of the production of bacterial sulfur compounds responsible for a halitosis; for oral use in mouth rinses and toothpastes at a concentration of 0.03% in clinical trials – active against multidrug- resistant bacteria and MRSA strains – strong cytotoxic agent which intercalates with DNA – due to strong interaction with DNA leading to genotoxicity and mutagenic properties, it has been removed from mouthwashes	Genotoxicity, proteinuria, nephrotic syndrome, increase in capillary dilatation, cells' proliferation; causing intoxications in human when consumed with adultered mustard oil
Synthetic Derivatives		
Drotaverine 	– A selective inhibitor of phosphodiesterase 4 with no cholinergic effects – a semisynthetic derivative of papaverine used as antispasmotic for blood, urinary and bile vessels. – dosage: 120–240 mg in 2–3 doses per day	Rare: constipation, dizziness, headache, hypotension, allergies

(*Continued*)

TABLE 9.2 (Continued)

Alkaloid	Pharmacological Activity	Adverse Effects
Hydrocodone 	– A derivative of morphine used as an analgesic and antitussive drug of similar activity as morphine – administered orally, is characterized by 1.5 times stronger activity than morphine and 4.5 times stronger when administered intravenously	Allergic reactions, seizures, clammy skin, weakness, dizziness, unconsciousness, jaundice. It may cause euphoria and drowsiness.
Hydromorphone 	– An analgesic drug of opioid origin—a derivative of morphine. Sublingual administration	Drowsiness, vomiting, euphoria or dysphoria, analgesia
Mebeverine 	– Semisynthetic derivative of papaverine used as antispasmotic drug with no anticholinergic side effects; used in the treatment of abdominal cramping and irritable bowel syndrome (IBS)	Insomnia, anorexia, constipation, dizziness, slow heartbeat

Tramadol  (1*R*, 2*R*)-tramadol (1*S*, 2*S*)-tramadol	– A nonselective semisynthetic opioid receptor agonist drug marketed as an analgesic racemic mixture, often combined with paracetamol	Seizures, serotonin syndrome, drug addiction; constipation, nausea, itchiness
Heroin (Diacetylmorphine) 	– 2–4 times more potent than morphine, illegal to manufacture or possess – Abused as recreational drug; causes tolerance, physical and psychological dependence – Used in some countries as strong analgesic or in opioid replacement therapy	Dependence, constipation, severe withdrawal syndrome which includes sweating, malaise, priapism, depression, cramps

References Table 2: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0; [1] Gerhardt D, Bertola G, Bernardi A, Pires ENS, Frozza RL, Edelweiss MIA, Battastini AMO, Salbego CG. Boldine attenuates cancer cell growth in an experimental model of glioma *in vivo*. J Cancer Sci Ther 2013; 5(5): 194–99; [2] Kukula-Koch W, Mroczek T. Application of hydrostatic CCC–TLC–HPLC–ESI-TOF-MS for the bioguided fractionation of anticholinesterase alkaloids from *Argemone mexicana* L. roots. Anal Bioanal Chem 2015; 407: 2581–9; [3] Shao S, Li M, Du W, Shao F, Wang W. Galanthamine, an acetylcholine inhibitor, prevents prepulse inhibition deficits induced by adolescent social isolation or MK-801 treatment. Brain Res 2014;1589:105–111.

9.7.1 Tropane Alkaloids

Tropane alkaloids belong to the world's oldest plant medicines and their ethnopharmacological applications include analgesia, hallucinogens, and poisons. These ornithine-derived compounds are abundantly present in the Solanaceae, Erythroxylaceae, Convolvulaceae, Brassicaceae, and Euphorbiaceae families, and they comprise mono-, di-, and tri-esters, carboxylated and benzoylated tropanes [14]. Several of these alkaloids occur as chiral structures due to the presence of a tropic acid residue attached to the ecgonine nucleus as an ester. The former occurs naturally in its *R* form, however, racemic mixtures may appear, especially during alkaline extraction (e.g., the formation of (+)-atropine from (−)-hyoscyamine). Several acids were distinguished as being present in the tropane alkaloids, including: tropic, tiglic, acetic, isovaleric, isobutyric, benzoic, or anisic acids.

Tropane alkaloids are commonly used as anticolic and spasmolytic drugs (scopolamine) in both digestive and urinary tract spastic conditions. Also, atropine is commonly used in ophthalmological eyedrops to enlarge pupils, paralyze the accommodation reflex, and enable the ophthalmic examination. The juice from *Atropa belladonna* was extensively already used by women in the time of the renaissance to enlarge the pupils of the eyes so as to improve their looks.

However, tropane alkaloids are characterized by numerous contraindications and side effects. They are known to cause cardiac disorders, mainly related to heart rate disturbances, and also euphoric states, disorientation, depressive activity toward the CNS, and dryness of mucous membranes. They should be avoided in glaucoma, prostatic hypertrophy, patients with urinary tract diseases, and also during pregnancy [15−17].

Tropane alkaloids, due to their CNS activity are often abused. Among them cocaine is the compound of global significance. It is the second most popular psychostimulant (after cannabis), temporarily improving mental and physical functions. It inhibits serotonin, norepinephrine, and dopamine reuptake. In higher doses cocaine may evoke the blockage of sodium channels resulting in cardiac death. Chronic intake may cause serious transmitter level disorders leading to depressions, suicide attempts, insomnia, or psychomotor retardation [18]. Its abuse resulted in more than 4000 deaths in 2013.

Cocaine has scarce medical applications, which are limited to nasal or lacrimal surgeries, where it is used as an anesthetic. The dependence nature exhibited by this alkaloid is due to its ligand effect on the opioid receptors found in the CNS.

Reviews of the tropane alkaloid-containing plants and a detailed characteristic of their activity profiles are presented in Tables 9.3 and 9.4.

9.7.2 Pyrrolizidine Alkaloids

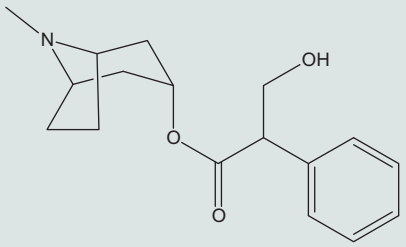
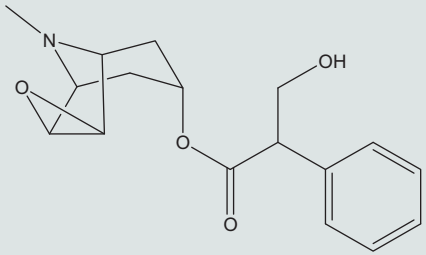
PAs are the esters of 1-hydroxymethylpyrrolizidines (necines) and necic acids (Fig. 9.3), and primarily originate from L-ornithine or L-arginine. Structurally, they are characterized by two conjoined five-membered rings with a bridgehead nitrogen atom. They are present in plants which belong to the Boraginaceae, Asteraceae, and Fabaceae families, often in the seeds and flowering parts of the plant.

A double bond at the C-1 and C-2 positions determines the hepatotoxicity of these alkaloids. In the absence of a double bond, no metabolic activation to the pyrrole nucleus, which induces the hepatotoxic or genotoxic actions, was observed. Necic acids are mono- or dicarboxylic acids with branched carbon chains composed of 5−10 carbon atoms, although double esterification can also occur and result in the formation of macrocyclic diesters composed of 11- to 14-membered ring systems (e.g., senkirkine). PAs often occur as *N*-oxides (except for the otonecine type of PAs), as they are produced in the roots of the plants from respective alkaloids [19].

The oral intake of unsaturated PAs is limited to 0.35 μg/day up to 14 days for adults, and for the representatives of sensitive groups, e.g., children: 0.007 μg PAs/kg body weight (bw), to prevent the occurrence of side effects [20]. The animal toxicity of these compounds is also significant and led to a thorough examination of their influence on grazing in animals. The effects of a single administration of PAs containing plants may progress to advanced liver disease, cirrhosis, or tumors in animals. Pigs and poultry are much more susceptible than cattle and horses, whereas sheep and goats are found to be relatively resistant to PAs evoked toxicity [13,21].

Contamination of foods or food supplements with PAs is commonly reported due to the structural variety of these alkaloids. More than 350 different PAs have been identified so far, and can be divided into three major groups: the derivatives of retronecine, heliotridine, and otonecine, as presented in Fig. 9.4 below. Several examples of plants containing PAs are presented in Table 9.4.

TABLE 9.3 The Most Significant Representatives of Tropane Alkaloids Used as Therapeutic Agents

Alkaloid	Pharmacological Activity	Adverse Effects
Hyoscyamine/atropine 	– Competitive and selective antagonist of muscarinic acetylcholine receptors in sweat and salivary glands, heart muscles, stomach, gastrointestinal tract, urinary tract and CNS – lowers blood pressure and heart rate – does not influence nicotine receptors Used to provide symptomatic relief for gastrointestinal disorders: ulcers, spasms, irritable bowel effects. Administered to Parkinson's patients and in palliative care in pain control Administered with opioid drugs, prevents constipation	Dizziness, dried mucous membranes, arrythmia, tachycardia, flushing, faintness, vomiting, hallucinations, euphoria, disorientation *Contraindications*: glaucoma, prostatic hypertrophy, urinary tract diseases, pregnancy
Scopolamine (hyoscine) 	92% of antimuscarinic potency when compared to atropine – central and peripheral antimuscarinic activity, spasmolytic action, antagonistic to salivary glands, heartbeat suppressing, sedative, depressive on CNS – after oral administration rapidly absorbed from the gastrointestinal tract and almost completely metabolized. Well absorbed through the skin, penetrates the blood–brain barrier – used in ear patches as antimotion sickness agents and antiemetic drugs	
Semisynthetic Drugs		
Scopolamine salts	Scopolamine hydrobromide—after oral administration is absorbed from digestive tract and metabolized in the liver. It crosses the brain–blood barrier and placenta Scopolamine quaternary derivatives: methyl bromide, butyl bromide, etc. exhibit spasmolytic properties only in digestive tract. They do not penetrate the CNS used in premedication, locomotor disease, as antiemetic, anticolic drugs also in the stomach ulcer, and as tranquillizers	As above Quaternary derivatives do not exhibit any CNS depressant activities

References Table 3: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0.

TABLE 9.4 A Review of Plant Species Containing Tropane Alkaloids

Chemical Group	Distribution	Examples	Major Pharmacological Activities	Pharmaceutical Applications	Dosage	Adverse Effects
Tropane alkaloids	*Atropa belladonna* L.—belladonna/deadly nightshade, Solanaceae, leaf *Datura stramonium* L.—jimson weed, Solanaceae, Leaf *Hyoscyamus niger* L.—henbane, Solanaceae, Leaf *Duboisia myoporoides* R. Br., *Duboisia leichhardtii* F.Muell.—corckwood, Solanaceae, leaf *Mandragora officinarum* L.—mandrake, Solanaceae, root *Scopolia carniolica* Jacq.—scopolia, Solanaceae, leaf	Atropine (racemic mixture), hyoscyamine, scopolamine, hyoscine	Parasympatholytic, mydriatic, spasmolytic	– Preparations are mainly used against spasms and colic-like pains in gastrointestinal and biliary tract – traditionally used as hallucinogenic, narcotic, anesthetic, and rheumatic pain relieving	In human: only standardized containing about 0.28 to 0.32% total alkaloids (i.e. 280 to 320 mg/100 g) calculated as hyoscyamine. The oral intake of a median single dose of 0.05—0.1 g prepared herb corresponds to 0.15 to 0.3 mg total alkaloids, the maximum single dose of 0.2 g contains 0.6 mg alkaloids and the maximum daily dose of 0.6 g up to 1.8 mg	Fever, tachycardia, tachypnea, hyperpexia, warm dry skin and mucous membranes, mydriasis, seizures, paralysis, respiratory depression
	Erythroxylon coca Lam.—Erythroxylaceae, leaf	Cocaine, hygrine, cinnamylcocaine, truxilline	Anesthetic, CNS stimulant, parasympathetic	– Applications totally confined to ophthalmic, ear, nose and throat surgery	–	Toxic and addictive properties
	Ipomoea polpha R. W. Johnson—giant sweet potato. Convovulvaceae, tuber *Convolvulus* spp.—morning glory, Convolvulaceae, herb	Calystegines	Inhibitors of glycosidases	– Calystegines are potent competitive inhibitors of the bovine, human and rat beta-glucosidase and alpha-glucosidase activities [1]	A threshold of toxicity is as low as 0.001% of the dry weight of the plant	Seizures, histologic lesions when overdosed in in vivo tests on rodents

Pyrrolizidine alkaloids	*Senecio* spp.—ragwort (fireweed), Asteraceae, root	Platyphylline	Atropine alike effects	– A poisonous plant	Toxic plants	Gradual weight loss, blindness, muscular coordination, abdominal straining, rectal prolapse, sudden death
	Tussilago farfara L.—coltsfoot, Asteraceae, leaf	PAs: senecionine and senkirkine; Mucous, inulin, flavonols	Anti-inflammatory, soothing, spasmolytic, antimicrobial, antiviral, expectorant	– Disorders of the respiratory tract, flu, fever, colds, rheumatism, gout	Oral intake is limited to 0.35 μg/day up to 14 days	Hepatotoxic, genotoxic, and carcinogenic properties
	Borago officinalis L.—starflower, Boraginaceae, seed	Gamma-linolenic acid (GLA) PAs: lycopsamine, amabiline Saturated PAs: thesinine	cardiotonic, diuretic, gastrointestinal regulator, spasmolytic in asthma, amebicidal action (*in vitro* $LD_{50} = 33$ μg/mL); source of seed oil rich in GLA	– respiratory disorders, – urinary tract inflammations, – arthritis	Oral intake is limited to 0.35 μg/ day up to 14 days	Hepatotoxic, genotoxic, and carcinogenic properties
	Symphytum officinale L.—comfrey, Boraginaceae, root	alantoin (purine alkaloid), PAs: intermedine, simphytine, lycopsamine	wound healing, antiallergic	– external application as poultice, ointments, or alcoholic digestion in burns and wounds	Oral intake is limited to 0.35 μg/day up to 14 days	Hepatotoxicity, genotoxic, and carcinogenic properties
	Petasites spp.—butterbur (sweet coltsfoot), Asteraceae root	Sesquiterpenes: petasin, isopetasin; PAs: petasitenine	Migraine preventive, antiallergic	Migraine headaches, hay fever, allergic rhinitis	Oral intake is limited to 0.35 μg/day up to 14 days	Hepatotoxic, genotoxic, and carcinogenic properties
Pyrrol and pyrrolidine	*Stachys officinalis* L., *S. sylvatica* L., *S. tuberifera*, Lamiaceae *Glycine max* L. (Merr.)—soya bean Fabaceae, seed	Stachydrine	Increases blood circulation, prevents stagnation of blood	– In vitro anticancer activity on solid tumor cells, prostate and breast cancer cells – in vivo reduction of uterine bleeding	–	–
	Erythroxylon coca Lam.—coca leaf, Erythroxylaceae, leaf	Hygrines	See tropane alkaloids applications	Anesthetic, analgesic	–	Causes addiction
	Broussonetia kazinoki Sieb.—Paper mulberry, Moraceae	Broussonetine K	Antifungal, antihyperglycemic, antityrosinase, antiglucosidase, antinociceptive, anti-inflammatory	– For bleeding – skin disorders, insect bites – the fruits are diuretic, ophthalmic, stomachic and tonic – increased glycemia		[2]

References Table 4: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0; [1] Asano N, Kato A, Matsui K, Watson AA, Nasj RJ, Molyneux RJ, Hackett L, Topping J, Winchester B. The effects of calystegines isolated from edible fruits and vegetables on mammalian liver glycosidases. Glycobiology 1997; 7(8): 1085—88. [2] Wang GW, Huang BK, Qin LP. The genus *Broussonetia*: a review of its phytochemistry and pharmacology. Phytother Res 2011;26:1—10.

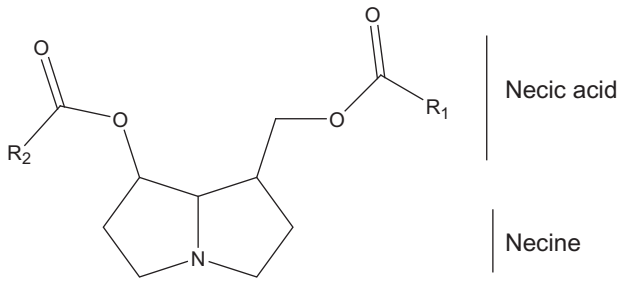


FIGURE 9.3 General structure of pyrrolizidine alkaloids.

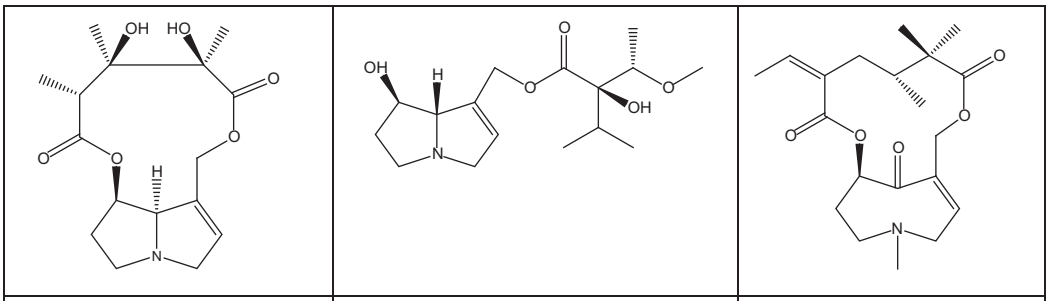


FIGURE 9.4 Commonly occurring types of pyrrolizidine alkaloids.

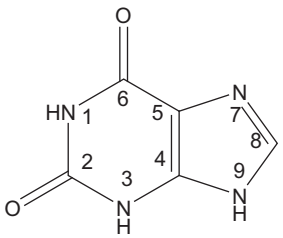


FIGURE 9.5 Chemical structure of xanthine.

9.8 ASPARAGINATE AND GLUTAMATE DERIVATIVES

9.8.1 Purine Alkaloids

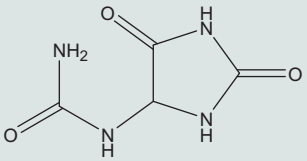
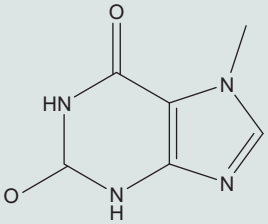
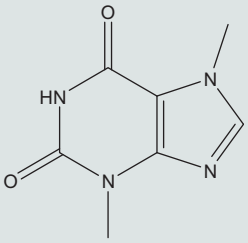
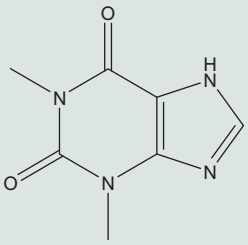
Purine and pyrimidine nucleotides, which are derivatives of xanthines, constitute the major structural units of purine alkaloids. Biosynthetically, they are derived from amino-ribose, asparaginate, and glutamate (see structure in Fig. 9.5). They are present in plants of the Sterculiaceae, Theaceae, Rubiaceae, Aquifoliaceae, and Sapindaceae families.

Purine alkaloids are known to undergo the murexide reaction (ammonium purpurate or MX). Purine nuclei-containing alkaloids are oxygenated in the presence of strong acids (e.g., nitric acid) and hydrolyze to dialuric acid, which is partially reduced to alloxane and to 5-aminobarbituric acid. Both compounds form an insoluble reaction product: alloxanthine, which, in the presence of ammonia forms murexide—ammonium salts of purpuric acid—which is characterized by a purple-red color.

Caffeine (1,3,7-trimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine) are the three most widespread alkaloids belonging to this group. Their pharmacological activities and adverse effects are listed in Table 9.5 [20].

Caffeine and catechin-containing plant extracts are introduced into foods and dietary supplements. They are distributed in the form of infusions and ready-to-drink beverages, and also in solid form as dietary supplements on the basis

TABLE 9.5 The Application of Purine Alkaloids

Alkaloid	Pharmacological Activities	Adverse Effects
Allantoin 	– An endogenous substance present in the body of both target animals and man—a breakdown product of uric acid – Used topically in external injuries to stimulate tissue regeneration – No accumulation expected – In healthy humans, created in the muscles during exercise, after urate oxidation.	Generally regarded as safe (GRAS)
Caffeine 	– Mild stimulant (CNS stimulatory activity) and diuretic – Astringent and antidiarrheal (due to tannin content) – Analgesic, producing stronger and quicker pain-killing actions when administered with painkillers – Antiasthmatic – Acid and pepsin secretion enhancer – Metabolism enhancer (increase in free fatty acid and glucose levels in plasma due to a selective blockade of adenosine receptors) – maximum daily dose: 135 mg – acute lethal dose 3–10 g	Caffeinism syndrome: CNS features: headache, anxiety, agitation, confusion, seizures Cardiovascular features: palpitations, chest pain, quick heart rate Gastrointestinal features: nausea, vomiting, abdominal pain, anorexia, diarrhea
Theobromine 	– Obtained from Cocoa kernels or seeds. – opposite to caffeine: strong diuretic, vasodilatory, blood pressure decreasing, antiedematous properties – Tranquilizing effect (depressive on CNS—weaker than caffeine) – A nonselective phosphodiesterase inhibitor: increase in cAMP concentration inside the cells, in PKA, decrease in leukotriene and TNF-alpha production (anti-inflammatory action) – Reported as causing addiction to chocolate [1] – Antitussive properties [2]	Loss of appetite, nausea, vomiting, withdrawal headaches, sleeplessness, tremors, restlessness, anxiety
Theophylline 	– Stronger and shorter diuretic activity in comparison with caffeine – β_1 and β_2 receptors stimulator (release of endogenous catecholamines) – Spasmolytic and antiasthmatic in obstructive pulmonary disorders – Acid and pepsin secretion enhancer	Hypokalemia, hyperglycemia, hypercalcemia, hypophosphatemia, acidosis when overdosed

References Table 5: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0; [1] William Gervase Clarence-Smith. Cocoa and Chocolate. London: Routledge 2000; 1765–1914: 10, 31. [2] Usmani OS, Belvisi MG, Patel HJ, Crispino N, Birrell MA, Korbonits M, Korbonits D; Barnes PJ. Theobromine inhibits sensory nerve activation and cough. *FASEB Journal* 2004; 19 (2): 231–233.

of green tea (*Camellia sinensis*) extracts or caffeine-containing plants. Green tea is present in the form of beverages prepared from instant green tea powder, or from decaffeinated green tea (see Table 9.6) as a source of antioxidant catechins. A 180 mL serving of tea contains approximately 60 mg of caffeine, whereas the same volume of coffee (*Coffea robusta*) delivers ca. 100 mg of this alkaloid. The amount of caffeine in tea-containing products is determined by the temperature, leaf size, and amount, and not by the type of tea (black, green, oolong, etc.) [22]. The amount of caffeine in coffee beans ranges around 1–2.5%, in cacao seeds (*Theobroma cacao*) around 0.05–0.36%, in maté leaf (*Ilex paraguariensis*) 0.2–2.0%, and in guarana (*Paullinia cupana*) seeds 2.5–7.0%.

TABLE 9.6 Purine Alkaloids in Plant Species

Distribution	Examples	Major Pharmacological Activities	Pharmaceutical Applications	Dosage	Adverse Effects
Cola acuminata Schott & Endl. (syn. *Sterculia acuminata*), *Cola nitida* Schott & Endl—Kola tree (syn. kola nut), Sterculiaceae, seed	Caffeine, theobromine, theophylline, Besides: polyphenols (catechins, chlorogenic and quinic acids tannins)	Tonic, physical and mental tiredness, stimulation of metabolism	– African traditional medicine used as suppressing hunger, thirst and sleep, strengthening gums, reducing pain, blocking dysentery, treating migraines, headaches, hang-over, and erectile dysfunctions; antitrypanosomal activity – increase in body metabolism; in low concentrations heart stimulating effects, in higher – heart rate reduction – 36% decrease in PSA secretion in prostate cells – nutraceutical use: teas, soft drinks (often with decocainized Coca leaves), liquors, baked goods, wines. It is GRAS as a food additive to a level of 0.2 mg/mL; OTC stimulant drug product Clinical studies: increases endurance in physical activity – caffeine increased self-rated alertness and jitteriness and blood pressure – consumption of green tea and coffee was inversely associated with diabetes type 2	Extracts (90% ethanol, caffeine + theobromine content = minimum 1.2%)—0.25–0.75 g daily, liquid extracts (70% ethanol percolates, C + T content = min. 1.2. %)—2.5–7.5 g daily, tincture (1:5, 70% ethanol, C + T = 0.25%) 10–30 g daily, cola wine 60–180 g daily Maximum daily dose: 9 g	Ulcer formation, increase in blood pressure, palpitations, nausea, abdominal pain, diarrhea, restlessness, excitement, irritability, loss of hair and appetite, and diuresis Antagonism with sedative drugs, increase in side effects caused by sympathomimetic drugs; methylating activity of Cola extracts
Coffea arabica L., *Coffea liberica* Hiern., *Coffea canephora var. robusta* Pierre ex A.Froehner—coffee seed, Rubiaceae, seed					
Theobroma cacao L.—cacao tree (syn. Cocoa tree), Sterculiaceae, seed					
Paullinia cupana Kunth.—guarana, Sapindaceae, seed					
Ilex paraguariensis A. St. Hil.—Yerba mate (Syn.: Mate leaf, Paraguay tea), Aquifoliaceae, leaf	caffeine, chlorogenic acid, vitamins, minerals		Mental and physical fatigue, weight loss, rheumatic pain, stimulant of bowel function, antidiabetic actions, diuretic actions Antiobesity effect: blood and hepatic lipid, glucose, insulin, leptin, body weight lowering action Chemopreventive role (EC_{50} at 57 mg/mL, EC_{100} at 74 mg/mL) Antiparkinsonian effect of the hydroalcoholic extracts Nutraceutical applications: 2–4 g/day		

Camellia sinensis (L.) Kuntze —tea plant, Theaceae, leaf	caffeine, theophylline, theobromine, flavanols (catechins: (−)-epicatechin, (−)-epicatechin-3-*O*-gallate, (−)-epigallocatechin, (−)-epigallocatechin-3-*O*-gallate; theaflavins: theaflavin and its gallates), flavonols (quercetin, kaempferol, myricetin), flavones (apigenin, luteolin), phenolic acids (chlorogenic acid, gallic acid), saponins (derivatives of barringtogenol C), vitamins, minerals, fluoride	Tonic, physical and mental tiredness, stimulation of metabolism	– relief of fatigue and sensation of weakness – treatment for weight control – problems with diuresis, edema – other: migraine, hyperdipsia, diarrhea, insufficient gastric secretion, – antimicrobial and antiviral activities Clinical studies: – ingestion of green tea-containing beverages results in the higher level of mental performance – green tea catechins consumed 300 mg/day (without caffeine) as a part of an exercise program for 8 weeks resulted in body mass reduction; – together with caffeine, catechins administered at a dose of 690 mg/day decreased the BMI, weight, waist circumference, body fat mass and LDL concentration. – green tea consumption reduces the relative risk of myocardial infarction – hypotensive properties among habitual tea drinkers in comparison to nondrinkers – consumption of green tea and coffee was inversely associated with diabetes type 2 – protective effect against the following cancer diseases of: breast, upper gastrointestinal tract, prostate, lungs, urinary bladder, and mouth cancer	– as herbal tea: 1.8−2.2 g of whole herbal substance in 100−150 mL of boiling water as a herbal infusion, 3−5 times daily – powdered herbal substance: – 390 mg, 3 to 5 times daily in adults over 18 years of age for a week	Gastric and duodenal ulcers, hypertension, arrhythmia, hyperthyroidism, sleeplessness when administered before bedtime; Antagonism with sedative drugs, increase in side effects caused by sympathomimetic drugs; Overdose effects may occur when a 300 mg caffeine dose is received Incidental hepatotoxicity, microcytic anemia in infants consuming more than 250 mL of green tea/day
Symphytum officinale L.— comfrey, Boraginaceae, root	Allantoin	Wound healing, antiallergic	Administration: sprays or balms containing 1.4−2% of allantoin 2−3 times per day	External application as poultice, ointments, or alcoholic digestion for burns and wounds	Toxic effects due to the presence of PAs in the extract

References Table 6: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0.

The daily dosage of caffeine should not exceed 400 mg (for a period of one week). A number of food products and dietary supplements from caffeine-containing plants are used for their body stimulant effects. According to the experiments performed by Michna and coworkers [23], the oral administration of this alkaloid enhanced the locomotor activity in rats by 24% (0.04% caffeine daily for 15 weeks).

Moreover, green tea products, as rich sources of catechins are said to play an important role as dietary supplements. Among numerous studies on the influence of green tea extracts on body weight reduction, there are still several reports of no effect. That is why the impact of catechins on weight loss is still inconsistent. Table 9.6 lists the most common sources of purine alkaloids.

9.9 L-TRYPTOPHAN DERIVATIVES

9.9.1 Indole Alkaloids

This group of nitrogen-containing compounds is not homogenous. Several subgroups of these compounds were distinguished, among them the following types: *Strychnos* alkaloids (strychnine, brucine, vomicine), yohimbans (yohimbine, reserpine, deserpidine), heteroyohimbans (ajmalicine, reserpiline), *Vinca* alkaloids (vinblastine, vincristine, vincamine), beta-carbolines (harmine, harmaline), kratom alkaloids (mitragynine), tryptamines (psilocybin, serotonin), ergolines/clavine alkaloids (ergine, ergotamine, lysergic acid), and *Tabernanthe iboga* alkaloids (ibogaine, voacangine, coronaridine).

Also monoterpene indole alkaloids contain an indole, dihydroindole, or oxindole skeleton coupled with a monoterpene unit derived from secologanin. They typically contain two nitrogen atoms, one indolic, and the second from the N^1-position of the indole ring.

Various groups of indole alkaloids have been isolated from more than thirty botanical families including the Apocynaceae, Rubiaceae, Loganiaceae, Passifloraceae, as well as several fungi.

Several have medical applications: vincamine and isovincamine from *Vinca minor* are used to treat hypertension; vincristine and vinblastine have antiproliferative and cytotoxic activities and are isolated from the leaves of *Catharanthus roseus*; and harmine from *Passiflora incarnata* is used as a tranquilizer [20] (see Table 9.7).

9.9.2 β-Carboline Alkaloids

The β-carbolines constitute a group of natural and synthetic alkaloids comprising a tricyclic pyrido[3,4-*b*]indole ring structure (Fig. 9.6) at different levels of unsaturation (dihydro-, tetrahydro, and aromatic β-carbolines). The pyridine nitrogen atom is characterized by a more basic character than the acidic indolic nitrogen.

The alkaloids are derived from *Peganum harmala* (Syrian rue, Zygophyllaceae), a plant used as an emmenagogue and alimentary tract medicine, which is also known to evoke hallucinations. However, recently their antiparasitic, antitumor, and antiviral properties are of greater interest compared to their defined CNS activity.

Surprisingly, the results of clinical trials confirm the increased levels of β-carbolines in the plasma of chronic alcoholics and heroin-dependent humans. They are also reported to increase the voluntary intake of alcohol [24].

Even though these alkaloids are richly represented in terrestrial species (see: *P. incarnata* in Table 9.8), a large group of β-carbolines was isolated from marine invertebrates: tunicates, sponges, soft corals, or bryozoans [24].

9.9.3 Manzamines

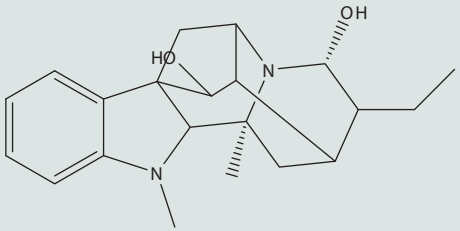
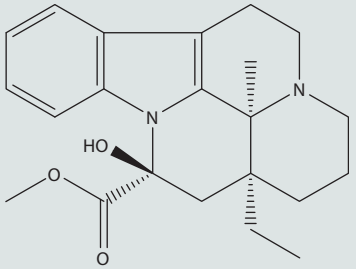
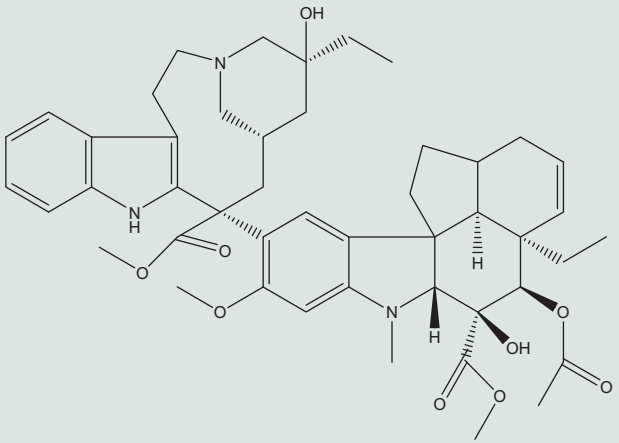
The manzamines are structurally complex components of several marine sponges, and are a well-studied example of marine alkaloids which contain a β-carboline moiety. The nucleus is attached to a pentacyclic diamine ring, which has either 8- or 13-membered rings incorporated on a pyrrolo[2,3-i]isoquinoline framework. These alkaloids are found in more than 16 species of marine sponges distributed from the Red Sea to Indonesia [25] and are known for their anticancer, antimalarial, and antileishmanicidal activities. Interestingly, their semisynthetic derivatives have not been proven to be of any significant activity.

A number of simple β-carboline derivatives are active psychostimulants [24].

9.9.4 Ergot Alkaloids

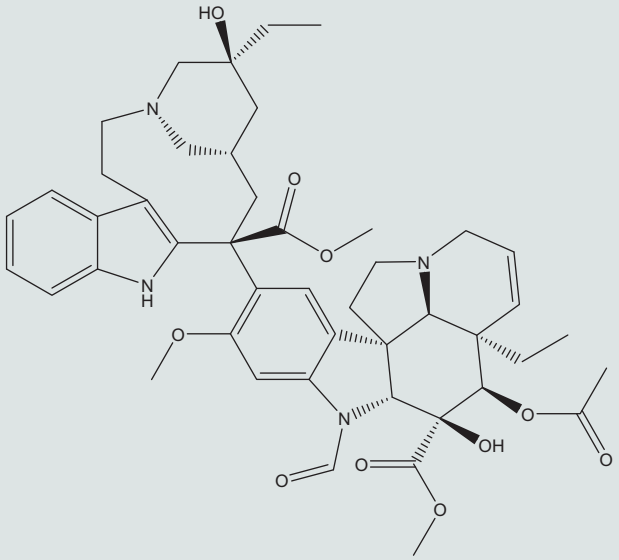
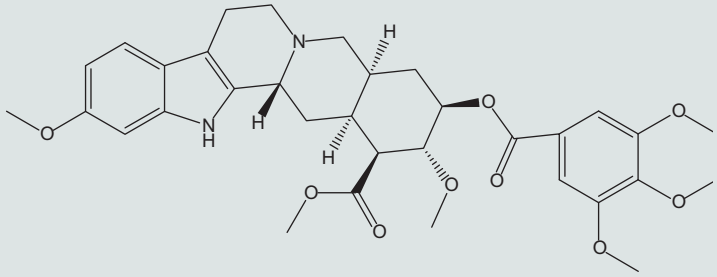
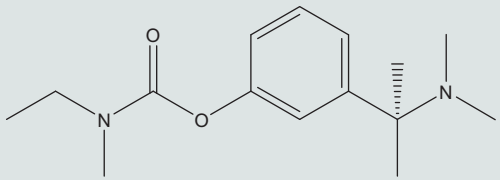
The ergot alkaloids belong to the group of indole alkaloids biosynthetically formed from tryptophan and a hemiterpene moiety. They are derived from the fungus *Claviceps purpurea* (Clavicipitaceae), which grows out from the rye's ovary,

TABLE 9.7 The Characteristics of the Most Prominent Indole Alkaloids

Alkaloid	Pharmacological Activity	Adverse Effects
Ajmaline 	Adrenolytic activity against alpha receptors which results in: – antiarrhythmic action – hypotensive effects – stimulation of peripheral circulation and cerebral circulation – in diabetes, intermittent claudication, and Raynaud's syndrome.	– Special precautions: pregnancy, breast-feeding – nasal congestion, diarrhea, vomiting, loss of appetite, convulsions, Parkinson's-like symptoms, coma – should not be used when driving or operating heavy machinery
Vincamine 	– Cerebral vasodilator in cerebral anoxia often used with papaverine or heptaminol – enhancer of global and regional blood flow in cerebral ischemia patients – characterized by reserpine-like noradrenaline depleting effect (similar strength to reserpine) – cardiovascular effect consist of a dose-dependent hypotension, – sedative effect as a result of its depressive activity toward CNS – therapeutic oral dose in humans: 40–80 mg/day for at least 20 days	– Stomach or gastrointestinal issues
Vinblastine 	– Inhibitor of cell cycle at the stage of mitosis; inhibits the assembly of microtubules after binding tubulin as an M phase arresting compound – used in the treatment of different cancer diseases: lung cancer, brain cancer, testicular cancer, bladder cancer – more effective when used with bleomycin	Severe allergic reactions, bone marrow toxicity, blood in urine, infection, severe bleeding, pain in bones, sudden shortness of breath, constipation, headache, vomiting, stomach pain, loss of appetite, deep ulcers

(*Continued*)

TABLE 9.7 (Continued)

Alkaloid	Pharmacological Activity	Adverse Effects
Vincristine 	– Inhibitor of mitosis in metaphase; binds to tubulin, inhibiting the polymerization and the formation of the mitotic spindle. – in the treatment of leukemia, lymphoma, nephroblastoma, soft tissue tumors, and neuroblastoma; often in combination with dexamethasone.	Hyponatremia, constipation, hair loss, peripheral neuropathy (dysesthesia, impaired neuromuscular transmission)
Reserpine 	– Sympatholytic presynaptic drug with psychotropic properties used in several formulations as it is responsible for dopamine depletion from brain and intestines – central sedative activity – when administered at 0.25 mg, it induces significant hypotension	High dosage: – depression – psychotic condition
Semisynthetic Derivatives		
Rivastigmine 	– A semisynthetic derivative of physostigmine from Calabar beans – used in the treatment of Alzheimer's disease as a noncompetitive acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitor, proportionally with the dose, and in Parkinson's disease – available as an oral and transdermal preparation. Oral dosage: twice daily 1.5–6 mg each time; transdermal: once daily 4.6–13.3 mg – Maximum daily dose 13 mg, administered with food	Nausea, vomiting, severe skin redness, irritation, inability to urinate, heavy sweating, seizures, diarrhea

References Table 7: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0.

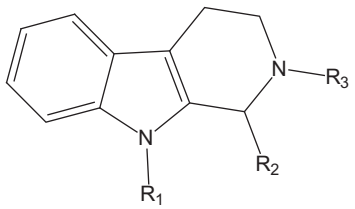


FIGURE 9.6 The β-carboline alkaloid skeleton.

creating *Secale cereale*. There are 36 representatives of the *Claviceps* genus, and *C. purpurea* is the most widespread species, parasitizing the widest range of monocotyledonous plants (ca. 600 species), mainly rye, barley, and wheat. Intoxication, in the form of either delirium or vasoconstriction, by ergot alkaloids has been known for at least a thousand years, however, modern wheat control has minimized its uncontrolled growth [26].

The alkaloids present in the ergot of rye can be divided into three subgroups: D-lysergic acid and its simple derivatives (e.g., the simple amides: ergometrine = ergobasine), the clavine alkaloids and the ergopeptines. The pharmacological properties of these groups differ significantly. Only D-lysergic acid derivatives are pharmacologically active. Ergopeptines (80% of the total alkaloid complex) contain lysergic acid attached to a peptide moiety (ergocristine, ergocornine, ergocriptine) and can be divided into the ergotamine and ergotoxine groups. In the ergometrine group, lysergic acid is linked to an amino alcohol [20].

Specific alkaloids are included in medicines for migraine headaches, hypertension, sexual disorders, or Parkinson's disease. Due to their similarity to noradrenaline, dopamine, and serotonin, they are peripheral α_1-adrenergic inhibitors (except for ergometrine), and may be used as bleeding inhibitors, especially in gynecology as drugs shrinking the uterus after childbirth, in postpartum hemorrhage, or after placental expulsion. Their administration induces a long tonic contraction of the uterus [26,27]. Ergotamine tartrate is also included into complex drugs for tranquilizing and analgesic applications.

Natural ergot alkaloids are missing selectivity toward the 5-HT receptor, which has resulted in the semisynthesis of more selective ligands [28].

Ergotamine and ergotoxine are used for the production of 9,10-dihydrogenated derivatives, which are stronger muscle relaxants than the starting alkaloids. Dihydroergotamine inhibits α-adrenergic and serotonin receptors. It is used for the treatment of migraine-type headaches (often with caffeine) and for orthostatic hypotension.

Since ergot alkaloids are dopamine receptor agonists, some of them (bromocriptine, cabergoline) are used as anti-Parkinson agents. However, due to a risk of fibrotic incidents, they are not regarded as frontline medicines [29].

Dihydrogenated derivatives of ergotoxine and ergocristine (Fig. 9.7) relax the peripheral vessels leading to hypotension. In addition, ergotoxine is included in geriatric treatments of stroke patients and those with cognitive impairment. Dihydroergocristine is an ingredient of hypotensive drugs and is used for the treatment of impaired peripheral circulation, often together with flavonoids. However, the hydrogenated forms do not exhibit smooth muscle stimulating properties [20].

Intoxication with ergot alkaloids results in the syndromes of ergotism, which are characterized by burning sensations in the limbs, hallucinations, and irrational behavior, convulsions, vasoconstriction, and even death.

A semisynthetic derivative of lysergic acid, LSD (lysergic acid diethylamide), was synthesized by Dr. Albert Hofmann from ergot in 1938, and accidentally was shown to have hallucinogenic effects. Later, it was considered as a psychiatric drug applicable to mind control. As a psychedelic drug, the compound induced altered thinking, visual effects, synesthesia, and spiritual experiences. It also induced psychiatric reactions, such as paranoia or delusions, and was prohibited in the early 1960s [30].

Adverse reactions of ergot alkaloids are generally gastrointestinal, and are limited to nausea and vomiting (1−10%), muscle weakness, fatigue, tightness in the chest, and diarrhea. A majority of ergot alkaloids are substrates of CYP3A4 metabolism and interact with other medicines metabolized by liver enzymes (including protease inhibitors, some macrolide antibiotics, quinolones, azole antifungals, etc.). Concomitant use with these strong CYP inhibitors is contraindicated due to possible acute ergot toxicity.

Dosage: ergometrine: intravenous (i.v.) solution (0.2 mg/mL) and oral tablets (0.2 mg); ergotamine tartrate: oral (1 mg) and sublingual (2 mg) tablets, rectal suppositories (2 mg), often with caffeine, which enhances its absorption (oral dosage: 2 mg at the onset of the migraine attack, maximum: 6 mg/day or 10 mg/week); dihydroergotoxine mesylate as a cognitive enhancer: over 60 years of age, oral capsules, tablets, solutions, or sublingual tablets, 1 mg.

TABLE 9.8 A Review of Indole Alkaloid-Containing Plant Species

Indole Alkaloid Type	Distribution	Examples	Major Pharmacological Activities	Pharmaceutical Applications	Dosage	Adverse Effects
Ergot alkaloids	*Claviceps purpurea* (Fr.) Tul.—ergot fungus, Clavicipitaceae on *Secale cereale*—rye	Ergotamine, ergocristine, ergotoxine, ergometrine	Central sympatholytic, peripheral α1-adrenergic blockade, smooth muscle stimulator, uterine contractor	– In gynecology to contract uterine – in migraine-type headaches – dehydrogenated derivatives as antihypertensive, cognitive in dementia	Only purified alkaloids are used instead of whole plant preparations.	
Pyrroloindole alkaloids	*Pausynistalia yohimbe* (K. Schum.) Pierre ex Beille (syn. *Corynanthe johimbe*)—yohimbe, Rubiaceae; Bark	Yohimbine	Sympatholytic, hypotensive, libido enhancer,	– In the treatment of male impotence	6 mg, 3 times daily	Allergy, hypertension (when administered with tricyclic antidepressants), arrhythmia, nausea, vomiting, hallucinations.
Quinoline alkaloids	*Cinchona* genus—quina, Rubiaceae, Bark	quinine, quinidine, cinchonine, cinchonidine	antipyretic, analgesic, uterine contractions inducing properties, antimalarial, astringent; quinidine: antiarrhythmic; a	– Quinine: malaria (*P. falciparum*), fever, common flu – quinidine: heartbeat disorders, atrial fibrillation, malaria – indigestion In veterinary: for gastric complaintsNutraceutical application: quinine is an ingredient of tonic drinks (bitter agent) at doses of ca. 70 mg/L	As a bitter stomachic, for malaria and common flu—1 g per dose, 3 g per day – single alkaloids against malaria and arrhythmia, respectively: quinine 600 mg 3 times per day, quinidine 0.8—2.0 g per daylethal dose: 2—8 g of quinine	Allergic reactions;cinchonism/quinism: sweaty skin, impaired hearing, blurred vision, dizziness, nausea, vomiting, diarrhea. Contraindicated in ulcers, pregnancy.
	Remijia genus, Rubiaceae, Bark	quinidine, cupreine	Antiarrhytmic	Heartbeat disorders	As for quinidine	As above

Simple indole alkaloids	*Strychnos* spp. L., Loganiaceae, curare;	strychnine, brucine, curare	Arrow poison; muscle relaxant	In veterinary medicine in mono-preparations or in combination products as bitter, digestive and nerve tonic – to stimulate ruminal motor activity after digestive disorders in sheep, goats, and cattle	2–7 days, per os; 100 g of product (i.e. 3 g strychni semen) per day for adult cattle, 12.5 g product per day for sheep and goats, 15 g per day for calves, 5 g per day for weanling sheep	Toxic! First symptoms: anxiety, restlessness, vomiting, nausea; later: convulsive retraction of the corners of the mouth, twitching of the facial musculature, increased sensitivity to touch and noise, symmetric cramps inhibiting respiration, anoxia, death. Lethal oral dose for cattle and horses: 0.5 mg/kg bw; in humans—0.5–1.0 mg/kg bw.
	Physostigma venenosum Balf.—Calabar bean (syn. ordeal beans), Fabaceae, Fruit	physostigmine, serine (see: rivastigmine Table 9.7)	contracting the eye pupil, reversing the effects of sedative drugs, enhances cognition	mild cognitive impairment, intoxications with tranquilizers (i.v.)	physostigmine salicylate in 1 mg/mL injections in AD	rapidly hydrolyzed by cholinesterases; duration of action: 45–60 min; Renal function impairment; additive effects with cholinergic agonists; bradycardia convulsions
	Vinca minor—common periwinkle, Apocynaceae, herb	vincamine, isovincamine	cerebral and cardiovascular vasodilatory drug	impairment of cognition, stupor, cerebral hypoxia, menorrhagia, hemorrhoids, cerebral ischemia, atherosclerosis	Due to marked differences in the alkaloid content in the plant, purified alkaloids are used instead of whole plant preparations	
	Catharanthus roseus (L.) G. Don—rosy periwinkle (syn. Madagascar periwinkle), Apocynaceae, herb	vincristine, vinblastine	antineoplastic, cytostatic	Purified alkaloids used in standard chemotherapy in lymphoma, testicular cancer, breast cancer, uterine cancer, Kaposi's sarcoma.	–	Severe allergic reactions, blood in urine, infection, severe bleeding, pain in bones, sudden shortness of breath, constipation, headache, vomiting, stomach pain, loss of appetite.
	Physostigma venenosum Balf.—calabar	physostigmine, serine,	contracting the pupil of the eye, reversing the effects of sedatives, inducing cognition (cholinesterase	Cognition impairment (Alzheimer's disease),	–	seizures, loss of control over the bladder, bowels and respiratory system

(*Continued*)

TABLE 9.8 (Continued)

Indole Alkaloid Type	Distribution	Examples	Major Pharmacological Activities	Pharmaceutical Applications	Dosage	Adverse Effects
	bean, ordeal bean, Fabaceae, fruit	calabatine, calabacine	inhibitor—semisynthetic derivative: rivastigmine); antagonist of atropine	mydriatics, poisoning with atropine		
Terpenoid indole alkaloids	*Rauwolfia serpentina* (L.) Benth. ex Kurz—rauvolfia, Apocynaceae; Root	ajmaline, reserpine, rescinnamine, aricine, serpentinine, yohimbine	Antiarrhythmic, antihypertensive, antimetabolic, antiovulatory, temperature decreasing properties	Mild hypertension, anxiety, psychomotor tension, Raynaud's phenomenon; in certain neuropsychiatric disorders Acts at postganglionic sympathetic nerve endings; induces the CNS storage of catecholamines.	CNS effects are dose-related, occurring more frequently with doses exceeding 500 mcg (0.5 mg) per day. Oral daily dose: 50 to 200 mg as a single dose or in two divided daily doses	depression, tiredness, impotence, anorexia, shortness of breath, bradycardia, impotence, lack of energy; interactions with: *Digitalis* glycosides (induces arrhythmic incidents), barbiturates, with other sympathomimetic drugs and alcohol; promotes breast cancer from previously formed cells [1]
β-Carboline alkaloids	*Passiflora incarnata* L.—passion-flower, Pasifloraceae; Herb	Flavonoids: vitexin essential oils, Alkaloids —: harmane, harmol, harmalol	tranquilizer, MAO inhibitor,	Irritation, mental stress, headaches, heartbeat disorders, anxiety, sleeplessness, mild depression. Parkinson disease Over 12 years of age Clinical studies: hallucinogenic in humans after oral, i.v. and s.c. administration (oral dose 4 mg/kg of harmaline) [24]	Comminuted herbal substance: 1—2 g in 150 mL boiling water 1—4 times daily; Powdered herbal substance: 0.5—2 g, 1—4 times daily Liquid alcoholic extracts:0.5—4 mL up to 4 times a day Administration in adults	Allergy, nausea, tachycardia, vasculitis, hypertension
β-Carboline alkaloids	*Haliclona* spp.	Manzamine alkaloids—manzamine	antileishmanial, antimalarial, antitumor, antiviral	—	—	Bleeding, fever, pain [25]

References Table 8: European Medicines Agency (EMA) monographs (www.ema.europa.eu); Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0; [1] Gerber JG, Freed CR, Nies AS. Antyhypertensive pharmacology. West J Med 1980; 132(5): 430—439; [24]; [25].

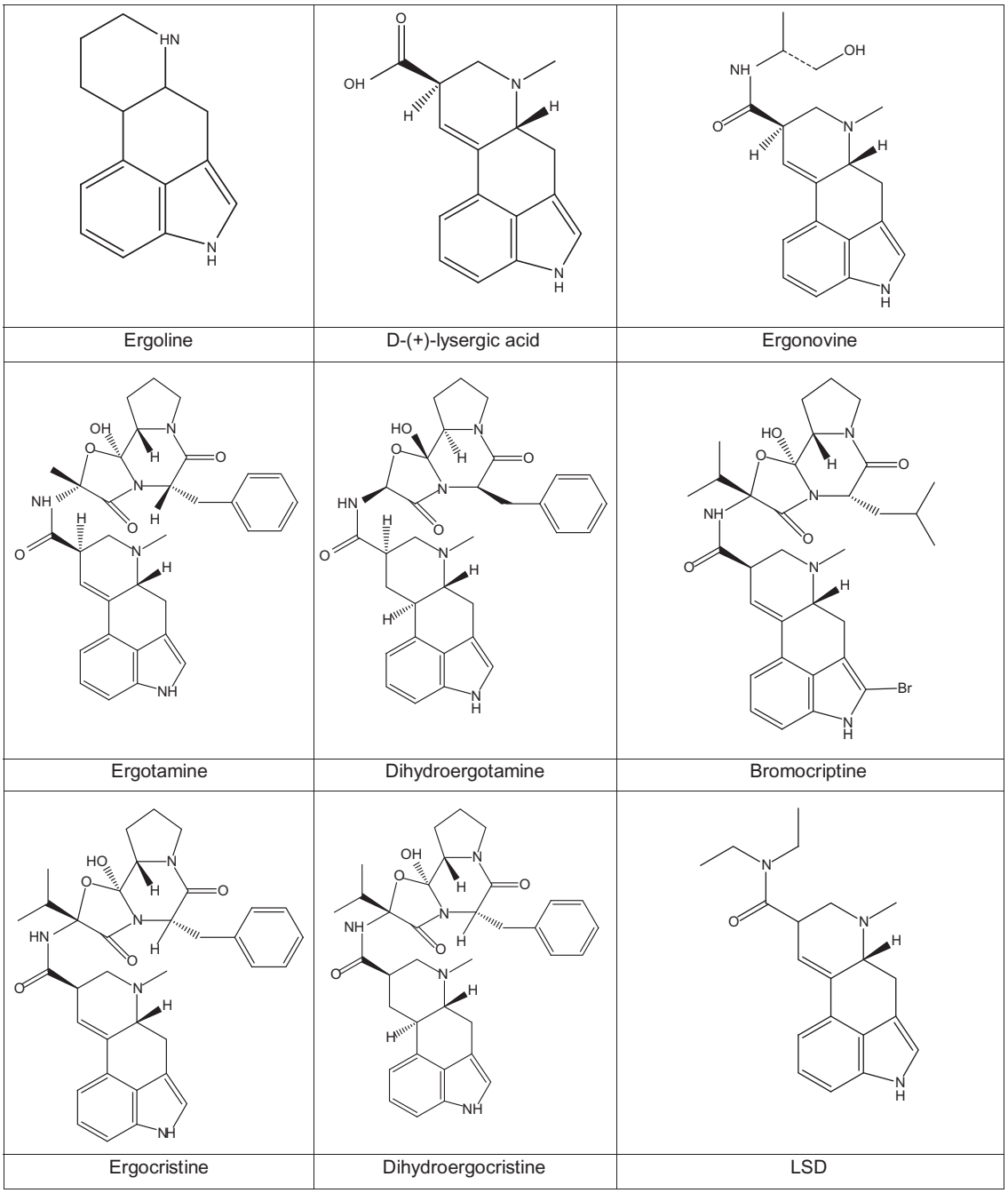


FIGURE 9.7 Chemical structures of selected ergot alkaloids.

9.9.5 Quinoline Alkaloids

The quinoline alkaloids are typically derivatives of anthranilic acid (except for the *Cinchona* alkaloids and camptothecin which are both tryptophan-derived). Their presence in extracts of several species of the Rutaceae, Rubiaceae, and Asteraceae was described, and also in fungal (*Penicillium* species) and bacterial (*Pseudomonas* species) extracts.

The most significant and pharmacologically important alkaloids are derived from *Cinchona* species (Fig. 9.8).

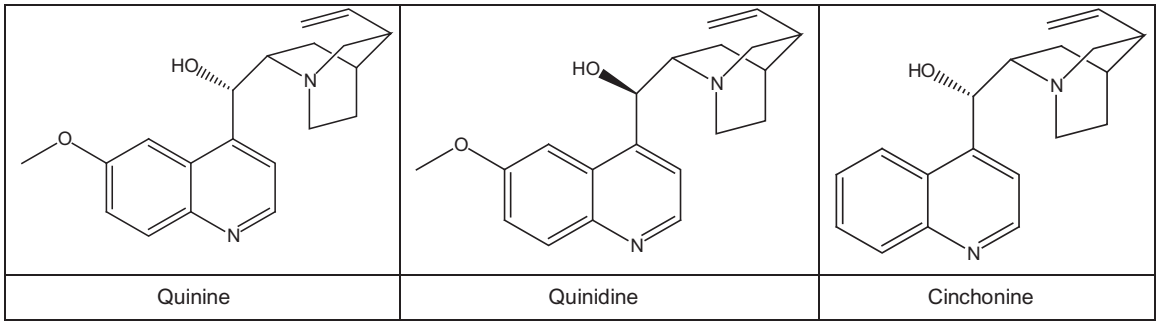


FIGURE 9.8 Structures of quinolone alkaloids.

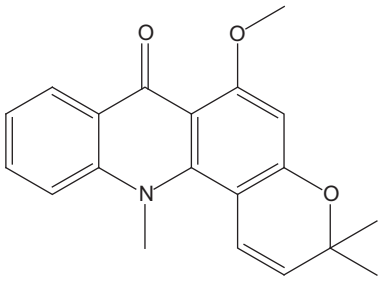


FIGURE 9.9 Structure of acronycine.

9.10 ANTHRANILIC ACID DERIVATIVES

9.10.1 Furoquinoline Alkaloids

Furoquinoline alkaloids are anthranilic acid derivatives found to exhibit spasmolytic and photosensitizing properties in the extracts of Rutaceae species. Dictamnine, skimmianine, or fagarine are responsible for the mutagenic properties of plant extracts, and also for their anticholinesterase, antiviral, antiplasmodial, and antibacterial activities [31].

9.10.2 Acridone Alkaloids

Acridone alkaloids constitute a small group of alkaloids with the most significant representative being acronycine (see Fig. 9.9), isolated from Australian shrub *Sarcomelicope simplicifolia* (formerly *Acronychia baueri*) from the family Rutaceae. The alkaloid and its derivatives are used as antineoplastic agents against colon and lung carcinoma (Table 9.9) [32].

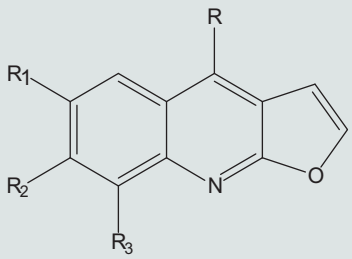
9.11 L-LYSINE DERIVATIVES

9.11.1 Pyridine and Piperidine Alkaloids

Piperidine alkaloids have been identified as containing a saturated piperidine ring, and are mostly derived from lysine. Generally, for the simple α-substituted piperidines, lysine constitutes the point of α-side chain attachment. The alkaloids are widespread among the Apiaceae, Solanaceae, Chenopodiaceae, Fabaceae, Crassulaceae, Lycopodiaceae, and Asteraceae.

The different alkaloids possess various pharmacological activities. Among them, poisonous compounds such as lycopodine from *Lycopodium* species, or nicotine from *Nicotiana tabacum* were described. The most significant alkaloids from a pharmacological point of view include the derivatives of lobeline and piperine, which are described in Tables 9.10 and 9.11 [20].

TABLE 9.9 Chemical Structures of Furoquinoline Alkaloids



	R	R_1	R_2	R_3
Furoquinoline	H	H	H	H
Dictamnine	OMe	H	H	H
Fagarine (=skimmianine)	OMe	H	H	OMe
Kokusagine	H	OMe	OMe	H

9.12 HISTIDINE DERIVATIVES

The most important alkaloid from this group is pilocarpine, whose biosynthesis may involve histidine and threonine. It is produced by the neotropical plant, *Pilocarpus jaborandi* in the family Rutaceae, native to Brazil, where it is cultivated (Table 9.12).

9.13 OTHER ALKALOIDS

Other alkaloids consist of basic compounds, whose biosynthesis is not related to an amino acid-derived pathway. They contain nitrogen atoms inside a heterocyclic ring or in a chain, however, the major carbon skeleton is derived from monoterpenes, steroids, or acetic or propionic acids [3].

9.13.1 Terpenoid Alkaloids

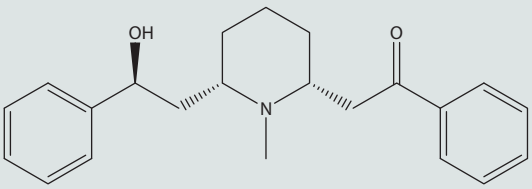
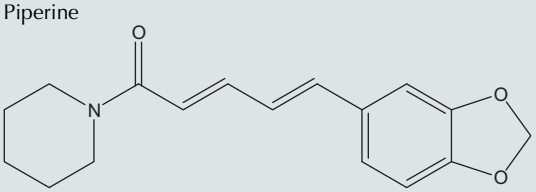
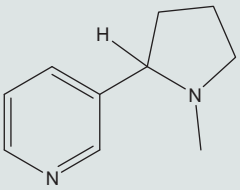
Terpenoid alkaloids, derived from an isoprene moiety, may be divided into three groups: monoterpene, sesquiterpene, and diterpene alkaloids. Diterpene alkaloids are the most abundant group, and comprise structures of medicinal interest, such as aconitine, paclitaxel (taxol), and delphinine. They are derived from tetra- or pentacyclic diterpenes joined with amines or aminoalcohols. They are present in the Ranunculaceae, Cornaceae, and Asteraceae. The most significant among the terpene alkaloids is without doubt, paclitaxel.

This alkaloid has been used extensively as a potent antineoplastic agent. It was originally found in extracts of the bark of the Pacific yew (*Taxus brevifolia*, Taxodiaceae) in scarce quantities, however, its efficient semisynthesis has been elaborated from 10-deacetylbaccatin isolated from the leaves of *T. baccata*. Paclitaxel stabilizes microtubules and blocks their subsequent depolymerization to tubulin, a protein related to cell division processes.

Its clinical applications in the therapy of ovarian, pulmonary, prostate, and breast cancer are vast because of its high efficiency rate. It is used in monotherapy (metastatic breast cancer), but more often in combination with other chemotherapeutic agents. For example, paclitaxel with carboplatin is the first-line treatment of nonsmall cell lung cancer in adult patients who are not candidates for potentially curative surgery. Recently, its presence in hazelnut shells and leaves was reported [33].

Daphniphyllum alkaloids are also important representatives of this group of compounds. They are characterized by a bridged or fused penta- or hexacyclic scaffold. They have been isolated from dioecious evergreen Asian plants and are widely used in traditional Chinese herbal medicine. Recent studies report their significant anticancer, antioxidant, vasorelaxing, and neurite growth-stimulating activities [34].

TABLE 9.10 Examples of Pyridine and Piperidine Alkaloids

Alkaloid	Pharmacological Activity	Adverse Effects
Lobeline 	Structural similarity to nicotine Exerts nicotine-like effects on choline receptors in autonomous ganglia, neuromuscular junctions, aorcid and carotid bodies; no activity on CNS cholinoceptors Increase of pulmonary and systemic blood pressure In veterinary medicine: For respiratory tract examination at a dose of 0.1 mg/kg bw (i.v.) and 0.2 mg/kg bw (i.m. or s.c.) Rapid (3–12 min) increase of respiratory frequency, and tidal volume for a few minutes, Therapeutic doses in human medicine: 0.06 mg/kg (i.v.), or 0.1 mg/kg bw (s.c.); single oral dose: 2 mg/person (ca. 0.3 mg/kg bw) As smoking deterrent: daily oral dose of 6 mg (3 × 2 mg), ca. 0.1 mg/kg bw.	6 weeks of administration for humans mostly
Piperine 	Antidiarrheal antipyretic, analgesic, insecticidal, antitumor, anti-inflammatory, and antidepressant properties. cytotoxic against cancer cell lines increased catecholamine secretion metabolized by cytochrome P450, it increases the concentration of several medicines (rifampin, propranolol, phenytoin, theophylline)	Gastrointestinal irritation [1]
Nicotine 	Liquid alkaloid, a CNS poison Spasmolytic in digestive system, increases the secretion of HCl and saliva Hypertensive due to contraction of peripheral vessels In controlled amounts used in smoking cessation	Mutagenicity, inflammation of peripheral nerves, memory impairment Stomach ulcer formation, dizziness, nausea, diarrhea, headaches 50 mg dose is lethal

References Table 10: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0; [1] Ahmad N, Fazal H, Abbasi BH, Farooq S, Ali M, Khan MA. Biological role of *Piper nigrum* L. (black pepper): a review. Asian Pac J Trop Biomed 2012;1–10.

TABLE 9.11 Plant Extracts Rich in Pyridine and Piperidine Alkaloids

Type	Distribution	Examples	Major Pharmacological Activities	Pharmaceutical Applications	Dosage	Adverse Effects
Piperidine alkaloids	*Lobelia* spp.—lobelias, Campanulaceae, Flower, herb	Lobeline	Stimulation of respiration; analeptic properties,	– In smoking cessation programs – bronchoconstriction	2–4 g of flowers daily in an antitussive tea	Nausea, vomiting, dizziness at high doses (8 mg lobeline sulfate); *Contraindications*: pregnancy, don't use for children
	Areca catechu L.—areca palm (syn. betel palm, Indian nut), Arecaeae, seeds	Arecoline	Parasympathomimetic; in veterinary anthelmintic and laxative agent	Seeds (betel nuts) chewed as a euphoric agent in Asia	Stains teeth and gums red	When chewed: oral submucous fibrosis which can progress to malignant oral cancer, asthma, spastic states of air passages
Quinolizidine alkaloids	*Punica granatum*—pomegranate, Lythraceae, Rind of fruit, bark	Pelletierine, pseudopelletierine and their tannates	Anthelmintic, astringent	Diarrhea, juice: as bitter agentin veterinary: in tapeworms, diarrhea	–	Dizziness, visual impairment, spasmsDoses higher than 80 g of bark in humans may cause severe vomiting, fever, tremor and collapse; temporary blindness may occur, mydriasis, headache, muscle cramps;
	Piper nigrum –black pepper, Piperaceae, unripe fruit	Piperine, piperettine	Condiment, secreting bile acids, insecticidal, antirheumatic, antiphlogistic, antibacterial	Bronchitis, indigestion, gonorrhea	–	Digestive tract irritation [1]
	Cytisus scoparius (L.) Link, dyer's broom, Fabaceae, Herb	Sparteine, lupanine, scoparin	Diuretic, antiarrhytmic, antioedematic	Aqueous decoctions are mild diuretic	Rarely used	Hipotension
	Lupinus luteus L.—lupine, Fabaceae, Herb	Lupinine, sparteine	As above			Poisonings: fever, high heart rate, tremors, dizziness, dry mouth, anxiety
	Nicotiana tabacum—tobacco, Solanaceae, Leaf	Nicotine, anatabine, anabasine, tabacine, choline	Insect repellent	Traditionally used in asthma, bronchitis, indigestion, or ulcers	Nicotine chewing-gums, nasal sprays or patches intended to help give up smoking because of nicotine dependence	Mutagenicity

References Table 11: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0; [1] Ahmad N, Fazal H, Abbasi BH, Farooq S, Ali M, Khan MA. Biological role of *Piper nigrum* L. (black pepper): a review. Asian Pac J Trop Biomed 2012;1–10.

TABLE 9.12 *Pilocarpus jaborandi* as the Most Significant Representative of Histidine Alkaloids

Type	Distribution	Examples	Major Pharmacological Activities	Pharmaceutical Applications	Dosage	Adverse Effects
Imidazole alkaloids	*Pilocarpus jaborandi* Vahl.—Pernambuco jaborandi; Rutaceae, leaf	Pilocarpine	An atropine antagonist, diaphoretic and pyretic agent, inducing the production of sweat saliva and digestive juices; muscarinic receptor agonist	Dry mouth in Sjogren's Syndrome and head and neck cancer, in eyedrops against glaucoma	5 mg twice daily	Changes in vision, dizziness, rush, tachycardia, headache, runny nose, hypertension, nausea, redness of face; interactions with seizure, blood pressure, muscle control or Parkinson's disease medications, allergy

References Table 12: European Medicines Agency (EMA) monographs (www.ema.europa.eu); Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0.

9.13.2 Steroidal Alkaloids

Steroidal alkaloids are biosynthesized by the inclusion of one or two nitrogen atoms into a preformed steroid molecule. *Solanum* and *Veratrum* alkaloids are representatives of this class of alkaloid, and are perceived as pharmacologically important alkaloids, and also as precursors of semisynthetic steroid derivatives. The alkaloids of *Veratrum* may be divided into two classes: jerveratrum alkaloids containing 1−3 oxygen atoms, and having antiparasitic activity, and ceveratrum alkaloids having a higher level of hydroxylation (7−9 oxygen atoms), which are responsible for the hypotensive activity of *Veratrum* species (e.g., germine or protoverine) (Table 9.13) [35].

9.14 NONHETEROCYCLIC ALKALOIDS

Nonheterocyclic alkaloids are compounds derived from amino acids or biogenic amines, which do not contain a heterocyclic nitrogen moiety. Ephedrine and capsaicin are the most important representatives of this group. They are widely studied and administered to patients. However, the group of protoalkaloids also includes compounds such as hordenine from *Hordeum distochon* (Poaceae), and mescaline from *Lophophora williamsii* (Cactaceae) [3] (Tables 9.14 and 9.15).

9.15 CONCLUSIONS

The variety of the types of plant-derived alkaloids described herein confirms the vast chemical diversity of this group of metabolites. The differences in their chemical structure markedly influence the bioactivity of the described compounds, which makes alkaloids of significant clinical use as medicines for the treatment of many different diseases. Numerous reports describe their novel bioactivity and clinical applications, and show the influence of semisynthetic structure modifications leading to an increase of activity, better pharmacokinetics, and a higher level of safety.

Even though the majority of alkaloids described in the chapter are commonly administered to patients or used in the traditional medicines of different countries, it should not be forgotten that they are often very potent in their action, and the thin line between their therapeutic and toxic doses should be remembered.

9.16 SELF EVALUATION QUESTIONS

1. List the alkaloid-containing plants used as hypotonic, rubefacient, and antiarrhythmic.
2. List the plant species affecting the digestive and secretomotory systems and indicate the major alkaloid responsible for this action.
3. What are the major side effects and indications of tropane alkaloids?

TABLE 9.13 A Review of Terpenoid and Steroid Alkaloids in Selected Plant Species

Major Precursor	Group of Compound	Distribution	Examples	Major Pharmacological Activities	Pharmaceutical Applications	Dosage
Geranyl-Geraniol	Terpenoid alkaloids	Aconitine—diterpenoid alkaloid	*Aconitum napellus* L.—aconite (syn. monkshood, wolfsbane), ranunculaceae, root	Blocks peripheral musculoskeletal synapses; local anesthetic.	Poison, lethal dose: 10 mg; formerly used as antineuralgic; nowadays only in homeopathic doses	Gastrointestinal signs: vomiting, nausea, diarrhea; hypotension, bradycardia, paralysis of the heart and respiratory center, asystole [1]
		Paclitaxel	*Taxus baccata*—European yew, Taxaceae, aboveground parts	Antimitotic activity; breast, ovarian, prostate, nonsmall cell lung cancer		Edema, body weight gain, nausea, vomiting, anemia, hypotension, female infertility
Acetic acid	Sesquiterpene alkaloids	Evonine, euoverrine, eunonymol	*Euonymus sieboldiana* Blume—sindle tree, Celastraceae, herb	Insecticidal, antibacterial, antihyperglycemic properties	–	Vomiting, diarrhea, unconsciousness, mental disorder [2]
		Coniine	*Conium maculatum*—poison hemlockApiaceaeor*Sarracenia flava*—yellow pitcher plant, Sarraceniaceae	Disruption of peripheral nervous system nicotinic receptor blocker—curare-like effects	Poison (less than 0.2 g is lethal)	Neurotoxic properties and respiratory paralysis—hypoxic convulsions prior to death
Saponins	Steroid alkaloids	Solanidine	*Solanum tuberosum L.*—perennial nightshade, Solanaceae, tuber	Fungicidal and pesticidal properties due to cholinesterase inhibition, triggers apoptosis	Toxic, 3–6 mg. kg bw may be lethal	Teratogenic, intoxications with vomiting, cardiac dysrhythmia, hypothermia, jaundice, hallucinations, loss of sensation [3]
		Veratramine, germine, protoverine, veracevine	*Veratrum* spp. Bernh., Melanthiaceae	Hypotensive drugs with significant toxicity; sodium channels activators	Toxic, used in homeopathic drugs	Hypotension, bradycardia, seizures, sweating, abdominal pain [4]

References Table 13: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0; [1] Chan TY. Aconite poisoning. Clin Toxicol 2009;47(4):279–85. doi:10.1080/15563650902904407. [2] Kukula-Koch W, Widelski J, Koch W, Glowniak K. HPLC, Two-dimensional TLC determination of phenolic content, and an *in vitro* perspective to antioxidant potential of *Euonymus verrucosus* Scop. extracts. Acta Chromatogr, DOI:10.15556/AChrom.27.2015.4.11. [3] Friedman M, McDonald GM (1999). Postharvest changes in glycoalkaloid content of potatoes. Adv Exptl Med Biol 459:121–143. [4] Schep LJ, Schmierer DM, Fountain JS. Veratrum poisoning. Toxicol Rev 2006;25(2):73–78.

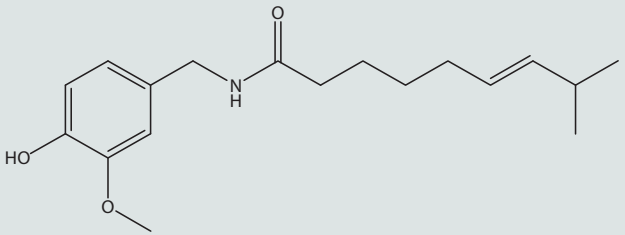
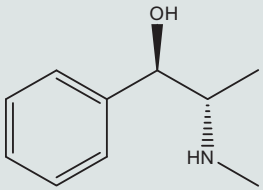
TABLE 9.14 A Review of Selected Nonheterocyclic Alkaloid-Containing Plants

Distribution	Examples	Major Pharmacological Activities	Dosage	Adverse Effects
Capsicum annuum L.—bell peppers or chili peppers, Solanaceae, oleoresin	Capsaicin, capsaicinoids	Internally used in atonic gout, dyspepsia, and externally in muscle pain, rheumatism, muscle tension in the areas of shoulder, neck and lower back, max. 1 plaster a day for at least 4 and up to 12 h with interval of at least 12 h before the successive application; semisolid forms: 2–4 times daily	Ethanol (80% or 96%) extracts used as creams, ointments and medicated plasters	Irritation of intestinal tract and kidneys
Ephedra sinica Stapf.—ephedra, Ephedraceae, Herb	Ephedrine	Plant used as the source of ephedrine alkaloid	–	Tachyarrhythmias
Colchicum autumnale L.—autumn crocus, meadow saffron, Liliaceae, seeds, flowers, tuber	Colchicine and its glucoside—colchicoside	Gout (decreases the uric acid precipitation and inflammatory reactions) Familial Mediterranean fever; Antineoplastic properties (in vivo tests on breast, bowel, lung and prostate cancers with doxorubicin) [1]	At a single dose equivalent to 1 mg of colchicine, not more than 8 mg daily. Toxic dose: 10 mg of colchicine	Diarrhea, aplastic anemia, agranulocytosis, myopathy, vomiting,
Lophophora williamsii—mescal cactus, Cactaceae	Mescaline	Psychomimetic effects resulting in alteration of mood, changes in perception, rich visual hallucinations, increase of body temperature and blood pressure evoked by marked activity on serotoninergic and dopaminergic receptors [2]	0.3–0.5 g is the hallucinogenic dose	Serious toxicity: anxiety, racing heartbeat, diarrhea, severe vomiting and nausea

References Table 14: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0; [1] Atkinson JM, Falconer RA, Edwards DR, Pennington CJ, Siller CS, Shnyder SD, Bibby MC, Patterson LH. Development of a Novel Tumor-Targeted Vascular Disrupting Agent Activated by Membrane-Type Matrix Metalloproteinases. *Cancer Res* 2010; 70 (17): 6902–12; [2] Kyzar EJ, Collins C, Gaikwad S, Green J, Roth A, Monnig L, El-Ounsi M, Davis A, Freeman A, Capezio N, Stewart AM, Kalueff AV. Effects of hallucinogenic agents mescaline and phencyclidine on zebrafish behavior and physiology. Progr Neuro-Psychopharmacol Biol Psychiat 2012;37:194–202.

4. What are some nutraceutical applications of purine alkaloids?
5. List the pyrrolizidine alkaloid (PAs) containing plants, define their toxic doses and the chemical structure.
6. Describe the activity profiles of berberine, galanthamine, caffeine, vincamine, ajmaline, codeine, papaverine, ephedrine, and colchicine.
7. List the constituents of *Camellia sinensis* and describe their influence on the activity of the total extract.
8. Describe the pharmacological activity of ergot alkaloids considering the level of hydrogenation of the basic skeleton.
9. List the most toxic alkaloids and those used in cancer therapy strategies.
10. Describe the activity and applications of opium alkaloids and their semisynthetic derivatives concerning their addictive potential.
11. List the botanical sources of caffeine.
12. Describe the pharmacological profile of the manzamines.
13. What are the differences in pharmacological action of the diastereoisomers quinine and quinidine?
14. What are the pharmacological activity and pharmaceutical applications of piperidine alkaloids?
15. Describe the indications of colchicine, its mechanism of action and its side effects.
16. What are the indications for ephedrine administration?

TABLE 9.15 The Pharmacology of Selected Nonheterocyclic Alkaloids

Alkaloid	Pharmacological Activity	Adverse Effects
Capsaicin 	– An agonist of vanilloid receptor (calcium channels) located on the ends of axons, which contain substance P, responsible for pain sensation Topical application of capsaicin results in the heat induced desensitization of the receptors due to substance P removal from axon endings, and in analgesic properties with the accompanying morphological damages on the neurons – Capsaicin is responsible for a significant rise in the production of HCl in the gastric juice, and for the inhibition of interleukin production in *H. pylori* infections – Other in vitro tests describe the influence of capsaicin on the apoptosis of tumor cells. After the addition of capsaicin, the levels of reactive oxygen species in tumor cells decreased and the apoptosis–inducing factor level was elevated – Various clinical studies confirm the analgesic properties of capsaicin after the third application, which is apparent by visible flare, temperature and blood flow increase in the application area. Moreover, capsaicin was effective in aborting cluster headache attacks.	Gastrotoxicity, burning, itching, swelling, cough, shortness of breath, headache, dizziness
Ephedrine 	– Ephedrine acts on the CNS, stimulates the cerebral cortex and autonomic centers, resulting in psychomotor excitement and stimulation of the respiratory center. – Ephedrine is one of the active substances of adrenomimetic action. It works directly, stimulating β-adrenergic receptors. In contrast to endogenous adrenaline it does not decompose in gastrointestinal tract. – increases blood pressure, accelerates the heart rate, works as spastic agent on peripheral blood vessels, increases the contractility and excitability of the heart muscle. – ephedrine reduces the bronchial and digestive system smooth muscle tension, acting as a spasmolytic drug. – stimulates the respiratory center, directly and indirectly through the β-adrenergic receptors in the bronchial tree, causing bronchodilation and inhibition of mucosal secretory function (antiasthmatic action). Pure alkaloid in the form of the hydrochloride is used in collapses, asthma, and emphysema. Synthetic derivative of ephedrine—ephetonine is used in asthmatic conditions.	The alkaloid may interact with beta-blockers, COMT inhibitors, MAO inhibitors, antidepressants, ergot alkaloids or cardiac glycosidesIt may cause dizziness, insomnia, blood sugar disturbances, several allergic reactions, stomach irritation, loss of appetite

References Table 15: [13]; Monographs of the European Scientific Cooperative on Phytotherapy (ESCOP); European Pharmacopoeia 8.0.

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Chapter 10

Tannins

E. Sieniawska and T. Baj
Medical University of Lublin, Lublin, Poland

Chapter Outline

Learning Objectives

- The structural and chemical characterization of tannins as plants secondary metabolites.
- To name the types of tannins and give examples.
- Identifying typical plants and plant families containing tannins.
- Examples of the bioactivities of tannins used as pharmaceuticals.
- Chemical tests used to identify tannins.
- The most ideal extractions methods for tannins.

10.1 DEFINITION

Based on the classical Bate-Smith definition, tannins are a group of plant secondary metabolites that have the ability to tan or convert animal skin into leather. These compounds are classified as being water soluble phenolics with a molar mass between 300 and 3000, and with the ability to precipitate alkaloids, gelatins, and other proteins This definition, however, excluded more recently identified larger molecular compounds with masses up to 2000 Da, with similar structures [1]. In the last few decades, the isolation of bioactive stilbenoids, thought to be responsible for the tannin activity of the bark of spruce trees, various resveratrol oligomers, and phlorotannins from brown algae have expanded the field of known tannins, by way of incorporating within the label, the tannin polyphenols [2].

10.1.1 Biosynthesis

There are two major biosynthetic routes that yield tannins of different structural complexity.

Pharmacognosy. DOI: http://dx.doi.org/10.1016/B978-0-12-802104-0.00010-X

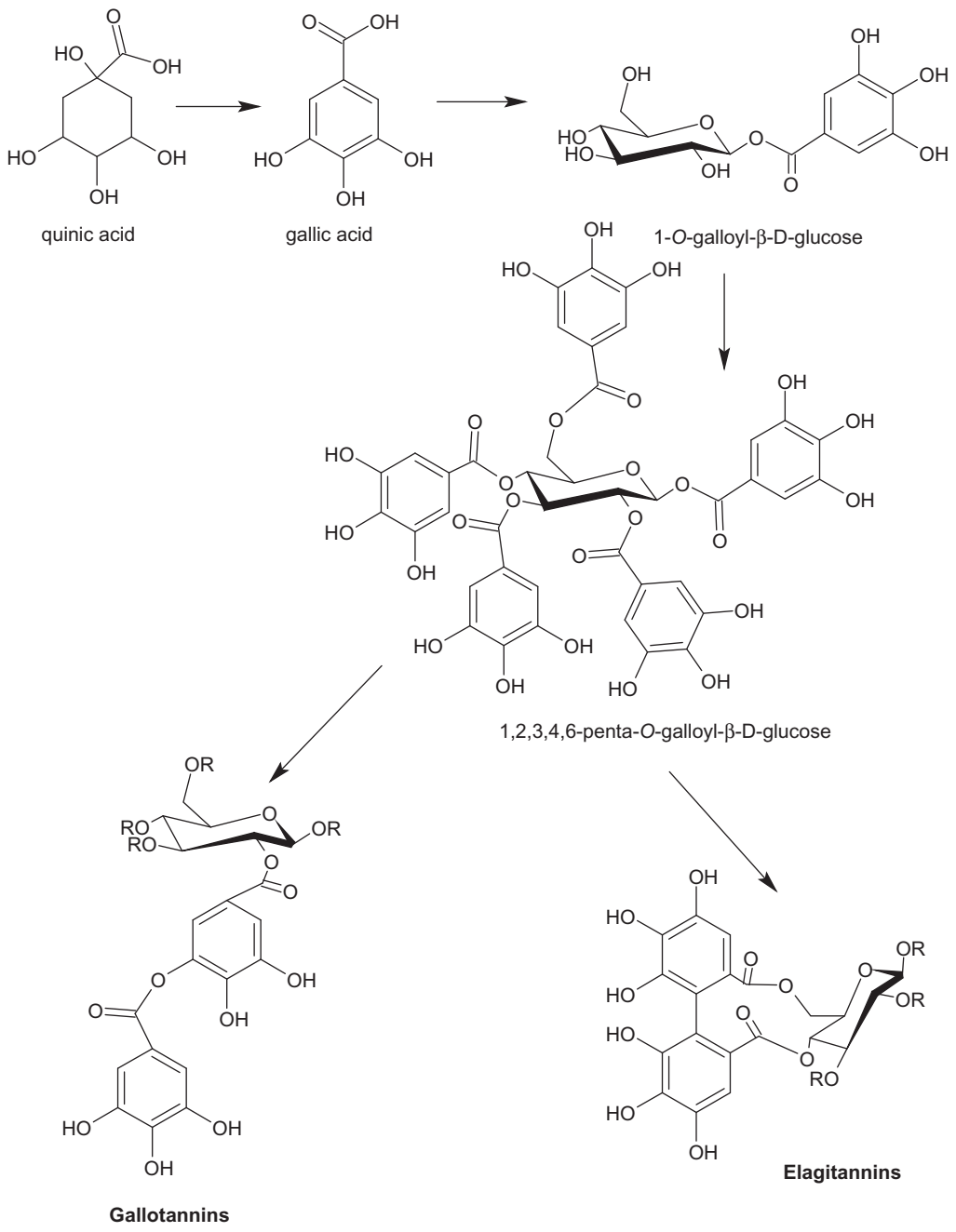


FIGURE 10.1 Biosynthetic route of hydrolyzable tannins.

The first one (Fig. 10.1), which is comprised of three steps, is derived from quinic acid, and yields gallic acid. Herein, a galloyl unit is used in the formation of 1-galloyl-β-D-glucose, a basic intermediate and a key-metabolite in the hydrolyzable tannins (HT) biosynthesis.

1. In the first step, 1-galloyl-β-D-glucose functions as an acyl acceptor and acyl donor, in order to form di-, tri-galloylglucoses, and, finally, pentagalloylglucose (the products are "simple" galloylglucose derivatives). The 2,3,4,6-*t*etra-*O*-*g*alloyl-D-*g*lucopyranose (TGG) and 1,2,3,4,6-*p*enta-*O*-*g*alloyl-β-D-*g*lucopyranose (β-PGG), found in many plant families, are key intermediates in the biosynthesis of nearly all hydrolyzable plant polyphenols.

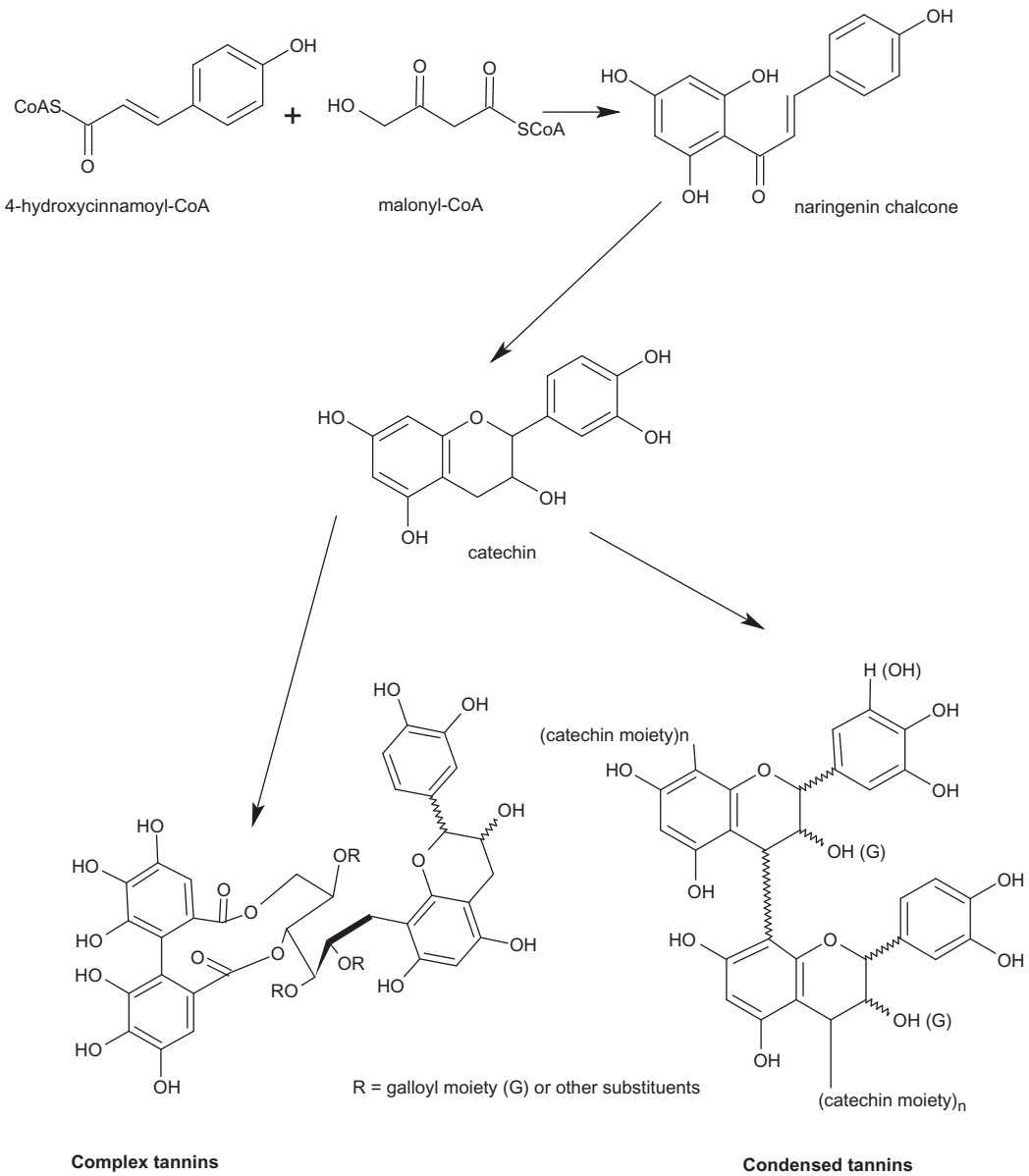


FIGURE 10.2 Biosynthetic route of complex and condensed tannins.

2. In the second step, the galloylation of pentagalloylglucose continues to yield hexa-, hepta-, octa-, etc.—galloylglucose derivatives, and to form an esteric link between two galloy moieties (gallotannins or depsidic metabolites).
3. The third step is via oxidation, leading to C−C linkages between suitably orientated galloyl residues of glucogalloyl molecules that form hexahydroxydiphenoyl (HHDP) units (ellagitannins) [3].

In the second biosynthetic route (Fig. 10.2), p-coumaroyl-CoA, when condensed with malonyl CoA, gives chalcone. This is a precursor of naringenin and then flavan-3-ols (e.g., catechin) [4].

1. The first step in this process is the glycosylation of a catechin unit to a gallotannin or an ellagitannin unit (both complex tannins).
2. The second step involves the oligomerization of these catechin units through the linkage of the *C*-4 of one catechin unit, with the *C*-8 or the *C*-6 of the next catechin unit (condensed tannins) [1].

10.2 TYPES OF TANNINS

The classical division of tannins was based on their resistance or not, to hydrolysis in the presence of hot water or the enzymes tannases (which catalyze hydrolysis reactions among the digallates). As a result, tannins were grouped as either:

1. Hydrolyzable
2. Nonhydrolyzable/condensed.

The HT encompass the polyesters of gallic and hexahydroxydiphenic acid (gallotannins and ellagitannins, respectively), whereas condensed tannins include the oligomers and polymers composed of favan-3-ol nuclei (proanthocyanidins) [5].

In more recent times, tannins are categorized according to their structural characteristics, into four major groups:

1. Gallotannins,
2. Ellagitannins,
3. Complex tannins,
4. Condensed tannins [1].

Gallotannins—These are polymers of galloyl units bound to diverse polyol units. The widely distributed polyol residues are derived from D-glucose, and the hydroxy functions of the polyol residues may be partly or fully substituted with galloyl units. In *meta*-depsides, galloyl residues are esterified with the polyol residue and also with one or more linked galloyl units in the metaposition relative to the galloyl units' carboxyl groups. Gallotannins in which the polyol residues are coupled to cinnamoyl or coumaroyl groups are rare [1−3,6,7]. Examples of these and their related structures can be viewed in Table 10.1.

Tannic acid—Its chemical structure is given as $C_{76}H_{52}O_{46}$, which corresponds with that of decagalloyl glucose, but in fact, it is in actuality, a mixture of polygalloyl glucoses or polygalloyl quinic acid esters, with the number of galloyl moieties per molecule ranging from 2 up to 12 (depending on the plant source used for the extraction of the tannic acid). Tannic acid is usually extracted from *Caesalpina spinosa* (Molina) Kuntze, *Rhus semialata* Murray, *Rhus coriaria* L., and *Quercus infectoria* Oliv [8].

Chinese gallotannin (*Rhus chinensis* Mill.) (Fig. 10.3) and *Turkish gallotannin* (*Quercus infectoria* Oliv.)—the number of galloyl moieties per molecule range from 8 up to 10, depending on the plant source. *Turkish gallotannin* contains penta-, hexa-, and heptagalloylglucose, while *Chinese gallotannin* mainly holds decagalloylglucose [2,8].

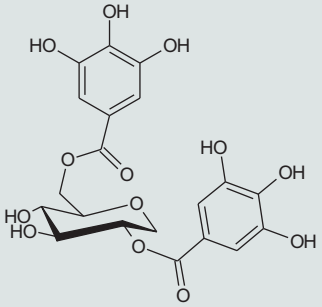
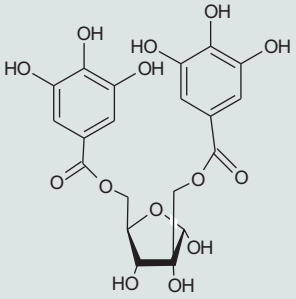
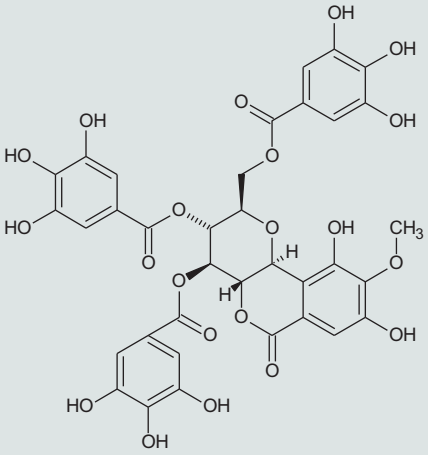
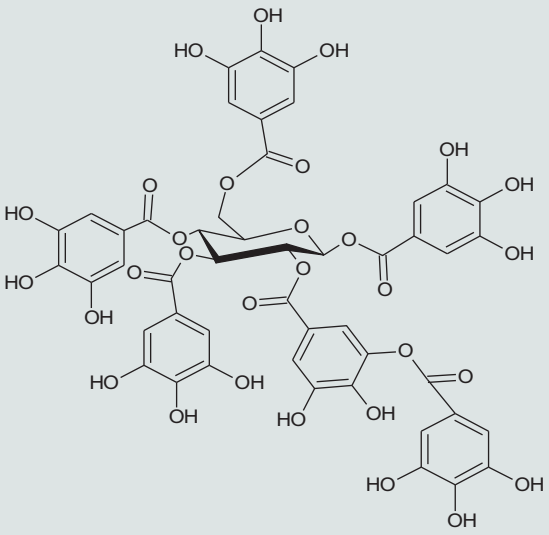
Ellagitannins— The characteristic unit of all ellagitannins, the HHDP group, is the product of the first-stage biogenetic oxidation of galloyl groups. Linking one or two additional galloyl group(s) to the HHDP unit via C−O or C−C bond formation gives rise to several variations of the HHDP group [1,9−11]. The exemplary ellagitannins are described in the Table 10.2.

Complex tannins—Herein, a catechin unit is bound glycosidically to a gallotannin or to an ellagitannin unit [1,2]. The exemplary complex tannins are described in Table 10.3.

Oak complex tannins: *Acutissimin A* and *eugenigrandin A*—In these, the ellagitannin moiety of vescalagin is connected through a C−C bond to (+)-catechin and to (+)-gallocatechin, respectively. The **acutissimins A**and **B** were isolated from the bark of several *Quercus* species and the bark of *Castanea crenata* (Siebold & Zucc.) while eugenigrandin A and **guajavin B** have also been found in the bark of *Psidium guujava* L. [1,2].

Condensed tannins (proanthocyanidins)—in such, all oligomeric and polymeric proanthocyanidins are formed by the linkage of the *C*-4 of one catechin (flavan-3-ol) with the *C*-8 or *C*-6 of the next monomeric catechin (Fig. 10.4). While monomeric catechins and leuko-anthocyanidins have no tanning properties, when converted into oligomers and polymers, they do hold tanning properties by way of acidic and enzymatic action. Biosynthetically, the condensed tannins are formed by the successive condensation of the single building blocks, with a degree of polymerization between two and greater than fifty blocks being reached. Oligomers and polymers consisting of two to ten catechin units are also known as flavolans [1]. Derivatizations as *O*-methylation, *C*- and *O*-glycosylation and *O*-galloylation are frequently reported, and a structural complexity is most prominently present in the rearrangement products of proanthocyanidins. The variation in hydroxylation pattern brings about a classification of the proanthocyanidins into several subgroups: propelargonidins (3,4′,5,7-OH), procyanidins (3,3′,4′,5,7-OH), prodelphinidins (3,3′,4′,5,5′,7-OH), proguibourtinidins (3,4′,7-OH), profsetinidins (3,3′,4′,7-OH), prorobinetinidins (3,3′,4′,5′,7-OH), proteracacidins (4′,7,8-OH; found only as a synthetic), promelacacidins (3′,4′,7,8-OH), pro-apigeninidins (4′,5,7-OH), and proluteolinidins (3′,4′5,7-OH). Of these subgroups, procyanidins are the most common [5]. The exemplary condensed tannins are described in Table 10.4.

TABLE 10.1 Examples of Gallotannins

Gallotannin	Structure	Sources	Reference
Acertannin 6-digalloyl-1,5-anhydro-D-glucitol, crystalline compound		*Acer ginnala* Maxim	[2,3]
Hamamelitannin digalloylhamamelose		*Hamamelis, Castanea,* and *Sanguisorba* species	[2,3]
3,4,11-tri-O-galloylbergenin C-glycosidic gallotannin		*Mallotus japonicas* Mull. Arg	[6]
2-O-digalloyl-1,3,4,6-tetra-O-galloyl-b-D-glucopyranose hexagalloylated gallotannin		*Paeonia suffruticosa* Andrews	[7]

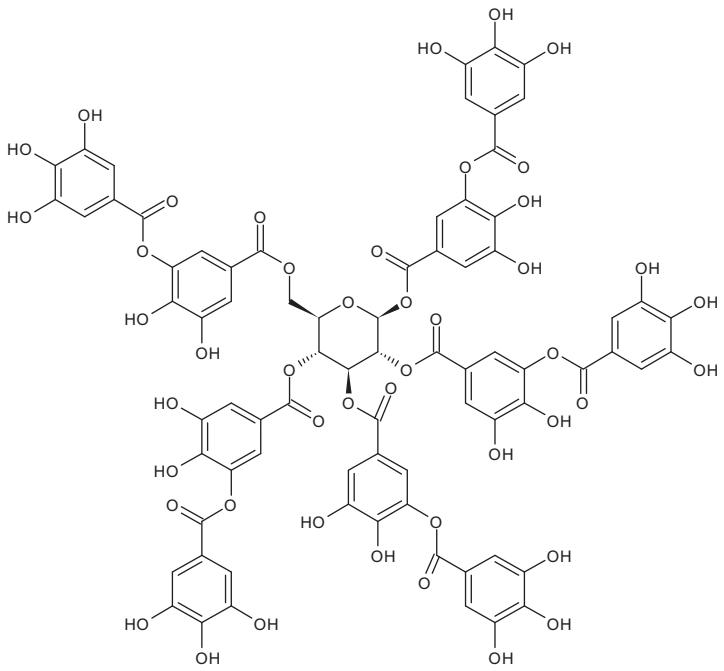


FIGURE 10.3 The chemical structure of *Chinese gallotannin*.

10.2.1 Plants Containing Tannins

High tannin concentrations are found in nearly every part of many plants, such as in the bark, wood, leaves, fruit, roots, plant galls, and seed. In stem tissue, tannins are often found in the growth areas of trees, such as the secondary phloem and xylem and the layer between the cortex and epidermis. Frequently, an increased tannin production can be associated with some sickness of the plant. Therefore, it is assumed that the biological role in the plant of many tannins is related to provision of protection against microbial infection, insect or animal activity [1].Condensed tannins are synthesized and stored inside tannosomes, a chlorophyllous organelle enclosed within tonoplasts in the vacuoles. In these sites, they do not interfere with plant metabolism and do not interact with proteins. Only after cell breakdown and death can they act and engender certain metabolic effects [12]. Typical plant materials containing tannins [2,13–36] are described in Table 10.5.

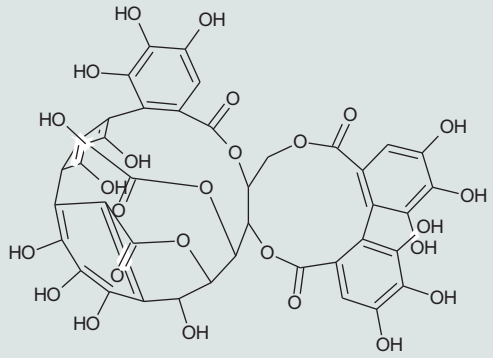
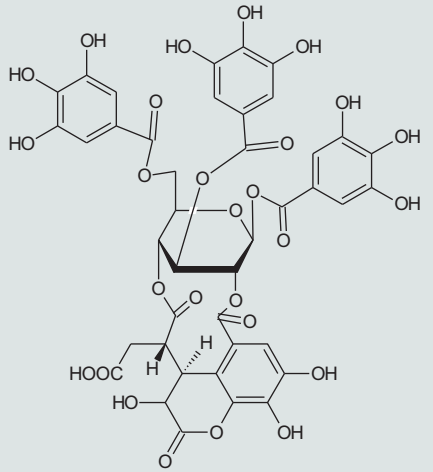
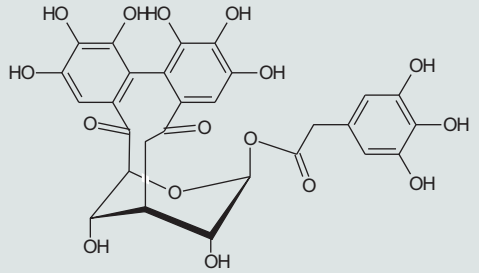
10.3 BIOACTIVITY OF TANNINS

There are a lot of epidemiological data which suggest that tannins are useful in the external treatment of skin inflammation and injuries, and that the intake of tannins may prevent the onset of chronic diseases [37]. The biological effects of tannins have been extensively studied using various in vitro or animal models, however, clinical data on humans is still limited to several plant extracts alone. Tannins may exert their biological effects in two different ways: as *unabsorbables*, these are usually complex structures with binding properties which may produce local effects in the gastrointestinal tract (antioxidant, radical scavenging, antimicrobial, antiviral, antimutagenic, and antinutrient effects), or as *absorbables*, these are usually low molecular weight structures which are easily absorbed, and produce systemic effects in various organs [37].

10.3.1 Bioactivity In vitro

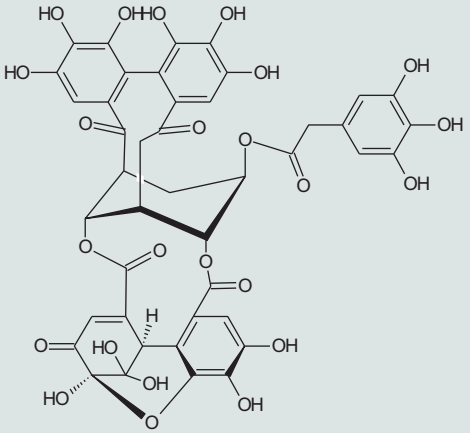
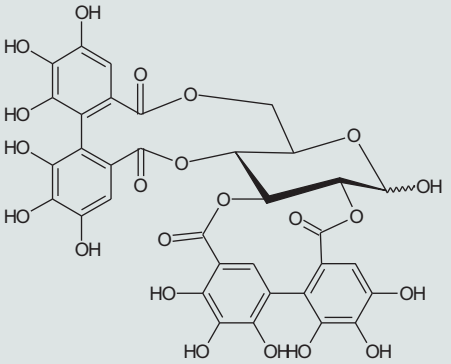
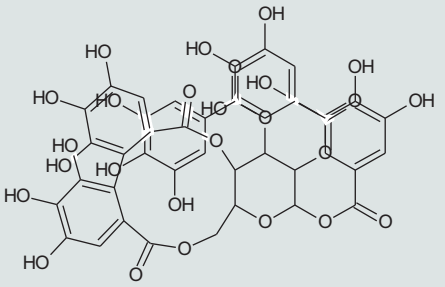
Tannins possess various in vitro bioactivities, among which antioxidant and antimicrobial properties were the most extensively studied. Tannins are known to inhibit lipid peroxidation and to have the ability to scavenge the free radicals that are important in cellular prooxidant states. Most of the activities of tannins, including their free radical-scavenging capacity, largely depend on their structure and degree of polymerization [8,38–40]. What is more, tannins seem to

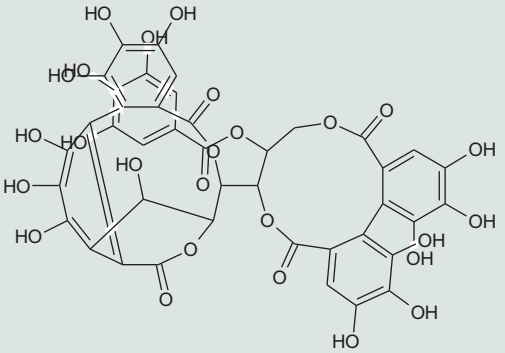
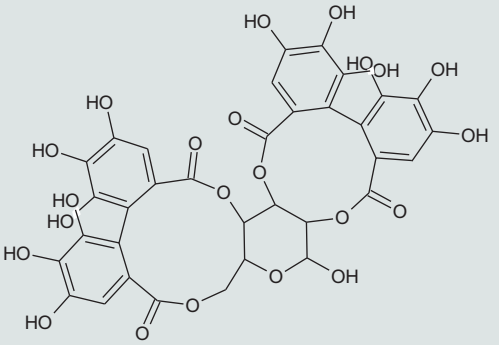
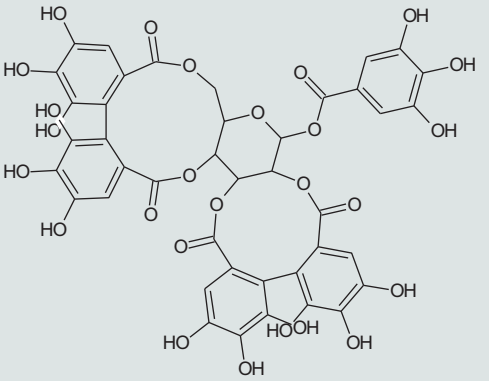
TABLE 10.2 Examples of Ellagitannins

Compound	Structure	Sources	Reference
Monomeric ellagitannins—All compounds are based on cyclization and modification of penta-galloyl-3-D-glucopyranoside [9]			
Castalagin		Found in Oak and chestnut wood; and in the stem barks of *Anogeissus leiocarpus* (DC.) Guill. & Perr and *Terminalia avicennioides* Guill. & Perr	[10]
Chebulinic acid has a polyphenolic group which is regarded as a product of further oxidation of the dehydrohexahydroxydiphenyl (DHHDP) group		*Terminaria chebula* Retz	[2].
Corilagin primary part of the structures of several ellagitannins and dehydroellagitannins; total synthesis of corilagin was reported		*Caesalpinia coriaria* (Jacq.) Wild;*Punica granatum* L.	[2]

(*Continued*)

TABLE 10.2 (Continued)

Compound	Structure	Sources	Reference
Geraniin dehydroellagitannin, crystallizable compound		*Geranium thunbergii* Siebold ex Lindl. & Paxt, in which it accounts for over 10% of dry weight of leaf of the plant; *Geranium*, Hippomaneae, Acalypheae, and Euphorbiaceae species	[10]
Pedunculagin has exclusively two *S*-HHDP groups on the glucose core		*Casuarina* and *Stachyurus* species	[2]
Tellimagrandin II an isomer of punicafolin or nupharin A, but the hexahydroxydiphenoyl group is not attached to the same hydroxyl groups in the glucose molecule		*Geum japonicum* L. *Syzygium aromaticum* (L.) Merrill & Perry	[9]

C-glycosidid ellagitannins Ellagitannins undergo a wide variety of postcoupling modifications, the opening of the glucose ring with formation of a C-glucosidic bond as well as a flavogallonyl moiety. These C-glycosidic compounds are the most abundant ellagitannins found in nature [9,10].			
Casuarinin isomer of casuarictin		Pericarp of *Punica granatum* L., Casuarina ans Stachyurus species, *Alnus sieboldiana* Matsum.	[11]
Pedunculagin formed from casuarictin via the loss of a gallate group		*Casuarina* and *Stachyurus* species	[9]
Potentillin		*Agrimonia japonica* (Miq.) Koidz	[9]
Oligomers and macrocyclic oligomers Often oligomeric ellagitannins are the main components of such plant species as *Geranium thunbergii* Siebold ex Lindl. & Paxt., *Agrimonia pilosa* Ledeb., *Oenothera erythrosepala* Borbás,or *Coriaria japonica* A. Gray. The pharmacological activity of these plants is primarily attributable to these components. Oligomeric ellagitannins are usually accompanied by smaller amounts of the monomers that comprise of dimers and higher oligomers [10].			

(*Continued*)

TABLE 10.2 (Continued)

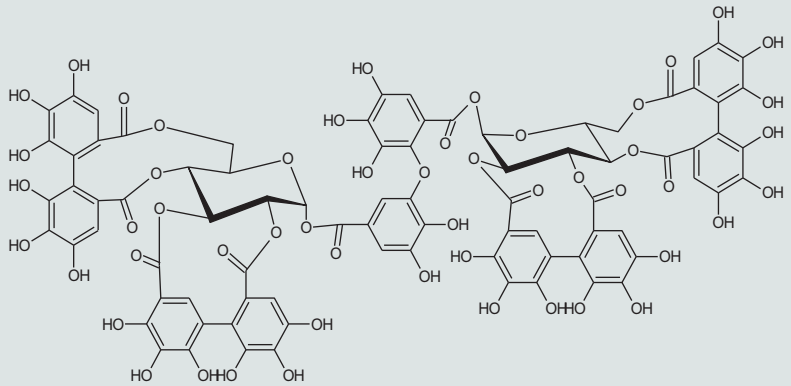
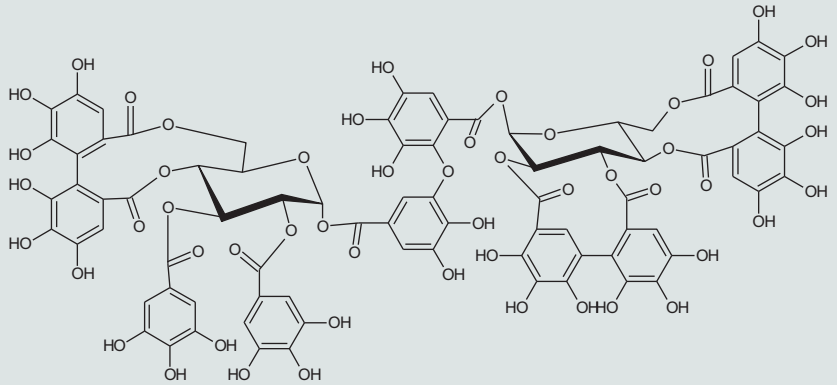
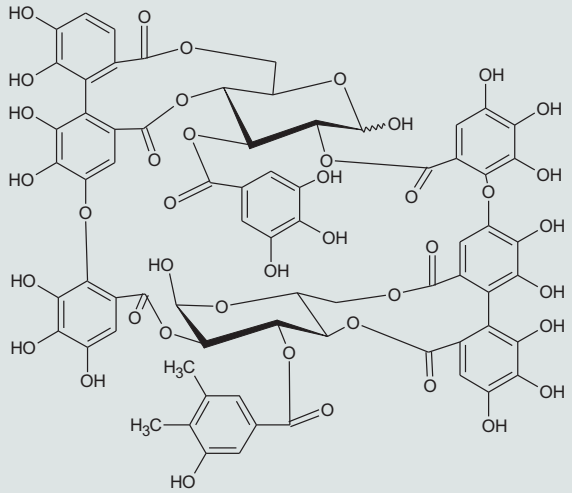
Compound	Structure	Sources	Reference
Agrimoniin first hydrolyzable tannin oligomer identified; dimer having α-glucosidic linkages		*Agrimonia pilosa* Ledeb. *Potentilla kleiniana* Wight & Arnott *Agrimonia, Rosa*, and *Potentilla* species	[2]
Gemin A *dimer*		*Geum japonicum* Thunb	[2]
Oenothein B macrocyclic dimer		*Oenothera erythrosepara* Borbás, *Lythrum anceps* (Koehne) Makino, and *Woodfordia fruticosa* Kurz	[2]

TABLE 10.3 Examples of Complex Tannins

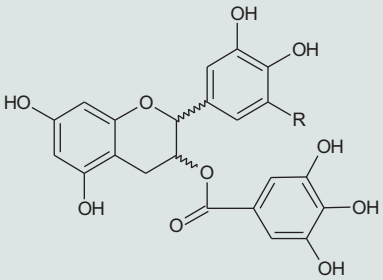
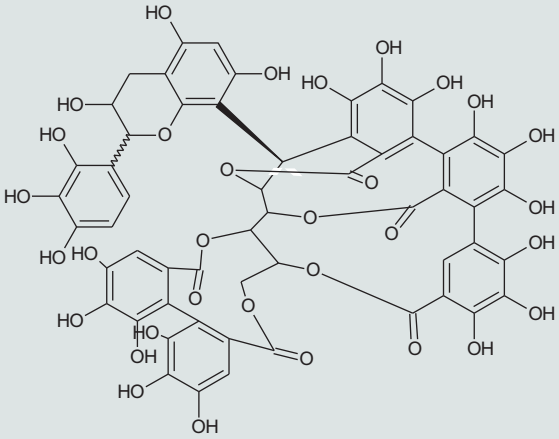
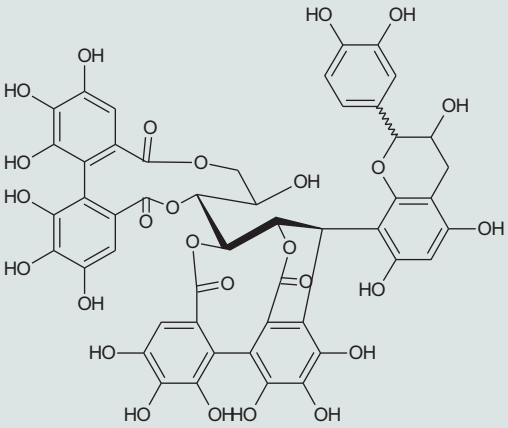
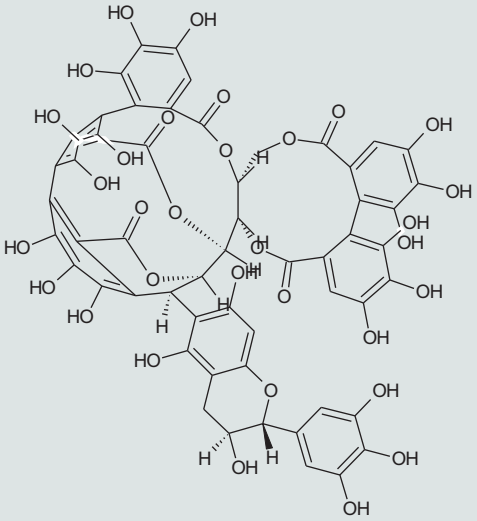
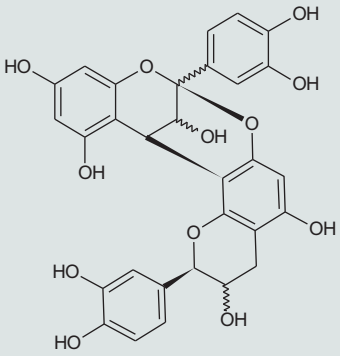
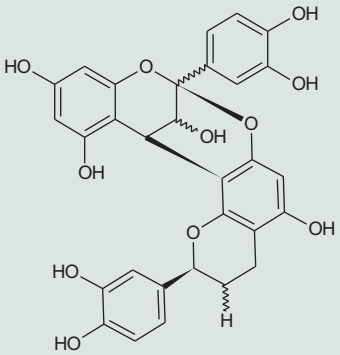
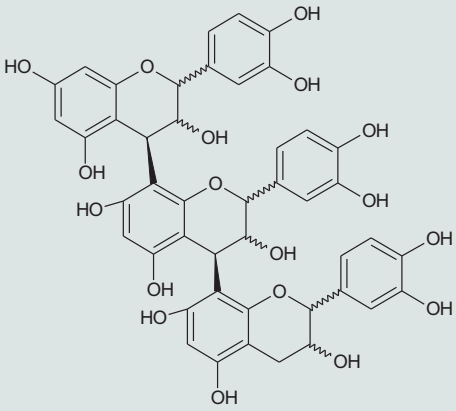
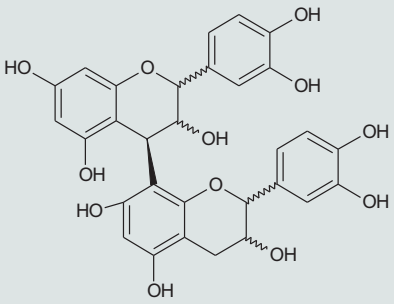
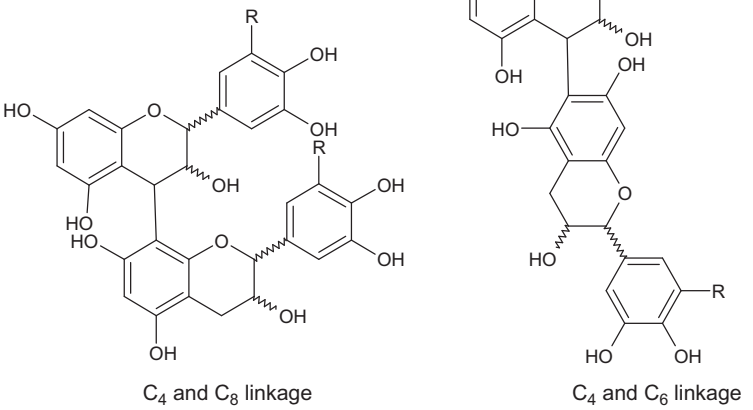
Compound	Structure	Sources	Reference
(−)-Epigallocatechin gallate(EGCG) and *(−)-epicatechin gallate*(ECG) EGCG accompanied by smaller amounts of ECG is the main component in the green tea tannins and largely responsible for the tannin activities of green tea	 Epigallocatechin gallate R = OH Epigcatechin gallate R = H	*Camelia sinsnsis* (L.) Kuntze	[2]
Acutissimin A		*Quercus* species and the bark of *Castanea crenata* Siebold & Zucc.	[1,2]
Camelliatannin A ha a flavan at *C*-1 of an open-chain glucose		*Camellia japonica* L.	[2]
Guajavin B		Bark of *Psidium guujava*L.	[1,2]

TABLE 10.4 Examples of Condensed Tannins

Compound	Structure	Sources	Reference
Proanthocyanidin A1 [epicatechin-(4β 8,2β *O* 7)-catechin],		*Rhododendron spiciferum* Franch, *Urceola micrantha* (Wall. Ex G.Don) D. J. Middleton and peanut skins	[1,5]
Proanthocyanidin A2 [epicatechin-(4β 8,2β *O* 7)-epicatechin]		*Aesculus hippocastanum* L., *Vaccinium oxycoccos* L.	[1,5]
Proanthocyanidin C1 [epicatechin-(4β 8)-epicatechin-(4β 8)-epicatechin)		*Vitis vinifera* L.	[1,5]
Procyanidin B2 [epicatechin-(4β 8)-epicatechin]		*Crataegus monogyna* Jacq, *Vitis vinifera* L., *Cinchona pubescens* Vahl.	[1,5]



C_4 and C_8 linkage

C_4 and C_6 linkage

FIGURE 10.4 Different linkage in polymeric proanthocyanidins.

TABLE 10.5 Plant Species Containing Tannins

Species	Plant Material	Indigenous/Endemic	Tannin Constituents	Indication	Ref.
Acacia katechu (L.) Willd Fabaceae	Bark		Catechins up to 40%: catechin, epicatechin, epicatechin-3-*O*-gallate, and epigallocatechin-3-*O*-gallate,	Cough, diarrhea, topically for skin ulceration	[13]
Acacia nilotica (L.) Willd. ex Delile. Fabaceae	Pod	Native to Egypt, across the Maghreb and Sahel, south to Mozambique and KwaZulu-Natal, South Africa, and east through Arabian Penisula to Pakistan, India, and Burma	Gallocatechin-gallate, methyl gallate, catechin, catechin gallate, galloylglucose, epicatechin	Fever, diarrhea, diabetes, sore gums, and skin diseases.	[14,15]
Agrimonia eupatoria L. Rosaceae	Herb		Catechin; procyanidin B3, procyanidin trimer, agrimoniin	Eye infections, diarrhea, and disorders of gall bladder, liver, and kidneys	[16]
Caesalpinia spinosa (Molina) Kuntze Fabaceae	Pod	Native to Peru	Oligomers of polygallic acid attached by an ester link to quinic acid, gallic acid up to 53%	Inflammation of tonsils; washing the wounds, fevers, colds, and stomach aches, production of gallic acid	[17]
Camellia sinensis (L.) Kuntze Theaceae	Leaves	Native to southeastern Asia – China, Tibet, and northern India	Catechin, epicatechin gallate, epigallocatechin gallate, epigallocatechin	Aiding digestion, blood purification, strengthening teeth and bones, boosting immune system, enhancing heart function, antiviral, lowering blood and sugar levels	[18]

(*Continued*)

TABLE 10.5 (Continued)

Species	Plant Material	Indigenous/Endemic	Tannin Constituents	Indication	Ref.
Diospyros kaki Thunb. Ebenaceae	Fruit	Native to China	Proanthocyanidin oligomers based on catechin, gallocatechin, catechin-3-*O*-gallate, gallocatechin-3-*O*-gallate	Antiseptic, lowering cholesterol, preventing cardiovascular andcerebrovascular diseases	[19]
Geranium thunbergii Siebold ex Lindl. & Paxt. Geraniaceae	Leaves		Geraniin	Intestinal disorders	[2]
Geum japonicum L. Rosaceae	Herb		*O*-galloyl-beta-glucoside, pedunculagin, 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose, tellimagrandin II, 2,6-di-*O*-galloyl-D-glucose, casuariin, and 5-desgalloylstachyurin	Diuretic and astringent, anticoagulant	[20,21]
Hamamelis virginiana L. Hamamelidaceae	Bark and leaves	Native to eastern North America, from Nova Scotia west to Minnesota, and south to central Florida to eastern Texas	Hamamelitannin, pentagalloylglucose, derivatives of epicatechin-(4B→8)-catechin; proanthocyanidins	Swellings, healing of wounds, burns external inflammations, and tumors	[22]
Krameria trianda L. Krameriaceae	Root	Native to the Andes Mountains in Bolivia and Peru.	Tannic acid: rhataniatannic acid, peculiar acid principle: krameric acid, phlobaphene, phloroglucin, oligomeric proanthocyanidins	Chronic diarrhea, dysentery, menorrhagia, incontinence of urine, hematuria, and passive hemorrhage from the bowels; sore throat; astringent wash for the mucous membrane of the eyes, nose, gums	[23]
Mallotus japonicas (L.) Mull. Arg. Euphorbiaceae,	Bark	Native to China	3,4,11-Tri-*O*-galloylbergenin	Gastrointestinal diseases such as gastritis, gastric ulcer, diarrhea, and constipation	[24]
Monochaetum multiflorum(Bonpl.) Naudin Melastomataceae	Leaves	Indigenous to Colombia	Pentameric ellagitannins: melastoflorins A–D oligomeric hydrolyzable tannins: nobotanins Q, R, S, T	Infections and skin injuries	[2,25]
Mouriri pusa Gardn. Melastomataceae	Leaves	Brazil	Catechins and condensed tannins	Gastritis and ulcers	[26]
Phyllanthus muellerianus (Kuntze) Exell Phyllanthaceae	Leaves stem bark	Western Africa	Geraniin, phenazine derivative of geraniin, corilagin, furosin.	Old, deep and chronic wounds as well as for boils and skin eruptions	[27]

(*Continued*)

TABLE 10.5 (Continued)

Species	Plant Material	Indigenous/Endemic	Tannin Constituents	Indication	Ref.
Potentilla erecta (L.) Rauschal Rosaceae	Roots	Central Europe, Italy, Sweden, Serbia and Montenegro, Russia, Bulgaria, Turkey	Pentadigalloylglucose, pedunculagin, agrimoniin, epigallocatechin, catechins, their dimers and trimers, proanthocyanidins	Inflammations, treatment of wounds, bleeding, dysentery, diarrhea, inflammatory bowel disease, bacterial, fungal, and viral infections, certain forms of cancer, antiseptic for the mouth and throat	[28]
Potentilla kleiniana Wight & Arnott Rosaceae	Aerial parts	China, Korea, Japan, Nepal, India	Agrimoniin, potentillin	Diarrhea, bleeding, influenza, cough, parotitis, lymphadenitis, hepatitis, scare, numbness of limbs, dysmenorrhea, ulcer	[28,29]
Quercus infectoria Oliv. Fagaceae	Gall (Turkish gall)		Tannic acid	Astringent, inflammation, local anesthetic, bacterial, fungal and viral infections,	[30]
Quercus robur L. Fagaceae	Bark	Native to Ireland	Grandinin, castalagin, glucogallin	Diarrhea, minor inflammation of the oral mucosa or skin, itching and burning associated with hemorrhoids	[31]
Rhus chinensis Mill. Anacardiaceae	Gall (Chinese gall)	Native to China and Southeast Asia	Structures containing 1 to 14 galloyl residues, yielding tri-, tetra-, penta-, hepta- and nonagalloylglucose up to 70 %, gallic acid and derivatives: 3-galloyl-gallic acid and 4-galloyl-gallic acid	Antiseptic, astringent, and hemostatic. chronic diarrhea, spontaneous sweating, night sweats, externally to burns, bleeding due to traumatic injuries, hemorrhoids, and ulcers in the mouth	[32]
Sanguisorba officinalis L. Rosaceae	Root	Native throughout northern Europe, northern Asia, and northern North America	Sanguiin H-6	Bloody dysentery, nosebleeds, burns, and insect bites	[33]
Syzygium cumini	Bark	Native from Africa to Madagascar	Corilagin and related ellagitannins	Sore throat, bronchitis, asthma, thirst, biliousness, dysentery, and ulcers	[34]
Terminalia chebula Retz. Combretaceae	Fruit		2,4-Chebulyl-β-D-glucopyranose, chebulinic acid, punicalagin, terflavin A, terchebin, tannic acid	Cold-related nagging coughs, ulcer	[35,36]

affect bacterial growth by way of several mechanisms. These are indirect, by way of the inhibition of extracellular microbial enzymes, as well as the deprivation of the substrates required for microbial growth. These can also be direct, by way of action upon the microbial metabolism through the inhibition of oxidative phosphorylation [2,5,37,41–51]. Antiviral activity is mainly via inhibition of virus absorption [2,52–56].

Other bioactivities include cardioprotective activity, histamine release inhibition, and cytotoxic activity. Tannins have been seen to have cardioprotective activity via induced stabilization of pericardial tissue, the inhibition of enzymatic degradation of elastin, and the reduction of the calcification of the glutaraldehyde-fixed aortic wall [57–63]. In conducted research, histamine release inhibition was described for HT, and found to be dependent on the number and composition of galloyl groups, HHDP groups and their analogs, but not simply on the number of phenolic hydroxyl groups present [64]. With regard to cytotoxic activity, procyanidins have been reported to be more cytotoxic than monomer flavanols in studies utilizing a variety of human cancer cell lines. Many studies which have compared the effectiveness of various monomer flavanols, have indicated that the presence of a galloyl residue on the 3 position on the C-ring enhances the cytotoxicity of these compounds [8,37,64–68].

Tannins also exhibit antidiabetic and antiobesity bioactivities. Tannins possess antidiabetic potential firstly due to their ability to lower blood glucose levels by delaying intestinal glucose absorption, and by inducing an insulin-like effect on insulin-sensitive tissues, and, secondly, by delaying the onset of insulin-dependent diabetes mellitus by regulating the antioxidant environment of pancreatic β-cells [37]. The lowering of glucose levels can be also be brought about through the inhibition of α-amylase, as well as by α-glucosidase activity. It has been suggested that the interaction between tannins and the human α-amylase depends on the possessed free hydroxyl groups that are able to participate in hydrogen bonding [69]. Tannins have also been shown to effectively inhibit intestinal α-glucosidase activity. In so doing, K_i values are seen to be in the same range as the synthetic inhibitors (acarbose and voglibose), which are already being used therapeutically to control noninsulin-dependent diabetes mellitus [70]. The extent of inhibition of α-glucosidase is related to the proanthocyanidin content, while α-amylase inhibition is induced by the presence of HT [71]. It has recently been suggested that tannins hold an insulin-like effect on insulin-sensitive tissues. These have been shown to act on cells by modifying or interacting with certain specific proteins found within important intracellular signaling pathways, and, hence, these affect their role in improving hyperglycemia. Of note, grape seed procyanidin extracts have exhibited insulinomimetic properties [37,72]. Furthermore, laboratory studies indicate that the antiobesity effects of polyphenol-rich diets may be attributed to the ability of polyphenols to interact, directly or indirectly, with adipose tissues (preadipocytes, adipose stem cells, and immune cells) [73–79].

Tannins are known for anti-inflammatory and wound- healing properties and many laboratory tests confirmed that they have the ability to inhibit hyaluronidase and elastase enzymes [80–83]. Among other activities of tannins, facilitation of pepsin digestion may be mentioned [84]. The detailed activity of tannins is described in Table 10.6.

10.3.2 Bioactivity In vivo

In most conducted in vivo experiments, extracts, juices, or fruits with described activity that is known or thought to be beneficial to health are mainly used [2,8,26,37,86–91]. The most studied activities are described in Table 10.7.

10.4 CLINICAL TRIALS

As shown in the previous sections, tannins have been widely investigated in order to ascertain if they have beneficial effects on human health. Pure tannins, as well as extracts and beverages incorporating tannins, have been subjected to several clinical trials. In such work, the influence of tannins on metabolic syndrome, cardiovascular disease, plasma lipid profiles, and inflammation have been studied, and some of these trials have demonstrated positive correlations, whereas others have produced nonsignificant findings [92–105]. The summary of clinical trials and meta-analysis of these trials is presented in Table 10.8.

10.5 EXTRACTION PROCESSES

The solvents used to extract HT from natural tissues are aqueous solutions of methanol, ethanol, or acetone, as well as ethyl acetate. Nonpolar organic solvents (*n*-hexane, petroleum ether) and solvents with low extraction strength (chloroform, dichloromethane) are usually used in sample pretreatment to remove lipids and chlorophyll and/or to prevent enzymatic reactions [3]. Of note, methanol treatment tends to be better for handling low molecular weight tannins or in the processing of matrices containing large amounts of enzymes (i.e., bark or fruit), while the use of acetone is preferred

TABLE 10.6 In vitro Bioactivity of Tannins

Tannin classification	Tannins	Bioactivity in vitro	Reference
Antioxidant Activity			
Hydrolyzable tannin	Geraniin	Potent inhibition of lipid peroxidation in the cell membranes of mouse eye lens caused by the xanthin–xanthine oxidase	[8]
Hydrolyzable tannin	Pentagalloylglucose	Potent inhibition of lipid peroxidation in the cell membranes of mouse eye lens caused by the xanthin–xanthine oxidase	[8]
Hydrolyzable tannins	Chebulinic acid, geraniin isoterchebin, mallotusinic acid, pedunculagin, pentagalloylglucose, tellimagrandins I and II,	Potent scavenging effects on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical	[8]
Ellagitannin	Pedunculagin, possessing two hexahydroxydiphenyl groups	Potent inhibition of lipid peroxidation in rat liver mitochondria stimulated by adenine 50-diphosphate and ascorbic acid	[8]
Complex tannin	Epigallocatechin gallate	Potent inhibition of lipid peroxidation in rat liver mitochondria stimulated by adenine 50-diphosphate and ascorbic acid	[8]
Condensed	Procyanidins B1 and B3	Exhibit stronger protection of linoleic acid in aqueous systems than ascorbic acid or a-tocopherol; antiradical power is observed with an increase in the degree of polymerization up to seven	[38,39]
Highly condensed	Kaki-Tannin	Significant inhibition of the auto-peroxidation of lipids in the liver homogenates from healthy mice Significant inhibition of the auto-peroxidation of lipids caused with either H_2O_2or Fe^{2+}/ ascorbic acid in liver homogenate	[40]
Antibacterial Activity			
Gallotannins		Inhibitors of water-insoluble glucan synthesis leading to bactericidal effects on *Streptococcus mutans*, *S. Salivarius*, and *Actinomyces viscosus*	[42,43]
Ellagitannins		Inhibitors of *Staphylococcus* bacteria, *Candida albicans*, and *Campylobacter jejuni*	[37]
Macrocyclic ellagitannin dimer	Oenothein B	Suppression in antibiotic resistance of MRSA	[2,44]
Hydrolyzable tannins and acid-treated hydrolyzable tannins		Promising narrow spectrum activity against *H. Pylori* with no influence on *E. coli*	[45]
Hydrolyzable tannins	Tellimagrandin I, rugosin B and corilagin	Noticeable reduction in MIC values of oxacillin for the methicillin-resistant *Staphylococcus aureus* (MRSA) strain	[2,44]
Complex tannins	(–)-Epigallocatechin gallate (–)-Epicatechin gallate	Antibacterial activity against *Helicobacterpylori* at 100 μg/mLconcentration level	[49]
Complex tannins	Catechin	Dose- and time-dependent growth inhibitory effect (as measured either in liquid culture medium or in semisolid agar plates) in *H. Pylori* and *Escherichia coli*	[46,47]

(*Continued*)

TABLE 10.6 (Continued)

Tannin classification	Tannins	Bioactivity in vitro	Reference
Complex tannins	(+)-Catechin	Inhibition of growth of *Clostridium histolyticum* and enhancement of growth of *E. coli* and members of the *Clostridium coccoides—Eubacterium rectale* group. The growth of *Bifidobacterium* and *Lactobacillus* spp. Remained relatively unaffected	[48]
Complex tannin	Theasinensin A (−)-Epicatechin gallate	Noticeable reduction in MIC values of oxacillin for the methicillin-resistant *Staphylococcus aureus* (MRSA) strain	[2,44]
Complex tannins	Theasinensin A	Reduction in MIC values of penicillin G and ampicillin as well as an aminoglycoside antibiotic streptomycin for the MRSA strain	[2,44]
Proanthocyanidins	Procyanidins B3 and B4	Reduction in MIC values of penicillin G and ampicillin as well as an aminoglycoside antibiotic streptomycin for the MRSA strain	[2,44]
Proanthocyanidins with unique molecular structure characterized by a repetition of catechin unit with one or more A-type linkages		Inhibition of *E. coli* adherence to human uroepithelium	[50,51]
Antiviral Activity			
Hydrolyzable tannins		Inhibition of *Herpes simplex* virus absorption. The antiherpetic activities of hydrolyzable tannins were dependent on the number of galloyl or hexahydroxydiphenoyl groups	[52,53]
Hydrolyzable tannins	Chebulagic acid and punicalagin	Effective in abrogating infection by human cytomegalovirus (hcmv), hepatitis c virus (hcv), dengue virus (denv), measles virus (mv), and respiratory syncytial virus (rsv), at micromolar concentrations and in dose-dependent manners without significant cytotoxicity	[54]
Dimeric ellagitannins	Oenothein B, coriariin A, and agrimoniin	Potent antihuman immune-deficiency virus (HIV) activity least partly mediated by adsorption inhibition (binding to components of the viral envelope)	[2,55]
Ellagitannin	Casuarinin	Antiherpesvirus activity in inhibiting viral attachment to cells and viral penetration, and also disturbing the late event(s) of infection.	[56]
Galloylated condensed tannins		Inhibition of *Herpes simplex* virus absorption; the antiherpetic activities of condensed tannins increased with the degree of condensation	[52,53]
Cardioprotective Activity			
Hydrolyzable tannins		Anti-ischemic activity and an endothelium-dependent vasorelaxant effect through the interplay of different factors such as cyclooxygenase pathway activation, TNF-alpha inhibition, endothelial nitric oxide synthase activation, and scavenging of free radical and reactive oxygen species	[57]

(*Continued*)

TABLE 10.6 (Continued)

Tannin classification	Tannins	Bioactivity in vitro	Reference
Hydrolyzable tannins	Purified tannins of *Geum japonicum*	Caused NO- and cGMP-mediated potent vasorelaxation in rat aortic rings that had been precontracted with the α1-adrenergic receptor agonist phenylephrine	[58]
Hydrolyzable tannins	Gallic acid, digallic acid, praecoxin, 1-desgalloyl rugosin F	Inhibitory effect on propranolol-induced negative inotropism; the galloyl group in the tannin structure is crucial for the negative inotropic action	[59]
Gallotannin	Tannic acid	Relaxed precontracted human coronary arteries and rat aortic rings in an endothelium and nondependent manner, accompanied by an increase in vascular cGMP levels	[60]
Condensed tannins		Dependently relaxed the norepinephrine precontracted vessels in rabbit pulmonary artery presumably via formation of endothelium-derived relaxing factor	[61]
Proanthocyanins		Long-lasting antihypertensive and vasorelaxing properties linked to endothelium-related factors in which nitric oxide is involved; Significant effect in the protection of heart against myocardial infarction induced by isoproterenol	[62,63]
Histamine Release Inhibition			
Monomeric hydrolyzable tannin	Geraniin	Significant inhibition of $k0_2$-induced histamine release	[64]
Dimeric hydrolyzable tannins	Agrimoniin, euphorbin c, and oenothein b	Showed the most potent inhibition of $k0_2$-induced histamine release	[64]
Cytotoxic Activity			
Hydrolyzable tannins	Geraniin and corilagin	Inhibition of the release of tumor necrosis factor-α (tnf-α)	[8]
Hydrolyzable tannins	Vescalagin, acutissimin A/B, epiacutissimin A/B, grandinin/ roburin E, hexagalloyl glucose and heptagalloyl glucose	Inhibition of α-amylase in the in vitro human starch digestion model; the type of enzyme inhibition was mixed non-competitive; inhibitory activity was significantly enhanced in the absence of proteins in the food matrix	[65]
Complex tannins	(+)-Gallocatechin-3-*O*-gallate (−)-epicatechin-3-*O*-gallate (−)-epigallocatechin-3-*O*-gallate (−)-epicatechin (0)-epigallocatechin (+)-catechin	In vitro and in silico inhibition of human pancreatic α-amylase Inhibit the growth of cancer cells; anticancer activity is likely resulted from the antioxidant activity and the direct binding to proteins, resulting in the modulation of multiple cellular signaling pathways	[66,67]
Proanthocyanidins		Effectively suppressed the epidermal growth factor receptor phosphorylation, inhibiting the growth of human colon carcinoma cell line ht29	[37]

(*Continued*)

TABLE 10.6 (Continued)

Tannin classification	Tannins	Bioactivity in vitro	Reference
Proanthocyanidins	Procyanidins b1 and b2	Reduction in cell number and an inhibition of cell proliferation in a variety of human cancer cell lines	[68]
Antiobesity Effects			
Hydrolyzable tannins	1,2,3,4,6-penta-*o*-galloyl-*b*-D-glucopyranose	Stimulation of glucose transport in adipocytes by direct binding to the insulin receptor and displacement of insulin from the insulin-binding site	[79]
Complex tannins	(−)-Epigallocatechin galllate (−)-epicatechin (−)-epigallocatechin	Induction of dose- and time-dependent decrease in adipocyte viability cell cycle arrest at the G0/G1 phase Induction of apoptosis in murine preadipocyte and mature adipocytes and increase of caspase-3 activity	[73–76]
Complex tannins	(−)-Epigallocatechin galllate	Induction of G2/M growth arrest in a dose-dependent manner in mature adipocytes Inhibition of preadipocyte differentiation and cellular triglyceride accumulation in adipocytes in a dose- and time-dependent manner	[77,78]
Anti-inflammatory and Wound-Healing Properties			
Gallotannins	Pentagalloylglucose Trigalloyl-glucose	Wound-healing properties	[81,82]
Gallotannins	Tannic acid	Prevents collagen matrix degradation by cross-linking fibrous collagen and inhibiting matrix metalloproteinase activity	[83]
Ellagitannins	Geraniin, corilagin, furosin	Strong wound-healing properties (stimulating effects against human keratinocytes and dermal fibroblasts)	[85]
Other Activities			
Catechins		Facilitation of pepsin digestion of major food allergens	[84]

for dealing with high molecular weight tannins and because it is less liable to react with them [106,107]. Furthermore, high temperatures for extended times may cause hydrolysis of the galloyl moiety attached to the glucose anomeric C-1 position and can also release ellagic acid from ellagitannins [107]. In addition, extraction with ethanol and/or methanol may produce ethyl or methyl esters of gallic acid, respectively [106,107]. Beyond the choice of extraction solvent, factors such as pH, temperature, solvent to material ratio, and the number and time intervals of individual extraction steps play an important role in the tannins separation. The following extraction types have been applied in tannin processing:

- Classic extraction by way of boiling solvent—the optimal conditions for extracting Assam green tea polyphenols are a water ratio of 1:20, at pH 4 and 5, utilizing fresh green tea leaves [108].
- Pressurized liquid extraction—with the solvent at an elevated temperature (usually between 50 and 200°C) and under higher pressure (between 10 and 15 Mpa). Herein, the best recoveries were obtained by way of employing this technique for releasing catechin and epicatechin from grape seeds, as well as from nonfermented tea leaves, medium-fermented tea leaves, and fermented tea leaves. Such release comes about within 10 min [109].
- Supercritical carbon dioxide extraction—using supercritical carbon dioxide (SC-CO_2) with critical point at 31.1°C and a state of pressure of 73.8 bar. Carbon dioxide is not normally a suitable solvent for extracting polyphenols/flavonoids because of its nonpolar nature, hence, to overcome this problem, a polar organic solvent such as a solution

TABLE 10.7 In Vivo Bioactivity of Tannins

Tannins	Bioactivity In vitro	Reference
Antiulcer Activity		
Tannins from *Mouriri pusa* Gardner ex Gardner	Cytoprotective and cicatrizing effect on gastric ulcers in rats after a 14-day treatment with 25 mg/kg of tannin fraction	[26]
Antioxidant Activity		
Tannin-rich cocoa	Protection ofliver injury caused by arsenic after 4 weeks of oral gavage administration of 30–300 mg/kg tannin-rich cocoa	[86]
Cocoa extracts	Antioxidant effects in the obese-diabetic rats during the 4 weeks supplementation of cocoa extracts (600 mg/kg body weight/day)	[87]
Antidiabetic Activity		
Syzygium cumini (L.) Skeels. Bark extracts	Reduction of blood glucose levels and increase of plasma insulin and C-peptide levels of both normal and diabetic rats after 45 days oral administration of extract in the dose 300 mg/kg of body weight	[88]
Kaki-tannin (a highly polymerized tannin composed mainly of epicatechin, epigallocatechin, epicatechin-3-*O*-gallate and epigallocatechin-3-*O*-gallate)	Prevention of rise in plasma total cholesterol, non-HDL cholesterol, triglycerides, and insulin in type 2 diabetic mice fed high fat diet; induction of genes related to cholesterol metabolism	[89]
Anti-inflammatory Activity		
Corilagin	Reduction of bleomycin-induced lung fibrosis number of apoptotic lung cells and prevention of lung epithelial cells from membrane breakdown after intraperitoneal administration of 10 and 100 mg/kg of corilagin	[90]
Protecting Hematopoietic Stem Cells of Bone Marrow		
Tannins from *Sanguisorba* root	Protection and treatment of cyclophosphamide-induced myelosuppression in mice after 10 days administration of extract by oral gavage at the dose of 20 mg/kg	[91]
Inhibition of Skin-Tumor Promotion		
1,2,3,4,6-penta-*O*-galloyl-β-D-glucose	Reduction of percentage of tumor-bearing mice to about 50%, and the average number of tumors per mouse to ca. 33% in a two-stage skin carcinogenesis assay using DMBA plus teleocidin	[8]
Tenophyllanin A and alienanin B and C	Antitumor promoting activity in the two stage mouse skin carcinogenesis assay with DMBA and 12-*O*-tetradecanoylphorbol-13-acetate	[8]
Epigallocatechin gallate	Reduction of number of the tumor bearing mice treated with 7,12-dimethylbenzo[a]anthracene (dmba) (a tumor initiator) plus teleocidin (a tumor promoter)	[8]
Tumor Inhibition in the Gastrointestinal Tract		
(−)-Epigallocatechin galllate	Reduction of number of the tumor bearing mice to less than one-third of the control group in a model system of mouse duodenal carcinogenesis with *n*-ethyl-*n*-nitro-*n*-nitrosoguanidine	[8]
Apple proanthocyanidins and red wine proanthocyanidins	Inhibition of tumor promotion in rats with azoxymethane-induced colon carcinomas	[37]
Host-Mediated Antitumor Activity		
Ellagitannin macrocyclic oligomers such as oenotheins A and B, camelliin B and woodfordins C, D, E, and F	Induction of the immune response of host animals, as shown by stimulation of interleukin 1 (IL-1) production achieved by tannins administration either before or after intraperitoneal inoculation of tumor cells	[2,8]

TABLE 10.8 A Summary of Clinical Trials of Tannins and Meta Analysis of These

Tannins	Clinical Trial/Meta-Analysis	Effects	Reference
Green tea	14 Healthy persons (mean age 30 y) 6-g Dose of green tea or a 125-mg dose of caffeine	Flow-mediated dilation of the brachial artery, a predictor of cardiovascular disease, was improved with green tea but not with caffeine alone	[92]
	32 Obese individuals (average BMI 32.2 kg/m^2) 625 mg of green tea catechins daily for 3 months	Subjects drinking the green tea extract did not lose more weight, but they had slightly lower triacylglycerol levels	[93]
	60 Subjects from 32 to 73 years of age who had serum hyperglycemia Tea with 544 mg/day of polyphenols for 2 months	The individuals who drank the tea showed a modest but significant lowering of glycated hemoglobin	[94]
	35 Individuals (average age 42.5 y) with an average BMI of 36 kg/m^2 Green tea in bags (four cups a day), green tea extract capsules (two a day, total 800 mg of cathechins) for 2 months	There were no differences in parameters of the metabolic syndrome and pro-inflammatory cytokine levels among groups	[95]
	Meta-analysis of six studies conducted outside Japan and eight studies conducted in Japan	Studies conducted outside Japan showed a mean difference (MD) in weight loss of −0.04 kg (532 participants) and MD in BMI of −0.2 kg/m^2 (222 participants), while	[96]
	Study length ranged between 12 and 13 weeks	Studies conducted in Japan showed MD in weight loss from −0.2 kg to −3.5 kg (1030 participants) and reduction in BMI ranging from no effect to −1.3 kg/m^2 (1030 participants) in favor of green tea preparations over control	
Grape seed products	27 individuals 25−80 years old with the metabolic syndrome 150−300 mg/day of grape seed extract for 1 month	A significant decrease in systolic and diastolic blood pressures in subjects taking either dose of grape seed extract. The higher was the baseline of oxidized ldl, the greater the decrease in oxidized ldl with a 300-mg dose	[97]
	44 women (premenopausal, mean age 39.7 y, and postmenopausal, mean age 58.5 y) 36 g/day of a 182-g lyophilized grape powder per day (equivalent of 1.5 cups/day of grapes) for 1 month	Pre- and postmenopausal women who drank a grape beverage showed lower triacylglycerols by 15% and 6%, respectively. There were no changes in the cytokines TNF-α, c-reactive protein, or IL-6	[98]
Polyphenol-rich cocoa	8 Patients with coronary artery disease Cocoa drinks containing a 375-mg dose of cocoa polyphenols twice a day for 1 month	Subjects brachial arteries had an improved vasodilatory capacity, and they were able to produce larger numbers of endothelial progenitor cells; the systolic blood pressure was decreased	[99]
	Meta-analyses of human trials	Induction of slight decrease in total cholesterol and LDL and recustion of systolic blood pressure by 4.5 mmHg and diastolic blood pressure by 2.5 mmHg	[100,101]
Cranberry products	Meta-analyses of data of all randomized controlled trials (RCTS) or quasi-RCTS	Cranberry products did not significantly reduce the occurrence of symptomatic urinary tract infections overall or for any the subgroups;	[102,103]
	Women with recurrent urinary tract infections; older people; pregnant women; children with recurrent urinary tract infections; cancer patients; or people with neuropathic bladder or spinal injury.	The effectiveness of cranberry was not significantly different to antibiotics for women and children.	

(*Continued*)

TABLE 10.8 (Continued)

Tannins	Clinical Trial/Meta-Analysis	Effects	Reference
Hawthorn extract	Meta-analysis of 14 clinical trials Ten trials including 855 patients with chronic heart failure (New York heart association classes i to iii) provided data that were suitable for meta-analysis	Tolerance were significantly increased by hawthorn extract. The pressure-heart rate product, an index of cardiac oxygen consumption, also showed a beneficial decrease with hawthorn treatment. Symptoms such as shortness of breath and fatigue improved significantly with hawthorn treatment	[104]
Pycnogenol®	Meta analysis. 15 trials with a total of 791 participants Asthma (two studies; $n = 86$), attention deficit hyperactivity disorder (one study; $n = 61$), chronic venous insufficiency (two studies; $n = 60$), diabetes mellitus (four studies; $n = 201$), erectile dysfunction (one study; $n = 21$), hypertension (two studies; $n = 69$) and osteoarthritis (three studies; $n = 293$	No definitive conclusions regarding the efficacy or safety of pycnogenol® were possible	[105]

of ethanol and water, can be used during extraction or prior to the extraction [110]. Ethanol as a cosolvent was successfully utilized in the extraction of green tea catechins [111,112].

- Microwave-assisted extraction with solvent directly heated by microwaves—the optimal performance of tea polyphenols extraction was obtained under a microwave intensity of 600 W, a microwave radiation time of 3 min, and a one time microwave radiate action, with a tea/water ratio of 1:20 [113].
- Ultrasound/sonication-assisted extraction—this method demonstrates excellent solvent penetration into cellular materials. In this process, optimal extraction conditions for several green tea HT came about by way of treatment with 19.7 % ethanol, for 26.4 min, at 24.0°C [114]. Of note, for deriving catechins from commercial Chinese tea samples, the novel dynamic ultrasound-assisted extraction method was found to be more effective than static ultrasound-assisted extraction [115].

10.6 CHEMICAL TESTS

The physiological and pharmacological interactions of tannins are, in principle, directly derived from the physical and chemical properties of the polyphenolic skeleton [5]. Tannins appear as light yellow or white amorphous powders or shiny, nearly colorless, loose masses, with a characteristic strange smell and astringent taste. Tannins have the ability to form complexes, both with metal ions and with macromolecules such as proteins and polysaccharides. In their extraction, complexes with proteins and metals precipitate in the solution. The catechins possessing ester binding properties have a greater ability to form precipitates with enzymes than do simple HT. This leads to the formulation of a cream [116] and the quantification of tannins is based on their binding activity referred to above. The classical hide-powder method is based on the binding with animal skin protein, and the relative astringency and relative affinity to methylene blue determinations are based on the binding with blood and methylene blue, respectively, under controlled pH. These properties of tannins are based on their chemical structures (which have two or three phenolic hydroxyl groups on a phenyl ring, in a molecule of moderately large size) [2]. The determination of type or degree of tannins present in a material is mainly based on colorimetric assays (Folin−Ciocalteu method, hide-powder method, vanillin−HCl method, butanol−HCl method, rhodanine assay, and Wilson and Hagerman assays), as well as by precipitation assays (PEG binding assay).

10.6.1 Colorimetric Assays

10.6.1.1 Folin−Ciocalteau Method

This reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid, which, after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced has a

maximum absorption in the region of 750 nm, and is proportional to the total quantity of phenolic compounds originally present. The method determines the total free phenolic groups, and is, therefore, a method suitable for determining *total soluble phenolics* (either HT and PA). Interfering compounds such as ascorbic acid, tyrosine, and possibly glucose are also measured [117].

10.6.1.2 Hide-Powder Method

This assay is based on the precipitation of tannins within a solution through binding with hide powder. The difference in the total polyphenols content measured with Folin–Ciocalteau reagent and amount of polyphenols seen after the tannin precipitation gives the yield of tannins from an examined material [118,119].

10.6.1.3 Vanillin–HCl Assay

This assay is specific for the analysis of condensed tannins. Vanillin reacts with the metasubstituted A-ring of flavanols to form a chromophore. The number of flavanols is proportional to the absorbance of the solution; however, low molecular weight flavanols overreact and large polymers underreact [117].

10.6.1.4 Butanol–HCl Assay

This assay is specific for ascertaining the presence of condensed tannins, and it involves the HCl catalyzed depolymerization of tannins in butanol so as to yield a red anthocyanidin product that can be detected spectrophotometrically. Of note, the tannins cleaved into dimers or trimers are underestimated. The degree of polymerization of the PAs can be estimated by combining the butanol–HCl assay with the vanillin assay. The acid butanol assay measures the total number of flavanoid residues present, and the vanillin assay measures the number of molecules. The butanol–HCl assay is also used to estimate the amount of insoluble tannins found within extraction residues [117].

10.6.1.5 Rhodanine Assay and Wilson and Hagerman Assay

The first assay is specific toward establishing gallotannins content, while the second one is specific for estimating ellagitannins presence. The sample is subjected to hydrolysis to release gallic acid or ellagic acid, respectively. The reaction between gallic acid and the dye rhodanine and between ellagic acid and the sodium nitrite produce an intense color that is measured spectrophotometrically [117].

10.6.2 Precipitation Assay

10.6.2.1 PEG Binding Assay

This assay identifies the tannins found within plant samples containing strong tannins–protein complexes in which extraction gives low tannins yields. Polyethylene glycol 4000 (PEG) has a greater affinity for tannins than for proteins, and it binds to a wide range of hydrolyzable and condensed tannins. The PEG–tannins complexes are stable over a pH range between 2 and 8.5, and are insoluble in boiling water, neutral and acid detergents, as well as in many organic solvents [120].

10.7 SPECTROSCOPIC DETERMINATIONS

The majority of tannins are beyond the range of gas chromatography (GC) analysis (unless they are derivatized), and high-performance liquid chromatography (HPLC) is necessary for the determination of complex tannin extracts. The coupling of HPLC with electron ionization (EI) and mass spectrometry (MS) gives researchers a powerful tool in the analysis of tannins from crude and purified extracts. In LC/EI/MS, effluent from an HPLC is introduced into an EI source, giving typical EI spectra that can be searched for within a common mass spectral library. Each method yields different fragmentation patterns, but in combination, provide additional information of a complementary nature for structural elucidation [121]. Hydrolyzable and complex tannins are usually easily determined; however, condensed

TABLE 10.9 Examples of Tannins Determined by Mass Spectrometry

Plant Material	Spectrometer	Identified Compounds	Reference
Rubus loganbaccus x baileyanus Britt.	ESI-IT-MS	Sanguiin H-10 (an isomer); Sanguiin H-6; Sanguiin H-2	Kool et al., 2010
	MALDI-TOF-MS	Galloyl−sanguiin H-6 (possible artefact of lambertianin C)	
Litchi chinensis Sonn.	ESI-Qe-FT-ICR-MS	A-series procyanidins from monomers to pentamers and B-series procyanidins including dimers and trimers	Li et al., 2012
Rubus idaeus L.	ESI-IT-MS	Sanguiin H-10; Lambertianin C; Sanguiin H-6; Nobotanin A-/malabathrin B-like	Mullen et al., 2003
Juglans regia L.	ESI-IT-TOF-MS	Pedunculagin; Tellimagrandin I; Praecoxin D; Praecoxin A methyl ester; Tellimagrandin II; Casuarictin; Procyanidin B2	Grace et al., 2014
Punica granatum L.	ESI-IT-MS	HHDP-gallagyl-hex (punicalagin); Gallagyl-hex (punicalin); Digalloyl-HHDP-gluc (punigluconin); Galloyl-HHDP-gluc (lagerstannin C); Galloyl-HHDP-DHHDP-hex (granatin B); Flavogalloyl-HHDP-gluconic acid (lagerstannin B); Galloyl-bis-HHDP-hex (casuarinin); Digalloyl-HHDP-hex (pedunculagin II); bis-HHDP-hex (pedunculagin I); Galloyl-HHDP-hex; HHDP-hex	Fischer et al., 2011

ESI-electrospray ionization; MALDI- matrix assisted TOF; FT-ICR-Fourier Transform-Ion Cyclotron ; Resonance; IT – ion trap; Qe- Hybrid quadrupole.
Kool M.M., Comeskey D.J., Cooney J.M., McGhie T.K., Structural identification of the main ellagitannins of a boysenberry (Rubus loganbaccus x baileyanus Britt.) extract by LC−ESI-MS/MS, MALDI-TOF-MS and NMR spectroscopy; Food Chemistry 119 (2010) 1535−1543.
Li S., Xiao J., Chen L., Hu C., Chen P., Xie B., Sun Z. Identification of A-series oligomeric procyanidins from pericarp of Litchi chinensis by FT-ICR-MS and LC-MS, Food Chemistry 135 (2012) 31−38.
Mullen W., Yokota T., Lean M.EJ., Crozier A. Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruits by LC−MSn Phytochemistry 64 (2003) 617−624.
Grace M.H., Warlick C.W., Neff S.A., Lila M.A. Efficient preparative isolation and identification of walnut bioactive components using high-speed counter-current chromatography and LC-ESI-IT-TOF-MS; Food Chemistry 158 (2014) 229−238.
Fischer U.A., Carle R., Kammerer D.R. Identification and quantification of phenolic compounds from pomegranate (Punica granatum L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD−ESI/MSn Food Chemistry 127 (2011) 807−821.

tannins need to be subjected to acid depolymerization before HPLC and MS analyses [122]. The exemplary tannins determined by MS are presented in Table 10.9.

10.8 NUTRACEUTICAL APPLICATION

10.8.1 Tannins as Functional Foods

Tannins, especially tea catechins, can be used as foods or food ingredients that provide a health benefit beyond satisfying traditional nutritional requirement. For example, tea polyphenols such as ECG, free theaflavins, theaflavins monogallate A, theaflavins monogallate B, and theaflavins digallate can be used alone or as food additives in the treatment of hypertension [123]. Moreover, HT, as well as procyanidins, both of which can retard starch digestion by inhibiting human α-amylase, are considered as being effective antidiabetic functional foods. Tannins delay carbohydrate digestion, glucose release, and absorption with attenuation of postprandial hyperglycemic secretion [124,125]. Furthermore, they interact with adipose tissue, inhibiting adipogenesis and directly enhancing insulin activity [126]. What is more, procyanidins has been shown to improve the pathological oxidative state of a diabetic situation [127]. Taken together, tannins consumed in fruits, vegetables, and beverages are potential candidates for the treatment of noninsulin-dependent mellitus.

10.8.2 Tannins in Nutraceutical Patents

Chewing gum containing tea polyphenols is claimed to prevent viral infections against influenza and to inhibit dissemination of this virus [128]. In addition, cocoa procyanidins and cocoa extracts which include procyanidin monomers and their oligomers, are claimed to modulate cytokine gene production, protein levels, and provide beneficial effects to subjects suffering from asthma or viral infections or at risk of viral infections [129].

10.8.3 Tannins in Dietary Supplements

There are number of dietary supplements containing plant extracts rich in hydrolyzable and condensed tannins, the consumption of which, may provide health benefits. Standardized to being a blend of ellagitannins, punicalagins, and polyphenols (Healing America Ellagitannin capsules; EllagicActive tablets; Buckeye Nutritionals Black Raspberry Ellagitannins 60 capsules), pomegranate extract from the whole seed is used in the prevention and the reduction of atherosclerosis (Pomegranate Pills with Ellagic Acid from Juice and Seeds Capsules; PomeGuard capsules; Pomegranate Standardized capsules; Living Pomegranate capsules; Endothelial Defense with Full Spectrum Pomegranate capsules). Moreover, decaffeinated green coffee extract abundant in chlogogenic acid is sold as a supplement toward enhancing loss of body weight (however, there is some controversy about its effectiveness) (Svetol Green Coffee Bean Extract capsules; Pure Health Green Coffee Bean capsules). For antioxidant purposes, a green tea extract containing catechins is widely used (Spring Valley Standardized Extract Green Tea Herbal Supplement Capsules). Furthermore, extracts from Acerin fruit and Canaigre root which are then activated with zinc and buffered with L-proline, support normal flora balance in the gastrointestinal tract (Viracin capsules). Similar effects can be obtained by way of taking in an extract of Swedish Birch bark containing condensed tannins. These are also known powerful antifungals. They work by binding to the surface of the yeast, thus preventing the yeast from adhering to the intestinal wall membrane and from colonization, while leaving the healthy probiotic bacteria intact (Tanalbit capsules). Finally, proanthocyanidines extracts from cranberries, as well as cranberry products inhibit bacterial infections in the urinary tract (Cranberry Concentrated capsules; CranRx capsules).

10.9 PHARMACEUTICAL APPLICATION

There are no pure compounds used in current pharmaceuticals except for tannic acid and tannalbin. Pycnogenol is a widely used patented extract of tannins, but the majority of medicinal applications is based on the crude plant extracts of plants rich in tannins (see Table 10.5).

10.9.1 Pycnogenol

Pycnogenol is a patented specific blend of procyanidins extracted from the bark of the pine,*Pinus pinaster* Aiton. (formerly known as *Pinus maritime* Mill.). Pycnogenol can be taken for treating circulation problems, allergies, asthma, ringing in the ears, high blood pressure, muscle soreness, pain, osteoarthritis, diabetes, attention deficit hyperactivity disorder (ADHD), a disease of the female reproductive system called endometriosis, menopausal symptoms, painful menstrual periods, erectile dysfunction, and an eye disease called retinopathy. It is also used for preventing disorders of the heart and blood vessels, including stroke, heart disease, and varicose veins. Moreover, Pycnogenol is employed to slow the aging process, maintain healthy skin, improve athletic endurance, and improve male fertility [130].

10.9.2 Tannic Acid

The product exhibits antibacterial, antienzymatic, antihistamine, antioxidant, antimutagenic, antitussive properties. It is astringent. It is used in treating diarrhea, ulcers, toothache, wounds, diaper and skin rashes, ingrown toenails, and for stopping bleeding [131,132].

Tannic acid should not be used continuously or in high quantities because it slows down the absorption of iron and possibly other trace minerals.

Tannic acid ingestion can also reduce the effectiveness of digestive enzymes [131,132].

It is the source for the production of tannate salts of certain antihistamines and antitussives. Tannic acid imparts increased stability or slow release properties to certain active pharmaceutical ingredients [133].

10.9.3 Tanalbin

Herein, tannic acid is complexed with proteins. The product has a milder effect.
It is insensitive to the gastric environment, and does not cause gastric irritation [131].

It is employed in treating diarrhea and bacterial and fungal infections via coagulation of proteins in the intestine and through arresting water loss, as well as through binding to the surface of the yeast and bacteria and preventing them from colonization, in addition to adhering to the intestinal wall membrane [131].

10.9.4 New Applications of Tannins

A-type and B-type procyanidins and derivatives thereof are patented for the treatment of inflammation and inflammation-related or associated diseases or conditions, and for the relief of pain, in individuals who are sensitive to selective cyclooxygenase-2 (COX-2) inhibitors, or are sensitive to COX-nonselective nonsteroidal anti-inflammatory drugs [134,135].

Sinecatechins as derived from green tea leaves are approved by the U.S. Food and Drug Administration and are an ingredient in an ointment compounded for treating genital and anal warts in adults. The exact mechanism of action of sinecatechins in the eradication of human papillomavirus-induced external genital and perianal warts is unknown, but may be due to induction of apoptosis, mediated by cell cycle deregulation [136].

10.10 ADVERSE EFFECTS

The main side effects associated with tannin-rich foods and tannin-rich plants or plant extracts consumption depends on the compound's ability to form complexes with proteins, starch, and metal ions. Tannins can have a large influence on the nutritive value of many foods eaten by humans and the feedstuff eaten by animals. HT and proanthocyanidins form tannin−protein complexes in a similar manner. Proteins thus bound are generally resistant to attack by proteases, and, hence, may be unavailable for livestock nutrition. However, it is hypothesized that HTs may have a less damaging effect on protein digestion because these tannins may hydrolyze within the acidic gastric environment and, hence, will release the bound proteins. When soluble tannins interact with proteins, both soluble and insoluble complexes are formed; their relative proportion depends on the concentration and size of both molecules [37,137]. Salivary proline-rich proteins form complexes with dietary HTs and will precipitate. The result of this action is an astringent sensation, which is perceived as a diffuse feeling of extreme dryness and roughness and is not confined to a particular region of the mouth or tongue [3]. Of note, several studies have shown that tannins decrease organic matter and fiber digestion, damage the mucosal lining of the digestive tract and influence the viability of gastrointestinal bacteria [138]. What is more, tannins may decrease the bioavailability of coadministered drugs. In one study, subjects consuming 1 g/day of EGCG supplements (more than 10 cups of green tea/day) suffered from stomach disorders [139]. It has also been shown that chronic intake of high doses of catechin (1.5 g/day) can bring about renal failure, hepatitis, fever, hemolytic anemia, thrombocytopenia, and skin disorders [139].

10.11 METABOLIC PROFILE OF WIDELY USED TANNIN PRODUCTS

Tannin permeability through the intestine depends mainly on the size and solubility and of the molecular form. However, the release from the food matrix and the interactions with macronutrients and other ingested compounds are not an indifferent effect on tannin availability. Complex tannins buffered by the food bolus are exposed to much less acidic conditions than that encountered in the in vitro experiments. This prevents their depolymerization [140]. Moreover, Flavan-3-ols are not metabolized by the intestinal cells. In addition, there were no metabolites evident during the few hours of the transport experiment in the Caco-2 cells model. What is more, in the same permeation model, (+)-catechin and a proanthocyanidin dimer and trimer had low permeability coefficients, indicating a preferential trans-epithelial transport through the paracellular route, because the seen permeability was similar or lower than that of mannitol, a marker of the paracellular transport. Furthermore, in the same study, it was evident that the proanthocyanidin oligomer, with an average polymerization of 6 (average molecular weight, 1740), was poorly absorbed, in comparison

TABLE 10.10 Metabolites of Tannins Produced by Microbes

Parent compound	Organism	Metabolites	Reference
Casuarictin	Human fecal microbiota	Ellagic acid	Daniel et al., 1991
(−)-Epicatechin	human fecal microbiota	3-(3′-hydroxy phenyl) propionic acid; 5-(3′,4′-dihydroxy phenyl)-γ-valerolactone; 5-(3′-hydroxy phenyl)-γ-valerolactone; phenyl acetic acid, 3-(3′,4′-dihydroxy phenyl) propionic acid	Stoupi S., et al., 2010
	Human fecal microbiota	3-Hydroxyphenylpropionic acid; 3-phenylpropionic acid; 3-hydroxyphenylvaleric acid.	Aura A.M., et al., 2008
	Human fecal microbiota	Pyrogallol, gallic acid, 5-(3′,4′-dihydroxyphenyl)-valerolactone, and 5-(3′,4′-dihydroxyphenyl)-valeric acid.	Meselhy, M.R., et al., 1997
(+)-Catechin,	Human fecal microbiota	3,4-Dihydroxyphenylpropionicacid; 3-hydroxyphenylpropionicacid; 3-phenylpropionic acid	Aura A.M., et al., 2008
Procyanidin dimer B2	Human fecal microbiota	Epicatechin; 3-(3′-hydroxy phenyl) propionic acid; 5-(3′,4′-dihydroxy phenyl)-γ-valerolactone	Stoupi S., et al., 2010
Proanthocyanidins (oligomers and polymers)	Human fecal microbiota	Phenylvalerolactones, phenylvaleric acids, phenylpropionic acids, phenylacetic acids, hippuric and benzoic acids	Manach C, et al., 2004.
Punicalagin	Human fecal microbiota	Urolithins A and B	Heber, 2008
Theaflavin-3,30-digallate	*Lactobacillus plantarum* 299 v and *Bacillus subtilis*	Theaflavin-3-gallate, theaflavin-30-gallate, theaflavin, gallic acid, and pyrogallol	Chen, H., et al., 2012

Aura AM, Mattila I, Seppanen-Laakso T, Miettinen J, Oksman-Caldentey KM, Oresic M. Microbial metabolism of catechin stereoisomers by human fecal microbiota: comparison of targeted analysis and a non-targeted metabolomics method. Phytochem Lett 2008;1:18−22.
Bode LM, Bunzel D, Huch M, Cho GS, Ruhland D, Bunzel M, Bub A, Franz CM, Kulling SE. *In vivo* and *in vitro* metabolism of trans-resveratrol by human gut microbiota. Am J Clin Nutr 2013;97:295−309.
Chen H, Hayek S, Rivera GJ, Gillitt ND, Ibrahim SA, Jobin C, Sang S. The microbiota is essential for the generation of black tea theaflavins-derived metabolites. PLoS ONE 2012;7:51001.
Daniel E.M., Ratnayake S., Kinstle T., Stoner G.D. The effect of pH and rat intestinal content on the liberation of ellagic acid from purified and crude ellagitannins. J Nat Prod 1991;54:946−52.
Heber D. Multitargeted therapy of cancer by ellagitannins.Cancer Lett 2008;269:262−68.
Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727−47.
Meselhy MR, Nakamura N, Hattori M. Biotransformation of (−)-epicatechin 3-gallate by human intestinal bacteria. Chem Pharm Bull 1997;45:888−93.
Ruotolo R, Calani L, Fietta E, Brighenti F, Crozier A, Meda C, Maggi A, Ottonello S, Del RD. Anti-estrogenic activity of a human resveratrol metabolite. Nutrition, Metabolism and Cardiovascular Diseases 2013;11:1086−92.
Stoupi S, Williamson G, Drynan JW, Barron D, Clifford MN. A comparison of the *in vitro* biotransformation of (−)-epicatechin and procyanidin B2 by human fecal Microbiota. Mol Nutr Food Res 2010;54:747−59.
Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. High absorption but very low bioavailability of oral resveratrol in humans. Drug Metab Dispos 2004;32:1377−82.
Wang D, Hang T, Wu C, Liu W. Identification of the major metabolites of resveratrol in rat urine by HPLC−MS/MS. J Chromatogr B, Analytical Technologies in the Biomedical and Life Sciences, 2005;829:97−106.

with the monomer, dimer, or trimer. These results suggest that proanthocyanidin trimers and dimmers could be absorbed in vivo, and that polymer bioavailability is limited in the gut lumen [141]. Nonglucosylated polyphenols are likely absorbed through diffusion. Their in vitro permeability can be compared with their recovery in urine after ingestion by humans. Recovery in urine of (+)-catechin ingested with tea and EC ingested with wine was of 5−6% [141]. Regarding hydrolyzable tannin bioavailability from the small intestine, gallic acid is permeated via a paracellular route in Caco-2 cells [142]. However, the intestinal absorption of gallic acid after oral administration in humans is relatively slow (t_{max}, 1.27 h) [143] and its metabolite is 4-*O*-methylgallic acid [144]. In addition, ellagic acid has been detected in human plasma in low concentrations between 0.5 and 3 h after oral administration of pomegranate juice, while no ellagitannins in intact forms were detected in plasma samples [145]. Metabolites of tannins produced by human fecal microbiota are described in Table 10.10. The metabolic pathways of digested tannins are shown in Fig. 10.5.

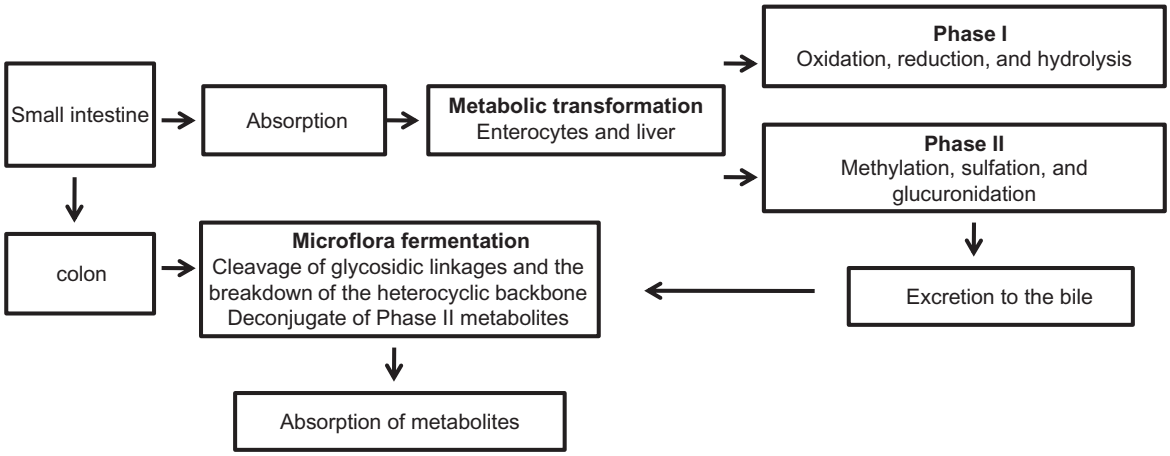


FIGURE 10.5 Metabolic pathways of digested tannins.

10.12 CONCLUSIONS

Tannins are a very large and important group of secondary metabolites. In this chapter, their structural and chemical characteristics, as well as their biological activities were described. Further, emphasis was placed on providing information on their in vitro/in vivo biological properties, and the results of clinical trials. The metabolism of tannins was discussed as well, and the role of human fecal microbiota was underlined in the biotransformation of tannins. Information on plants containing tannins and their traditional indications were also provided.

10.13 SELF-EVALUATION QUESTIONS

1. According to their structural characteristics, what types of tannins can be listed?
2. What are the main intermediates in the synthesis of hydrolyzable and condensed tannins?
3. List the plant families and plant organs that contain tannins.
4. What are the functions of tannins in plants?
5. Describe the mechanisms of action of the antibacterial and antiviral properties of tannins.
6. By what mechanism do tannins exhibit cardioprotective activity? Name the chemical group within their structures crucial for this activity.
7. Describe the mechanisms of tannins antidiabetic activity.
8. What is a kaki-tannin and what properties does it have?
9. What are the most suitable solvents used in the extraction of tannins?
10. What are the chemical tests used in the identification and determination of tannins? Which are those specific for HT and for proanthocyanidins?
11. What are the main nutraceutical and pharmaceutical applications of tannins? Moreover, what are the main indications of plants containing tannins?
12. How do tannins influence the nutraceutical value of foods?

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Chapter 11

Terpenoids

A. Ludwiczuk[1], K. Skalicka-Woźniak[1] and M.I. Georgiev[2]
[1]Medical University of Lublin, Lublin, Poland, [2]The Stephan Angeloff Institute of Microbiology, Plovdiv, Bulgaria

Chapter Outline

Learning Objectives

- Classify terpenoids based on number of isoprene units.
- Name the types of terpenoids and give examples.
- Define an essential oil.
- List typical plants and plant families containing terpenoids.
- Give examples of the activities of terpenoids used in pharmaceuticals, together with mechanisms of actions.
- Know the analysis of terpenoids for the purpose of their detection and isolation.

11.1 DEFINITION

Terpenoids, also known as isoprenoids, are the most numerous and structurally diverse natural products. The generic name "terpene" was originally applied to the hydrocarbons found in turpentine, the suffix "ene" indicating the presence of olefinic bounds.

Terpenoids are classified based on the number and structural organization of carbons formed by the linear arrangement of isoprene units followed by cyclization and rearrangements of the carbon skeleton with an empirical feature known as the isoprene rule [1]. Isoprene, the "building block" of terpenoids, is 2-methylbuta-1,3-diene (C_5H_8) (Fig. 11.1). The single isoprene unit, therefore, represents the most basic class of terpenoids, hemiterpenoids. The names of other terpenoid groups are shown in Table 11.1.

The so-called isoprene rule states that all terpenoids are derived from the ordered, head-to-tail joining of isoprene units. A head-to-tail fusion is the most common; however, nonhead-to-tail condensation of isoprene units also occur. Head-to-head fusions are common among triterpenoids and carotenoids, while some compounds are formed by head-to-middle fusions (e.g., irregular monoterpenoids) [2,3]. Fig. 11.1 shows the most common fusions of isoprene units, and how the isoprene units and the original backbone can be traced in three sample terpenoids.

Terpenoids are derived from the mevalonate (MVA) pathway, which is active in the cytosol, or from the plasticidal 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway (Fig. 11.2). Hemi-, mono-, di-, and triterpenoids are mainly

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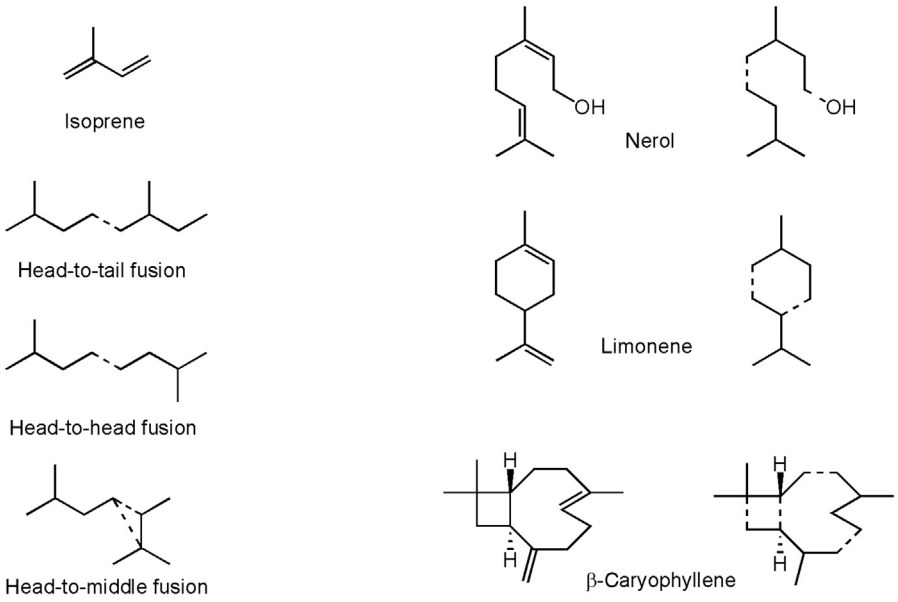


FIGURE 11.1 Head-to-tail coupling of isoprene units, and isoprene units in some terpenoid backbones.

TABLE 11.1 Classification of Terpenoids

Name	No. of Isoprene Units	No. of Carbon Atoms	General Formula
Hemiterpenoids	1	5	C_5H_8
Monoterpenoids	2	10	$C_{10}H_{16}$
Sesquiterpenoids	3	15	$C_{15}H_{24}$
Diterpenoids	4	20	$C_{20}H_{32}$
Sesterterpenoids	5	25	$C_{25}H_{40}$
Triterpenoids	6	30	$C_{30}H_{48}$
Tetraterpenoids (carotenoids)	8	40	$C_{40}H_{64}$
Polyterpenoids	>8	>40	$(C_5H_8)_n$

synthesized by the MEP pathway while sesqui- and triterpenoids by the MVA pathway, although there are exceptions and cross-talk between the two pathways [4,5].

Terpenoids are perhaps more familiar to us as major components of essential oils, which comprise the steam-, hydro-, or dry distillable fractions, or fractions obtained by means of mechanical treatment, responsible for the characteristic scent, odor, and/or smell of many plants [6]. Chemically, the terpene essential oils can be divided into two classes, the mono- and sesquiterpenoids, which differ in their boiling point range (monoterpenoids b.p. 140−180°C, sesquiterpenoids b.p. >200°C).

Isomerism is common among terpenoids, and pairs of the isomeric forms can be isolated from plants. Isomers are molecules which have the same empirical formulae, but differ in atomic bonding or arrangement in space. There are five principal forms of terpenoid isomerism: structural, positional, geometrical, conformational, and stereoisomerism as observed in examples shown in Fig. 11.3.

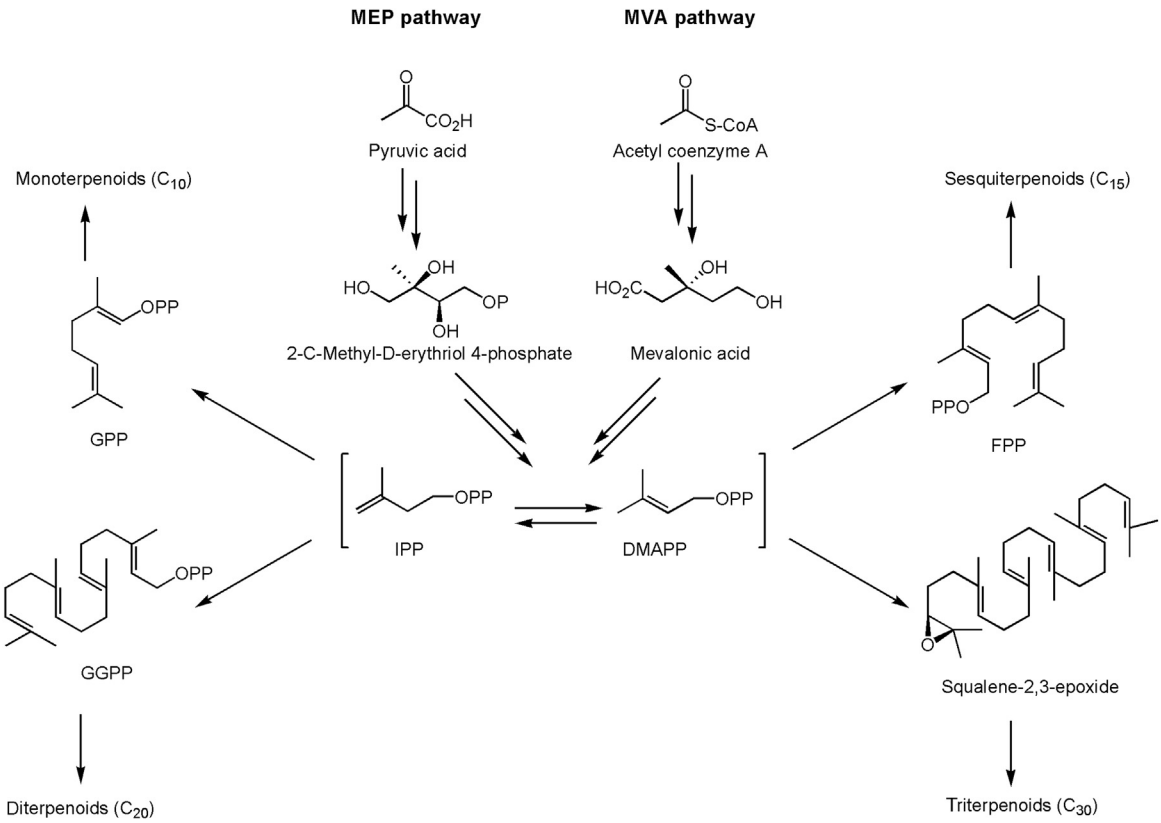


FIGURE 11.2 Biosynthesis of terpenoids.

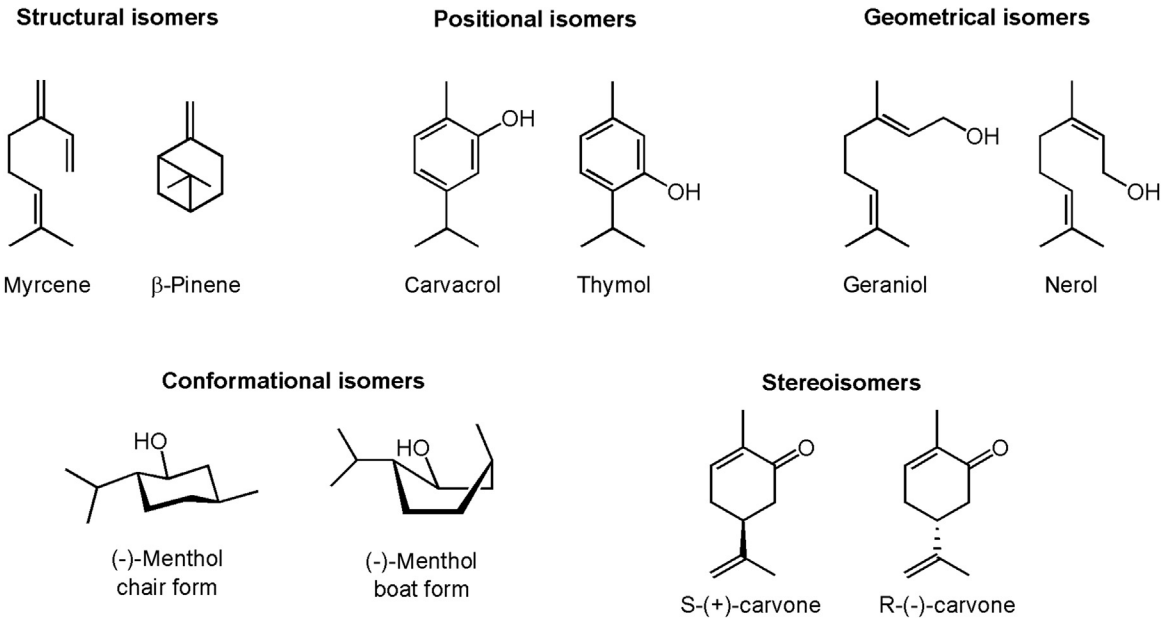


FIGURE 11.3 Principal forms of isomerism among terpenoids.

Terpenoids are most numerous and structurally diverse plant secondary metabolites.
The fundamental building block of terpenoids is the isoprene unit, C_5H_8.
Monoterpenoids are based on two isoprene units ($C_{10}H_{16}$). Other terpenoids have multiples of C_5 units, e.g., C_{15}, C_{20}, C_{25}, C_{30}, etc.

11.2 TYPES OF TERPENOIDS

11.2.1 Hemiterpenoids

Hemiterpenoids are the simplest among the terpenoids. The most prominent hemiterpene, isoprene (see Fig. 11.1) (boiling point 34 °C) is emitted from the leaves of many trees (including conifers, poplars, oaks, and willows) and herbs (e.g., *Hamamelis japonica*) [7]. Other known hemiterpenoids found in plants are tiglic, angelic, isovaleric, and senecioic acids, along with isoamyl alcohol [8] (Table 11.2).

11.2.2 Monoterpenoids

Monoterpenoids consist of a 10 carbon backbone (2 isoprene units) structure and can be divided into three subgroups: acyclic, monocyclic, and bicyclic. Within each group, the monoterpenoids may be simple unsaturated hydrocarbons or may have functional groups and be alcohols, aldehydes, and ketones. Common aliphatic examples include myrcene, citral, geraniol, lavandulol, and linalool. The important representatives of monocyclic monoterpenoids are α-terpineol, limonene, thymol, menthol, carvone, eucalyptol, and perillaldehyde. The bicyclic monoterpenes may be divided into three classes according to the size of the second ring. The first being a six-membered ring in each class while the second can be either a three, four, or five-membered ring. Thujone and Δ^3-carene are representatives of the group containing 6 + 3-membered rings, α- and β-pinene represent a 6 + 4 group, while borneol and camphor, a 6 + 5 group [9]. A few typical examples are show in Table 11.3.

11.2.3 Iridoids

Iridoids comprise a large group of monoterpenoids, characterized by skeletons in which a six-membered ring, containing an oxygen atom, is fused to a cyclopentane ring (iridane skeleton). These compounds most frequently occur in plants combined with sugar and so are classified as glycosides [10] that are further divided into four main groups. These are iridoid glycosides (aucubin, harpagoside), nonglycosylated or simple iridoids (loganin), secoiridoids (gentiopicroside), and bisiridoids, formed by dimerization of iridoids and secoiridoids [11] (Table 11.4).

TABLE 11.2 The Chemical Structures, Resources, and Habitats of Hemiterpenoids

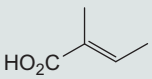
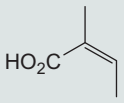
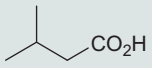
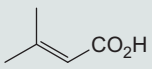
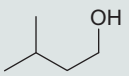
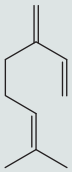
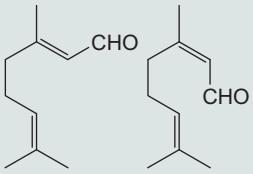
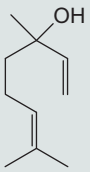
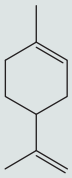
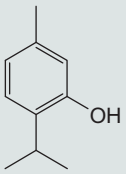
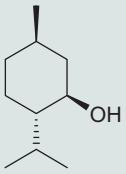
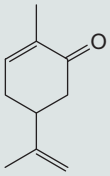
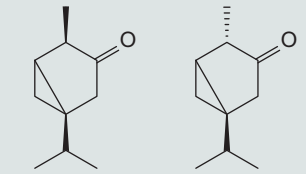
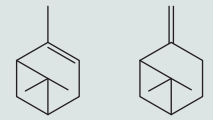
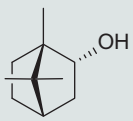
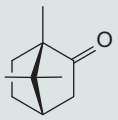
Name of Compounds	Structure	Resource	Habitat
Tiglic acid		*Schoenocaulon officinale* (Schltdl. & Cham.) A. Gray (Melanthiaceea) *Croton tiglium* L. (Euphorbiaceae)	Southern North America, Guatemala, Venezuela Africa, South America, Indian subcontinent
Angelic acid		*Archangelica officinalis* Hoffm. (Apiaceae)	Europe, the United States
		Peucedanum ostruthium (L.) Koch (Apiaceae)	Central and southern Europe
		Levisticum officinale Koch (Apiaceae)	Europe and southwestern Asia
Isovaleric acid		*Valeriana officinalis* L. (Caprifoliaceae)	Europe, northern Asia
Senecioic acid		*Senecio* sp. (e.g., *S. mikanioides* Walp.) (Asteraceae)	Worldwide distribution
		Ligularia sp. (Asteraceae)	Central and eastern Asia
Isoamyl alcohol		*Tuber melanosporum* (black truffle) (Tuberaceae)	Southern Europe: Spain, France, Italy

TABLE 11.3 The Chemical Structures, Resources, and Habitats of Monoterpenoids

Name of Compounds	Structure	Resource	Habitat
Acyclic Monoterpenoids			
Myrcene		Varbena oil (*Lippia citriodora* Kunth., Verbenaceae)	South and central America, tropical Africa
		Bay laurel oil (*Laurus nobilis* L., Lauraceae)	Mediterranean region
Citral		Lemongrass oil (*Cymbopogon flexuosus* (Nees ex Steud.) J.F. Watson	Southern India
		and *Cymbopogon citratus* (DC. ex Nees) Stapf, Poaceae)	Malaysia
Linalool		Ho leaf oil (*Cinnamomum camphora* (L.) J. Presl, Lauraceae) (**mainly R-(−)-linalool**)	China, Taiwan, southern Japan, Korea, Vietnam
		Coriander essential oil (*Coriandrum sativum* L., Apiaceae) (**mainly S-(+)-linalool**)	Southern Europe, north Africa, and southwestern Asia
Monocyclic Monoterpenoids			
Limonene		Major constituent of several citrus essential oils (Rutaceae): orange (*Citrus sinensis* (L.) Osbeck), lemon (*Citrus limon* (L.) Burm. f.), mandarin (*Citrus reticulata* Blanco), lime (*Citrus aurantifolia* (Christm.) Swing), and grapefruit (*Citrus paradisi* Macfad)	Southeast Asia (southern China, India, Indonesia, Malay Archipelago)
Thymol		Thyme oil (*Thymus vulgaris* L., Lamiaceae)	Southern Europe from the western Mediterranean to southern Italy
Menthol (the main form occurred in nature is (−)-menthol)		Peppermint oil (*Mentla piperita* L., Lamiaceae)	Indigenous to Europe and the Middle East
Carvone		Caraway oil (*Carum carvi* L., Apiaceae) (**mainly S-(+)-carvone**)	Native to western Asia, Europe, and northern Africa
		Spearmint oil (*Mentha spicata* L., Lamiaceae) (**mainly R-(−)-carvone**)	Native to much of Europe and Asia (Middle East, Himalayas, China)

(*Continued*)

TABLE 11.3 (Continued)

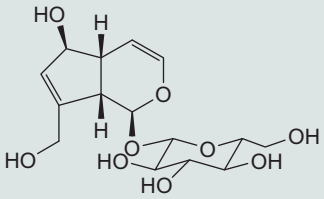
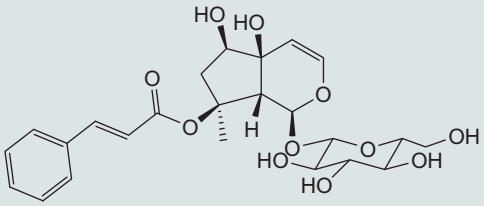
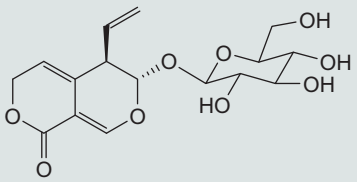
Name of Compounds	Structure	Resource	Habitat
Eucalyptol (=1,8-cineole)		Eucalyptus leaf oil (*Eucalyptus globulus* Labill., Myrtaceae)	Native to Australia and Tasmania
		Rosemary (*Rosmarinus officinalis* L., Lamiaceae)	Native to Mediterranean region
Bicyclic Monoterpenoids			
Thujone naturally occurs in two diastereomeric forms: (−)-α-thujone and (+)-β-thujone	 (-)-α-Thujone (+)-β-Thujone	Grand wormwood (*Artemisia absinthium* L., Asteraceae)	Native to temperate regions of Eurasia and northern Africa
α- and β-Pinene	 α-pinene β-pinene	They are usually sourced from turpentine (see above), and also occur in galbanum (see above) pines and other conifers;	Most regions of the Northern Hemisphere host some native species of pines
		(+)-α-pinene—mainly in *Pinus palustris* Mill.	Native to the southeastern United States
		(−)-β-pinene—mainly in *Pinus caribaea* Morelet and *P. pinaster* Aiton	Native to central America, Cuba, the Bahamas Native to the western and southwestern Mediterranean region
Borneol		Can be found in several species of *Artemisia* (e.g., *A. annua* L., Asteraceae),	Temperate climates of both hemispheres Tropical and subtropical zones of Asia, especially the Indian Subcontinent and southeast Asia
		Blumea balsamifera (L.) DC. (Asteraceae) and *Kaempferia galanga* L. (Zingiberaceae)	Indonesia, southern China, Taiwan, Cambodia, and India
Camphor		Camphor tree (*Cinnamomum camphora* T. Nees & Ebermeier, Lauraceae)	Indigenous to Japan, China, and Taiwan

11.2.4 Sesquiterpenoids

Sesquiterpenoids are derived from three isoprene units and exist in a wide variety of forms, including linear, monocyclic, bicyclic, and tricyclic frameworks. They are the most diverse group of terpenoids. The most characteristic examples of compounds belonging to each of the mentioned groups are presented in Table 11.5.

Sesquiterpene lactones are chemically distinct from other sesquiterpenoids by the presence of a γ-lactone system, and can be divided into two structural classes according to lactone ring annulations: 6,12- (e.g., costunolide, parthenolide, santonin, artabsin, matricin) and 8,12-olides (e.g., inunolide, alantolactone, thapsigargin, helenalin). Three major types of sesquiterpene lactones are germacranolides, eudesmanolides, and guaianolides (Table 11.6).

TABLE 11.4 The Chemical Structures, Resources, and Habitats of Iridoids

Name of Compounds	Structure	Resource	Habitat
Aucubin		*Euphrasia officinalis* L. *Euphrasia rostkoviana* Hayne (Scrophulariaceae)	Europe
		Plantago lanceaolata L. (Plantaginaceae)	Europe, Asia
Harpagoside		*Harpagophytum procumbens* (Burch.) DC. Ex Meisn. (Pedaliaceae)	Native to southern Africa
Gentiopicroside		*Gentiana lutea* L. (Gentianaceae)	Native to the mountains of central and southern Europe

11.2.5 Diterpenoids

Diterpenoids comprise of a chemically heterogenous group of compounds, all with a C_{20} carbon skeleton based on four isoprene units. They can be classified as linear, bicyclic, tricyclic, tetracyclic, pentacyclic, or macrocyclic diterpenes depending on their skeletal core. In nature, they are commonly found in a polyoxygenated form with keto and hydroxyl groups, these are often esterified by small-sized aliphatic or aromatic acids [12]. The representatives of each group are presented in Table 11.7. Ginkgolides are unique constituents of *Ginkgo biloba* and are found exclusively in this tree. The ginkgolides are diterpenes with a cage skeleton consisting of six five-membered rings: a spiro[4.4]-nonane carbocyclic ring, three lactones, and a tetrahydrofuran ring, e.g., ginkgolide A (see Table 11.7) [13].

11.2.6 Sesterterpenoids

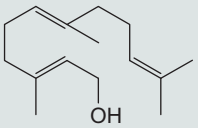
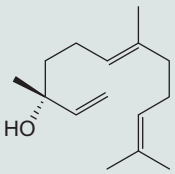
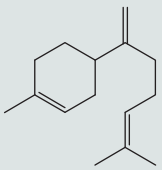
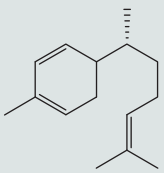
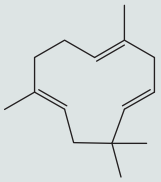
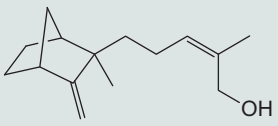
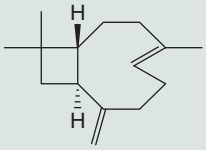
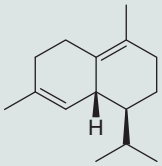
Sesterterpenoids consist of a 25 carbon backbone (5 isoprene units). They exists in a wide variety of forms, including linear, monocyclic, bicyclic, tricyclic, tetracyclic, and macrocyclic frameworks [14,15]. A few typical examples are show in Table 11.8.

11.2.7 Triterpenoids

Triterpenoids are compounds with a carbon skeleton based on six isoprene units which are derived biosynthetically from the acyclic C_{30} hydrocarbon, squalene. They have relatively complex cyclic structures, most being either alcohols, aldehydes or carboxylic acids [16].

Sterols are triterpenes which are based on the cyclopentane perhydrophenanthrene ring system. Plant sterols called "phytosterols," e.g., sitosterol, stigmasterol, and campesterol are widespread in higher plants. A less common compound, α-spinasterol (isomer of stigmasterol) was found in spinach, alfalfa, and senega root. Others occur mainly in lower plants, e.g., algae and liverworts [17]. The examples of most important triterpenes are shown in Table 11.9.

TABLE 11.5 The Chemical Structures, Resources, and Habitats of Sesquiterpenoids

Name of Compounds	Structure	Resource	Habitat
Acyclic Sesqiterpenoids			
Farnesol		Present in many essential oils, e.g., citronella (*Cymbopogon* species, Poaceae),	Southern India, Malaysia Native to Europe and the Mediterranean Basin east to Iran, with one species in Somalia
		Cyclamen (*Cyclamen* species, Myrsinaceae), tuberose (*Polianthes tuberoza* L., Asparagaceae)	Native to Mexico
β-Nerolidol		Neroli oil (*Citrus aurantium subsp. Amara* L., Rutaceae)	Native to southern Vietnam native to tropical and warm temperate regions of the Eurasia, Australasia, and Oceania
		Jasmine oil (*Jasminum officinale* L., Oleaceae)	
Monocyclic Sesquiterpenoids			
β-Bisabolene		Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) Cubeb (*Piper cubeba* L.f., Piperaceae)	Indigenous to southern China and was spread to other parts of Asia and subsequently to West Africa and Caribbean
			Java and Sumatra
α-Zingiberene		Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) Turmeric (*Curcuma longa* L., Zingiberaceae)	See above Native in southeast India
α-Humulene		Hope oil (*Humulus lupulus* L., Cannabaceae)	Native to Europe, western Asia, and North America
Bicyclic Sesquiterpenoids			
β-Santalol		Sandalwood (*Santalum album* L., Santalaceae)	It is a hemiparasitic tree, native to semiarid areas of the Indian subcontinent
β-Caryophyllene		Clove oil (*Syzygium aromaticum* (L.) Merrill & Perry; syn. *Caryophyllus aromaticus* L., Myrtaceae)	Native to the Maluku Islands in Indonesia
δ-Cadinene		Cade juniper (*Juniperus oxycedrus* L., Cupressaceae)	Native across the Mediterranean region from Morocco and Portugal, north to southern France, east to western most Iran, and south to Lebanon and Israel

(*Continued*)

TABLE 11.5 (Continued)

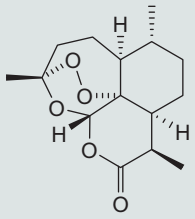
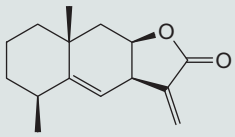
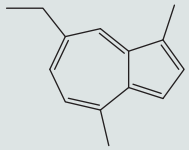
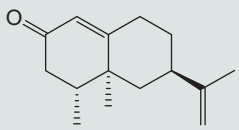
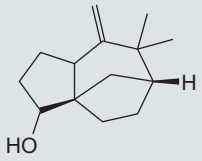
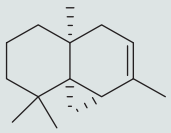
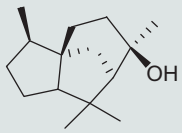
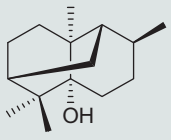
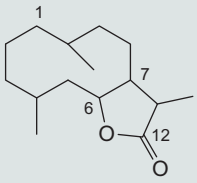
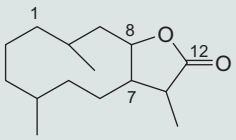
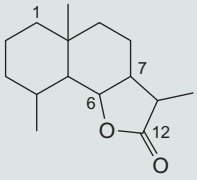
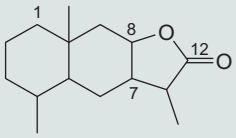
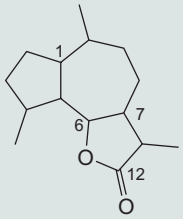
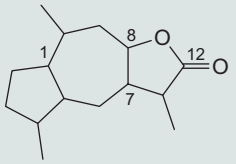
Name of Compounds	Structure	Resource	Habitat
Artemisinin		Sweet wormwood (*Artemisia annua* L., Asteraceae)	Native to temperate Asia
Alantolactone		Elecampane (*Inula helenium* L., Asteraceae)	Common in many parts of Great Britain, and ranges throughout central and southern Europe, and in Asia as far eastwards as the Himalayas
Chamazulene		Chamomile oil (*Matricaria chamomilla* L., Asteraceae)	Europe and temperate Asia
		Wormwood oil (*Artemisia absinthum* L., Asteraceae)	Native to temperate regions of Eurasia and northern Africa
		Yarrow oil (*Achillea millefolium* L., Asteraceae)	Native to temperate regions of Eurasia and northern Africa
Nootkatone		Grapefruit (*Citrus paradisi Macfad., Rutaceae*)	Southeast Asia (southern China, India, Indonesia, Malay Archipelago)
Tricyclic Sesquiterpenoids			
Khushimol		Vetiver (*Chrysopogon zizanioides* (L.) Roberty, Poaceae)	Native to India
Thujopsene		Cedarwood oil (trees from Cupresaceae family, e.g., *Juniperus* and *Cupressus* spp.)	Occurring in diverse habitats on all continents except Antarctica
β-Cedrol		See above	See above
Patchoulol		Patchouli (*Pogostemon cablin* (Blanco) Benth., Lamiaceae)	Native to tropical regions of Asia
		Spikenard (*Nardostachys jatamansi* (D. Don) DC., Caprifoliaceae)	Grows in the eastern Himalayas, primarily in a belt through Kumaon, Nepal, Sikkim and Bhutan

TABLE 11.6 Three Major Group of Sesquiterpene Lactones

Type	General Structure	Example	Occurrence
Germacranolides		Costunolide	Costus (*Saussurea costus* (Falc.) Lipsch., Asteraceae)
		Parthenolide	Feverfew (*Tanacetum parthenium* (L.) Sch. Bip., Asteraceae)
		Inunolide	*Inula racemosa* Hook.f., Asteraceae
Eudesmanolides		Santonin	Santonica (*Artemisia cina* Berg; *A. chamaemelifolia* Vill., *A. maritima* var. Stechmanniana Bess, Asteraceae)
		Alantolactone	Elecampane (*Inula helenium* L., Asteraceae)
Guaianolides		Artabsin	Absinthe wormwood (*Artemisia absinthium* L., Asteraceae)
		Matricin	Chamomile (*Matricaria chamomilla* L., Asteraceae)
		Thapsigargin	*Thapsia garganica* L., Apiaceae
		Helenalin	Arnica (*Arnica montana* L., Asteraceae)

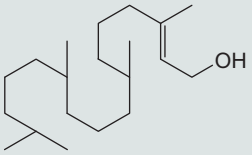
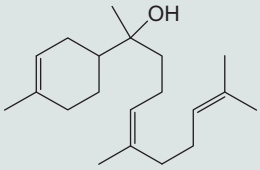
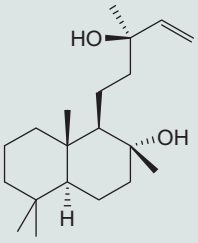
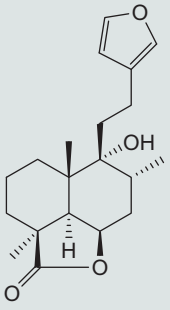
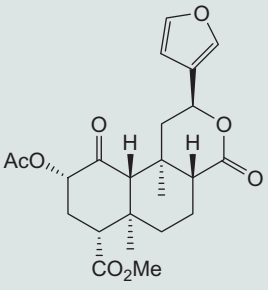
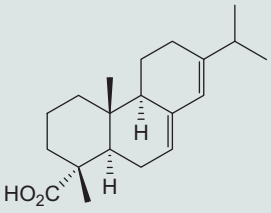
11.2.8 Tetraterpenoids

Tetraterpenoids consist of eight isoprene units and have the molecular formula $C_{40}H_{64}$. The most common tetraterpenoids are carotenoids, which are natural fat-soluble pigments. Structurally, carotenoids feature three aspects [18]:

- Most widely known carotenoids are either simple unsaturated hydrocarbons having the basic lycopene structure or their corresponding oxygenated analogs, known as xanthophylls (lutein, zeaxanthin).
- Eight isoprene units are found to be joined head to tail in lycopene to give it a conjugated system that is responsible for the chromophoric characteristic of the molecule, i.e., producing color.
- Cyclization of lycopene at both terminals of the molecule yields a bicyclic hydrocarbon commonly known as β-carotene, which occur most abundantly in higher plants.

Combined forms of carotenoids occur, especially in flowers and fruits of higher plants, and they are usually xanthophylls esterified with fatty acid residues, e.g., palmitic, oleic, or linoleic acids. Glycosides are normally very rare; in

TABLE 11.7 The Chemical Structures, Resources, and Habitats of Diterpenoids

Name of Compounds	Structure	Resource	Habitat
Acyclic Diterpenoids			
Phytol		Present as the ester attachementin the molecule of chlorophyll	
Monocyclic Diterpenoids			
9-Geranyl-α-terpineol		*Helichrysum heterolasium* Hilliard (Asteraceae)	Southern Africa
Bicyclic Diterpenoids			
Sclareol		Clary sage (*Salvia sclarea* L., Lamiaceae)	Native to the northern Mediterranean, along with some areas in north Africa and central Asia
Marrubiin		White horehound (*Marrubium vulgare* L., Lamiaceae)	Native to Europe, northern Africa, and southwestern and central Asia
Salvinorin A		Diviner's sage (*Salvia divinorum* Epling & Játiva, Lamiaceae)	Native to Sierra Mazateca of Oaxaca, Mexico, where it grows in shady and moist locations
Tricyclic Diterpenoids			
Abietic acid		Resin from coniferous trees, e. g., pine tree (*Pinus sylvestris* L., Pinaceae)	Native to Europe and Asia

(*Continued*)

TABLE 11.7 (Continued)

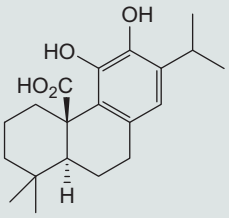
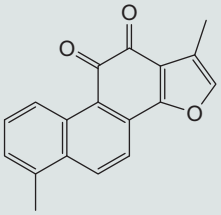
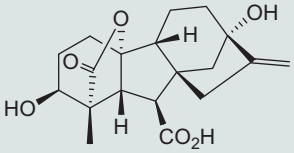
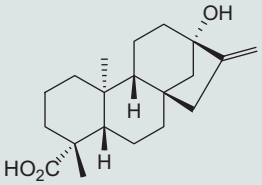
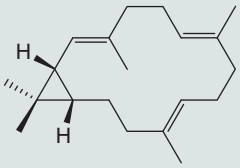
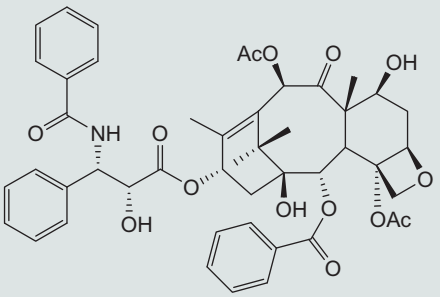
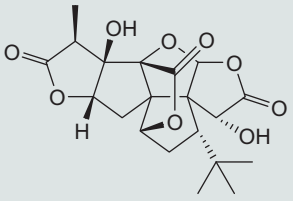
Name of Compounds	Structure	Resource	Habitat
Carnosic acid		Common sage (*Salvia officinalis* L., Lamiaceae) Rosemary (*Rosmarinus officinalis* L., Lamiaceae)	Both native to the Mediterranean region
Tanshinone I		Danshen (*Salvia miltiorrhiza* Bunge, Lamiaceae)	Native to China and Japan
Tetracyclic Diterpenoids			
Gibberellin A1		Belongs to gibberellins—plant hormones that regulate growth. First isolated from fungal strains (*Giberella fujikuroi*)	
Steviol		*Stevia rebaudiana* (Bert.) Bertoni, Asteraceae	Native to the valley of the Rio Monday in highlands of northeastern Paraguay in South America
Macrocyclic Diterpenoids			
Casbene		Castor bean (*Ricinus communis* L., Euphorbiaceae)	Indigenous to the southeastern Mediterranean Basin, Eastern Africa, and India
Taxol		Bark of the Pacific yew, *Taxus brevifolia* Nutt. (Taxaceae)	Native to the Pacific Northwest of North America
Miscellaneous Diterpenoids			
Ginkgolide A		*Ginkgo biloba* L., Ginkgoaceae	Native to China

TABLE 11.8 The Chemical Structures, Resources, and Habitats of Sesterterpenoids

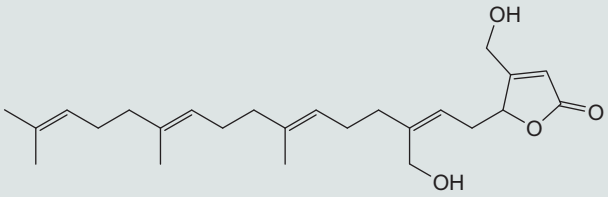
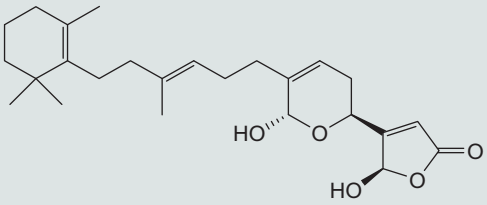
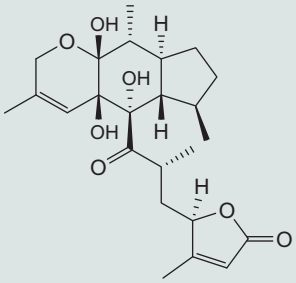
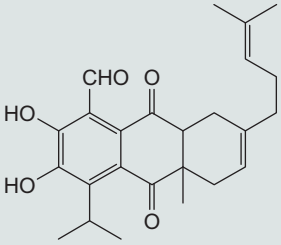
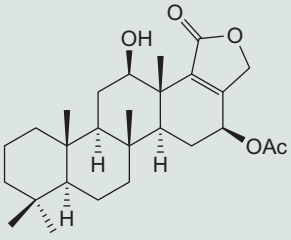
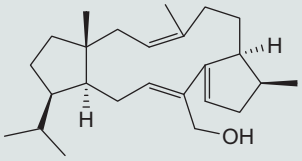
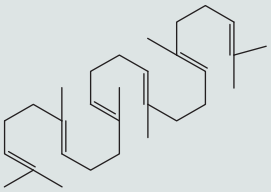
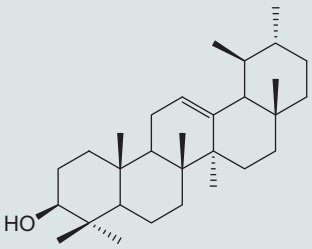
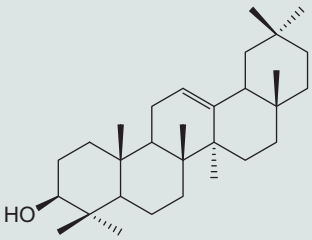
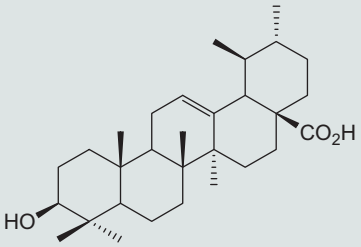
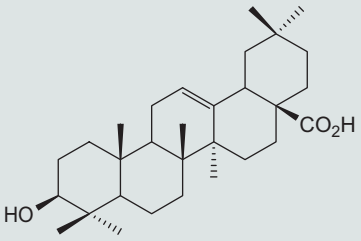
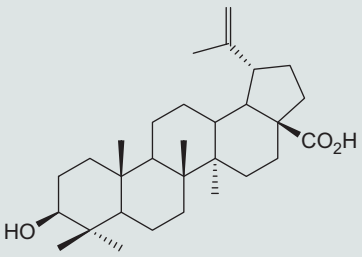
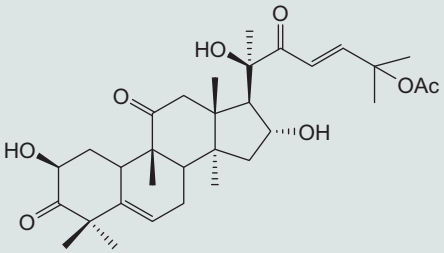
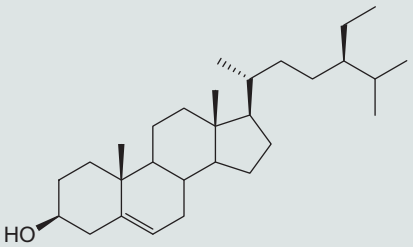
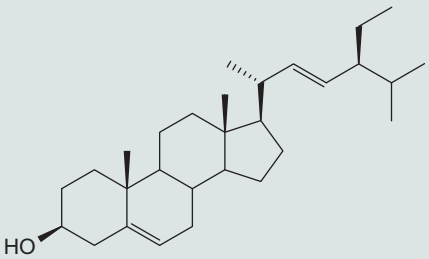
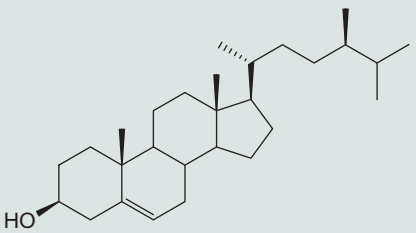
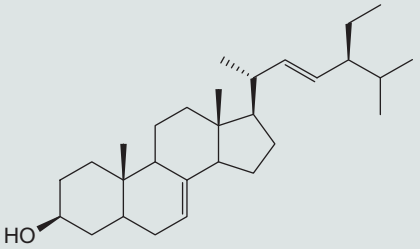
Name of Compounds	Structure	Resource	Habitat
Acyclic sesterterpenoids			
Hippolide E		Marine sponge *Hippospongia lachne* de Laubenfels (Spongiidae)	Distributed around the world in the western central Atlantic
Monocyclic sesterterpenoids			
Manoalide		Marine sponge *Luffariella variabilis* Polejaeff (Thorectidae)	Distributed widely through the Indo-Pacific
Bicyclic sesterterpenoids			
Leucosceptrine		*Leucosceptrum canum* Sm., Lamiaceae	Native to south western China (Sichuan, Tibet, Yunnan)
Tricyclic sesterterpenoids			
Heliocide H1		Upland cotton (*Gossypium hirsutum* L., Malvaceae)	Native to Mexico, the West Indies, northern South America, and Central America
Tetracyclic sesterterpenoids			
Sesterstatin 7		Marine sponge *Hyrtios erecta* Keller (Thorectidae)	Widely distributed (North Atlantic Ocean, Indian Ocean)
Macrocyclic sesterterpenoids			
Nitiol		*Gentianella nitida* (Griseb.) Fabris, Gentianaceae	Native to the high Andes of Peru

TABLE 11.9 The Chemical Structures, Resources, and Habitats of Triterpenoids and Sterols Representatives

Name of Compounds	Structure	Resource	Habitat
Squalene		Liver oil of shark (e.g., gray reef shark, *Carcharhinus amblyrhynchos*). Olive oil from the fruit of *Olea europaea* L., Oleaceae	Africa, the Mediterranean Basin from Portugal to the Levant, the Arabian Peninsula, and southern Asia as far east as China, as well as the Canary Islands, Mauritius and Reunion
α-Amyrin		Resins of *Bursera* and *Protium* species (Burseraceae)	Native (for many species endemic) to the Americas, from the southern United States south through to northern Argentina Native to the Neotropics, Madagascar, New Guinea, and southern Asia from Pakistan east to Vietnam
		Taraxacum officinale F. H. Wigg., Asteraceae	Temperate regions of the world
β-Amyrin		Lotus (*Nelumbo nucifera* Gaertn., Nelumbonaceae)	Native to Tropical Asia and Queensland, Australia
		Cuachalalate (*Amphipterygium adstringens* (Schltdl.) Standl., Anacardiaceae)	Central and southern Mexico
Ursolic acid		Holy basil (*Ocimum sanctum* L., Lamiaceae)	Native to Indian subcontinent
		Bilberry (*Vaccinum myrtillus* L., Ericaceae)	Native to Europe, northern Asia, Greenland, western Canada, and the western United States
		Rosemary (*Rosmarinus officinalis* L., Lamiaceae)	Native to Mediterranean region
Oleanolic acid		Olive leaves (*Olea europaea* L., Oleaceae)	See above
		Pot marigold (*Calendula officinalis* L., Asteraceae)	Native to southern Europe

(*Continued*)

TABLE 11.9 (Continued)

Name of Compounds	Structure	Resource	Habitat
Betulinic acid		White birch (*Betula pubescens* Ehrh., Betulaceae)	Native and abundant throughout northern Europe, Iceland, northern Asia, and Greenland
Cucurbitacin B		Various species from Cucurbitaceae family	Native to temperate and tropical areas
Sitosterol		Common nettle (*Urtica dioica* L., Urticaceae)	Native to Europe, Asia, northern Africa, and North America
		Nigella sativa L., Ranunculaceae	Native to south and southwest Asia
Stigmasterol		Calabar bean (*Physostigma venenosum* Balf., Fabaceae)	Native to tropical Africa
		Rape seeds (*Brassica napus* L. Brassicaceae)	Wild forms occur in Sweden, the Netherlands, and Britain
Campesterol		Banana (*Musa* species e.g., *Musa acuminata* Colla., Musaceae)	Native to tropical Indomalaya and Australia
α-Spinasterol		Spinach (*Spinacia oleracea* L., Amaranthaceae)	Native to central and southwestern Asia

higher plants, the best known in the water-soluble crocin, the gentiobiose derivative of an unusual C_{20}-carotenoid, crocetin (the yellow pigment of meadow saffron, *Crocus sativus* L.) [16]. The most characteristic representatives of carotenoids are presented in Table 11.10.

11.2.9 Polyterpenoids

Polyterpenoids are polymeric isoprenoid hydrocarbons, which consist of more than eight isoprene units. This class of compounds has customarily been confirmed to include the rubbers. The natural rubber molecule is a high-molecular weight polymer consisting of isoprene units in the *cis*-configuration. Some plants produce a polyisoprene with *trans* double bonds. These are gutta-percha from *Palaquium gutta* (Sapotaceae) and balata from *Mimusops balata* (Sapotaceae) (Table 11.11).

11.2.10 Irregular Terpenoids

11.2.10.1 Irregular Monoterpenoids

There are two major types of irregular monoterpenoids (Table 11.12) [19]:

1. The substituted cycloheptane monoterpenes, called tropones (e.g., nezukone). Such compounds most probably arise by an unknown ring expansion of the cyclohexane skeleton;
2. Compounds formed by head-to-middle condensation of isoprene units. Important members include artemisia ketone, chrysanthemic acid, and lavandulol. These compounds are found primarily in the Asteraceae and Lamiaceae families.

11.2.10.2 Ionones and Damascones

Ionones and damascones are compounds that belong to C13-norisoprenoids (norterpenoids), which are carotenoid-derived aroma compounds. The most known representatives are α- and β-ionone, and α- and β-damascone that occur in many essential oils (Table 11.12) [20].

Terpenoids can be cyclic or acyclic, with a large range of structural variations.

11.3 PLANTS CONTAINING TERPENOIDS

Terpenoids are a very diverse group of natural compounds which can be found in a number of plants. Monoterpenoids are chief components of the essential oils and are known for their aromatic properties. These compounds are the major constituents of galbanum (*Ferula gummosa* Boiss.) (80%), *Angelica* species (73%), hyssop (70%), rose (54%), peppermint (45%), juniper (42%), frankincense (40%), spruce (38%), pine (30%), cypress (28%), and myrtle (25%). In general, monoterpene hydrocarbons such as α- and β-pinene, limonene, Δ^3-carene, and myrcene are found as complex mixtures in most essential oils, particularly in those obtained from plant leaves. Flower and seed essential oils tend to have more specialized monoterpenoids present. When the molecule is optically active, the two enantiomers are very often present in different plants: (+)-α-pinene from *Pinus palustris* Mill; (−)-β-pinene from *Pinus caribaea* Morelet and *P. pinaster* Aiton; S-(+)-linalool from coriander (*Coriandrum sativum* L.); and R-(−)-linalool from *Cinnamomum camphora* (L.) J. Presl [9].

Iridoids are widely distributed in sympetalous plants within the dicotyledons. The presence of iridoids has been reported in approximately 50 plant families, such as Apocynaceae, Gentianaceae, Loganiaceae, Pedaliaceae, Plantaginaceae, Rubiaceae, and Scrophulariaceae [21].

Most of the sesquiterpenoids (especially hydrocarbons), as monoterpenoids, are considered to be essential oil components, since they belong to the steam distillable fraction often containing the characteristic odoriferous components of the plant [16]. Sesquiterpenoids are the principal constituents of cedarwood (98%), vetiver (97%), spikenard (*Nardostachys jatamansi* (D. Dan) DC) (93%), sandalwood (90%), patchouli (71%), myrrh (62%), and ginger (59%). Sesquiterpene lactones are a group of secondary metabolites found across the plant kingdom being most common in families such as Cactaceae, Solanaceae, Araceae, and the Euphorbiaceae. However, they are most prevalent in the Asteraceae, where they can be found almost ubiquitously [22].

TABLE 11.10 The Chemical Structures, Resources, and Habitats of Carotenoids

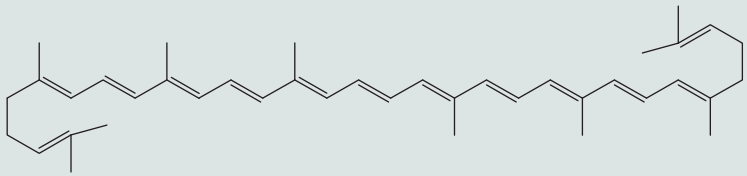
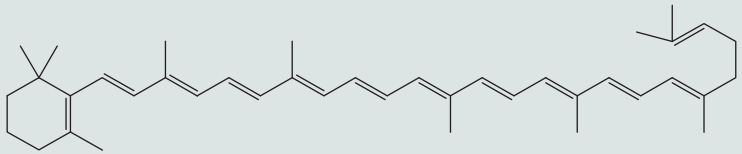
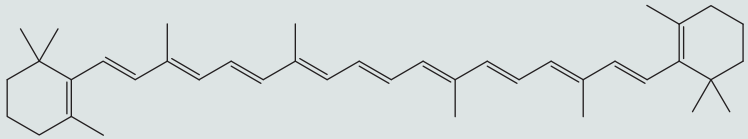
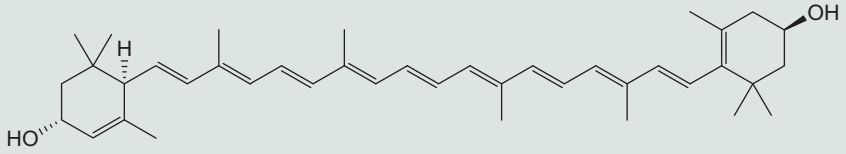
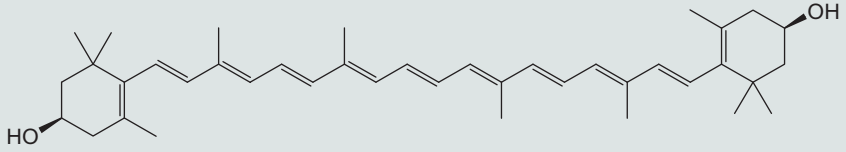
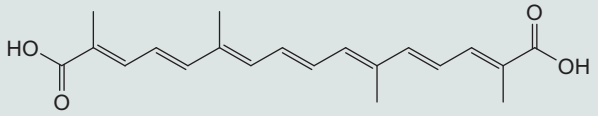
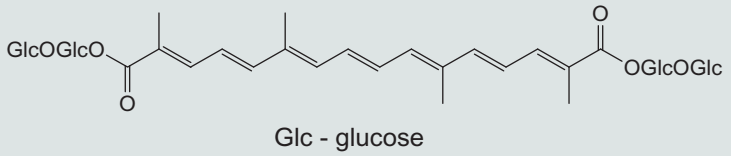
Name of Compounds	Structure	Resource	Habitat
Lycopene		Tomato (*Solanum lycopersicum* L., Solanaceae)	Originated in the South American Andes
γ-Carotene		Marigold (*Calendula officinalis* L., Asteraceae)	Native to southern Europe
β-Carotene		Carrot (*Daucus carota* L., Apiaceae)	Native to temperate regions of Europe and southwest Asia
Lutein		Spinach (*Spinacia oleracea* L., Amaranthaceae)	Native to central and southwestern Asia
Zeaxanthin		Paprica (*Capsicum annuum* L., Solanaceea	Native to southern North America and northern South America
Crocetin (unusual C_{20}-carotenoid)		Gardenia (*Gardenia jasminoides* J. Ellis, Rubiaceae	Originated in Asia and is most commonly found growing wild in Vietnam, Southern China, Taiwan, Japan, Myanmar, and India
Crocin		Meadow saffron (*Crocus sativus* L., Iridaceae)	Central and southern Europe, North Africa, and the Middle East

TABLE 11.11 The Chemical Structures, Resources, and Habitats of Rubbers

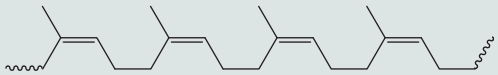
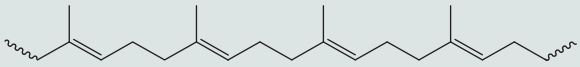
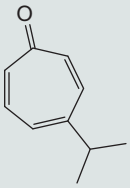
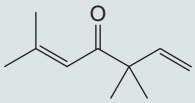
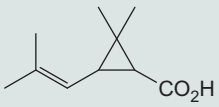
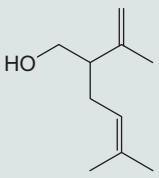
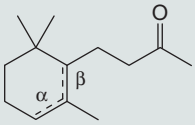
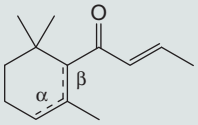
Name of Compounds	Structure	Resource	Habitat
Natural rubber (*cis*-configuration)		Rubber tree (*Hevea brasiliensis* Mull. Arg., Euphorbiaceae)	Native to Brazil (parts of the Amazon Basin and Matto Grosso) and the Guianas
Gutta percha or balata (*trans*-configuration)		*Palaquium gutta* (Hook.) Burck, Sapotaceae)	Found in Sumatra, Peninsular Malaysia, Singapore, and Borneo
		Manilkara bidentata (Aubl.) C.F. Gaertn., Sapotaceae)	Native to northern South America, Central America, and the Caribbean

TABLE 11.12 The Chemical Structures, Resources, and Habitats of Irregular Terpenoids

Name of Compounds	Structure	Resource	Habitat
Nezukone		*Thuja standishii* (Gordon) Carr. (Cupressaceae)	Native to southern Japan
Artemisia ketone		*Artemisia annua* L. (Asteraceae)	Native to temperate Asia
Chrysanthemic acid		*Tanacetum parthenium* (L.) Sch. Bip. (syn. *Chrysanthemum parthenium* (L.) Bernh.) (Asteraceae)	Native to Eurasia; specifically the Balkan Peninsula, Anatolia, and the Caucasus
Lavandulol		*Lavandula angustifolia* Mill. (Lamiaceae)	Native to the western Mediterranean
α- and β-Ionone		*Viola odorata* L. (Violaceae)	Native to Europe and Asia
α- and β-Damascone		*Rosa x damascena* Mill. (Rosaceae)	Originated in Middle East

In contrast to the mentioned mono- and sesquiterpenoids, the diterpenoids are of very limited distribution. The universally distributed diterpenes are gibberellic acid and phytol. The first one is a plant hormone and the second the side chain of chlorophyll-a [23]. Diterpenoids are of fungal or plant origin and are found in resins, gummy exudates, and in the resinous high-boiling fractions remaining after distillation of essential oils.

Sesterterpenoids are a relatively small group of terpenoids, but their sources are widespread. They have been isolated from terrestrial fungi, lichens, higher plants, insects, and various marine organisms, especially sponges. The structural conciseness and diverse bioactivity of sesterterpenoids have made them attractive targets for both biomedical and synthetic purposes [14,15].

Triterpenes are known to be of widespread distribution. This is true of the pentacyclic triterpenoids α- and β-amyrin and the derived acids, ursolic and oleanolic acids. These and related compounds occur especially in the waxy coatings of leaves and on fruits such as apple and pear. Triterpenoids are also found in resins and barks of trees and in latex. Certain triterpenoids are notable for their palate properties, particularly their bitterness. The most characteristic example is limonin, the bitter principle of *Citrus* fruits. This compound belongs to a series of pentacyclic triterpenes known as limonoids and quasinoids. They occur principally in the Rutaceae, Meliaceae, and Simarubaceae [16].

Carotenoids (tetraterpenoids) present in plants have two principal functions: as accessory pigments in photosynthesis and as coloring agents in flowers and fruits. In flowers (daffodil, pansy, marigold), they mostly appear as yellow colors, while in fruits they may also be orange or red (rose hip, tomato, paprika) [16].

Polyterpenoids can be found in rubber and occur as a colloidal suspension called latex in a number of plants, ranging from the dandelion to the rubber tree (*Hevea brasiliensis*, Euphorbiaceae). Especially rich in latex are the families of Moraceae, Apocynaceae, Euphorbiaceea, Papaveraceae, and Asteraceae. Rubber is absent in monocotyledons, gymnosperms, and lower plants.

The most important plants containing terpenoids, together with their location can be found in Table 11.13.

Mono- and sesquiterpenoids are chief constituents of the essential oils, while the other terpenoids are components of resins, waxes, and rubber.

11.4 BIOACTIVITY OF TERPENOIDS

Terpenoids are recognized as a very diverse group of natural compounds possessing a broad range of biological activities [24,25,26]. Most of them are listed in Table 11.13, however, the ones of heightened interest are mentioned here.

11.4.1 In vitro

- *Antimicrobial activity*—terpenoids have been shown to exhibit a broad spectrum of inhibitory activities against various Gram-positive and Gram-negative pathogenic bacteria. Being lipophilic, they easily permeate through the cell wall and cell membrane. Disruption of membrane integrity and potential, leakage of cellular contents, denaturation of cytoplasmic proteins, and inactivation of cellular enzymes lead to bacterial cell death [27]. The activity of terpenoids parallel their chemical structures and so the most bioactive ones tend to have a phenolic structure where the length of the aliphatic chains of terpene alcohols and the presence of double bonds are crucial. Bioactivity is increased by the presence of an aldehyde or ketone group; acetate moiety (e.g., geraniol and geranyl acetate) or the position of the hydroxyl group/s. Activity of terpenoids with a nonphenolic structure depends on the type of alkyl substituent. Also, α-isomers are less active compared to β-isomers (pinene) and *cis*-isomers compared to *trans*-isomers (e.g., geraniol and nerol) [28].

  Strong antibacterial activity is characteristic for thyme essential oil which also demonstrated activity against methicillin resistant *Staphyloccus aureus* and to several multidrug resistant bacterial strains. Even the vapor of the oil is highly effective against respiratory tract pathogens. The activity is mainly attributed to thymol and carvacrol. Thyme oil is also highly antifungal. Thymol interferes with the formation and viability of hyphae and induces morphological alterations in the envelope of *Candida albicans.* In addition, thymol in a dose-dependent manner exhibits anti-inflammatory effects by reducing the production and gene expression of the pro-inflammatory mediators [25,26].

TABLE 11.13 The Most Common Plants Containing Terpenoids [24,25,26]

Plant		Location	Terpenoid compounds	Activity
Artemisia absinthium L. (Asteraceae)	Herbs	Native to Europe, Asia and northern Africa	α-Thujone, (Z)-epoxyocimene, *trans*-sabinyl acetate and chrysanthenyl acetate, α-bisabolol, β-curcumen, and spathulenol	Digestive, spasmolytic, apetizer, antimicrobial
Boswellia serrate Roxb. ex Colebr. (Burseraceae)	Dried gum-resin	Native to India	Boswellic acids	Analgesic, anti-inflammatory
Carum carvi L. (Apiaceae)	Fruits	Native to Europe (Mediterranean region) and Asia	R-(+)-Carvone, limonene, carveol, and dihydrocarveol	Carminative, antispasmodic, antimicrobial
Chamomilla recutita L. Rauschert (Asteraceae)	Flowers	Europe	α-Bisabolol and its oxides A, B, C, matricin converted to chamazulene	Antispasmodic, anti-inflammatory
Cinnamomum camphora (L.) J. Presl (Lauraceae)	Wood	Native to Asian countries—China, Japan, Taiwan, Korea	Camphor	Circulatory stymulant, analeptic, antimicrobial, antiviral, antitussive, analgesics, and rubefacients
Cinnamomum verum J. S. Presl. (=*Cinnamomum zeylanicum* Nees) (Lauraceae)	Bark	Ceylon, Java, Sumatra	Cinnamaldehyde, cinnamyl acetate, eugenol, β-caryophyllene, linalool, 1,8-cineole	Antispasmodic, anti-inflammatory, antimicrobial
Coriandrum sativum L. (Apiaceae)	Fruits	Europe	Linalool, camphor, geranyl acetate	Spasmolytic, carminative
Eucalyptus globulus Labill. (Myrtaceae)	Leaves	Indigenous to Tasmania and south eastern Australia, cultivated in the coasts of Spain, Black sea and the Caucasus	1,8-Cineole, p-cymene, α-pinene, β-pinene, myrtenol, pinocarveol, α-terpineol	Antiseptic, carminative, expectorant
Euphrasia officinalis L. *Euphrasia rostkoviana* Hayne (Scrophulariaceae)	Herbs	Europe	Aucubin	Anti-inflammatory, antiphlogistic
Foeniculum vulgare Miller subsp	Fruits	Native to Europe (Mediterranean region)	Anethole, fenchone, estragole	Spasmolytic, carminative, secretolytic, expectorant, antimiocrbial, antioxidant, antitumor, estrogenic
vulgare var.*vulgare* (bitter fennel)				
Foeniculum vulgare Miller subsp				
vulgare var. *dulce* (Miller) Thellung (sweet fennel) (Apiaceae)				
Gentiana lutea L. (Gentianaceae)	Roots	Native to the mountains of central and southern Europe	Secoiridoid glycosides (sweroside, swertiamarin, gentiopicroside)	Digestive, hepatoprotective, analgesic, and wound-healing

Ginkgo biloba L. (Ginkgoaceae)	Leaves	Native to China	Ginkgolides A, B, C, J, bilobalide	Antioxidant, improving cognition, and memory
Hyssopus officinalis L. (Lamiaceae)	Herb	Native to southern Europe and Eurasia, North America	Marrubiin, oleanolic acid, pinocamphone, isopinocamphone, β-pinene, limonene	Expectorant, antiseptic
Inula helenium L. (Asteraceae)	Roots	Naive to Europe, Asia, Africa	Eudesmane, guaiane, germacrane types of sesquiterpenoids lactones	Antitumor, anti-inflammatory
Juniperus communis L. (Cupresaceae)	Pseudo-fruits	Europe	α-Pinene, limonene, myrcene, sabinen, terpinen-4-ol	Diuretic, antiseptic, anti-inflammatory
Kigelia africana (Lam.) Benth. (Bignoniaceae)	Root bark	South, central, and west Africa	Verminoside	Anti-inflammatory, antiphlogistic
Lavandula angustifolia Miller (Lamiaceae)	Flowers	Europe	Linalool, linalyl acetate, *cis*-ocimen, terpinen-4-ol, limonene, 1,8-cineole, camphor, lavandulyl acetate, lavandulol	Sedative, analgetic, anti-inflammatory, antibacterial
Melaleuca alternifolia (Maiden and Betch) Cheel (Myrtaceae)	Leaves	Australia	1,8-Cineole, terpinen-4-ol, γ-terpinene, α-terpinene,	Immunostimulant, anibacterial, anti-inflammatory, antiseptic
Melaleuca cajuputi Powell (Myrtaceae)	leaves	Australia, India, southeast Asia	1,8-Cineole	Antiseptic (mostly for respiratory track disorders)
Melaleuca quinquenervia (Cav.) S. T. Blake (niaouli) (Myrtaceae)	Leaves	Australia, southeast Asia, New Caledonia, Madagascar	1,8-Cineole, nerolidol, viridiflorol	Strong antiseptic
Melissa officinalis L. (Lamiaceae)	Leaves	Native to south-central Europe, north Africa, central Asia	Citral, neral, citronellal, β-caryophyllene, germacrene D, ursolic and oleanolic acids	Antispasmodic, sedative, antimicrobial, antiviral
Mentha x piperita L. (Lamiaceae)	Leaves	Native to Europe	Menthol, menthone, limonene, 1,8-cineole, menthofuran, isomenthone, menthyl acetate, viridiflorol	Digestive, carminative, spasmolytic, choleretic, antimicrobial
Myristica fragrans Houtt. (Myristicaceae)	Seeds	Native to Indonesia	Sabinene, α-pinene, β-pinene, limonene	Digestive, carminative
Ononis spinosa L. (Leguminosae)	Roots		α-Onocerin	Diuretic, antiseptic, and antimicrobial
Pelargonium graveolens (Geraniaceae)	Leaves	Native to South Africa, Zimbabwe, and Mozambique	Citronellol, geraniol, terpinen-4-ol, linalool, linalyl acetate, β-phellandrene, 1,8-cineole, limonene, citronellyl formate, isomenthone	Analgesic
Pimpinella anisum L. (Apiaceae)	Fruits	Native to Egypt, cultivate widely	Anethole, estragole, anisaldehyde, linalool, α-terpineol	Antispasmodic, carminative, antisecretolytic and expectorant, antimiocrbial, antioxidant, antitumor

(*Continued*)

TABLE 11.13 (Continued)

Plant		Location	Terpenoid compounds	Activity
Pinus silvestris L. (Pinaceae)	Resin obtained from live trees	Native to Europe and Asia	Turpentine (mixture of terpenes, mainly the monoterpenes α-pinene and β-pinene, Δ^3-carene, camphene, dipentene, terpinolene	Analgesic, anti-inflammatory, antiseptic, expectorant
Plantago lanceaolata L. (Plantaginaceae)	Leaves	Europe, Asia	Aucubin, catalpol, asperuloside	Anti-inflammatory, expectorant, antibacterial, antioxidant, immunostimulant
Rosmarinus officinalis L. (Lamiaceae)	Leaves	Native to Europe (Mediterranean region)	1,8-Cineole, α-pinene, camphor, carnosol	Carminative, spasmolytic, antiviral, antioxidant, anticancer
Salvia officinalis L. (Lamiaceae)	Leaves	Native to Europe (Mediterranean region)	Carvacrol, thujone, geraniol, camphor, 1,8-cineole, borneol, α- and β-pinene, ursolic and oleanolic acid	Anti-inflammatory, estrogenic, antiChE, crminative, antimicrobial, antihidrotic
Stevia rebaudiana (Bert.) Bertoni (Asteraceae)	Leaves	Native to Paraguay and sections of Argentina and Brazil	Stevioside, steviol, rebaudioside A–F, steviolbioside, dulcoside A,	Anticancer, insulinotropic, glucagonostatic, and antihyperglycemic
Syzygium aromaticum (L.) Merill et L. M. Perry (Myrtaceae)	Flowers	Indigenous to the Moluccas and southern Philippines, cultivated in many tropical areas	Eugenol, acetyl eugenol, β-caryophyllene	Analgesic, antimicrobial, antifungal, antiviral
Thymus vulgaris L., *Thymus zygis* L. (Lamiaceae)	Herb	Native to Europe (Mediterranean region)	Thymol, carvacrol, p-cymene, γ-terpinene, linalool, β-myrcene, terpinen-4-ol, ursolic and oleanolic acids	Expectorant, secretomotoric, spasmolytic, antibacterial, anti-inflammatory
Tripterygium wilfordii Hook. f. (Celastraceae)		Native to south and east China, Japan, Korea and Myanmar	Triptolide (structurally unique diterpene triepoxide)	Anticancer, anti-inflammatory, immune modulation, antiproliferative, and proapoptotic activity
Verbascum hapsus L.	Flowers	Europe, northern Africa, and Asia	Catalpol, aucubin, their acylated derivates, harpagide and harpagoside	Antiseptic, astringent, and expectorant
Verbascum phlomoides L. (Scrophulariaceae)				
Zingiber officinale Roscoe (Zingiberaceae)	Rhizome	Originates from Southeast Asia	α-Zingiberene, β-sesquiphellandrene, β-bisabolene, α-farnesene, zingiberol, camphene, β-phellandrene, 1,8-cineole, geraniol, *ar*-curcumene	Digestive, carminative, anti-inflammatory, antiemetic

Due to the strong antibacterial activity of *Caryophylli floris aetheroleum* against a series of bacteria and yeasts, it is popularly used in dentistry for the treatment of toothache, and minor infections of the mouth. Eugenol, the main constituent, significantly reduced the number of colony forming units sampled from the oral cavity. It reversibly activates calcium and chloride ion channels that may be responsible for the analgesic activity. Eugenol may exert its antinociceptive effect via the capsaicin receptor located on the sensory terminals in the spinal cord. Anti-inflammatory effects of eugenol are related to dose-dependent inhibition of the NO production [26].

- *Anticancer activity*—the mechanism of action is based on the prevention of tumor cell proliferation through necrosis or through induction of apoptosis. Their anticancer effects are associated with a decrease in inflammation and oxidative stress; however, some of them, like β-caryophyllene, thymol, or eugenol, produce oxidative stress in cancer cells without increasing oxidative stress in normal cells. Terpenoids play an important role in depolarization of the membrane of cancer cells and especially in the membrane of mitochondria, activation of apoptosis by caspases, or inactivation of the PI3K/Akt/NF-κB pathway and inhibition of angiogenesis. Terpenoids are also known to demonstrate significant anticancer capabilities in combination with chemotherapy agents. β-Caryophyllene facilitates the passage of paclitaxel through cancer cell membranes and thus potentiates its anticancer activity [29]. Eugenol is one of the most active and then most studied. It induces apoptosis of cervical cancer cells without toxicity to healthy cells, at a concentration of 0.5 μM and inhibits cell growth by 50% after 24 h in melanoma cell lines. Administrated 125 mg/kg twice a week intraperitoneally in mice (melanoma xenograft model) it reduces the size of tumors by 40% compared with control animals [29].
- Menthol—the main compound of essential oils is well known for its cooling effects due to its ability to chemically activate the cold-sensitive transient receptor potential cation channel. It is worth noting that (1 R,3 R,4 S)-(−)-menthol was able to amplify the pain threshold, while (+)-menthol did not exhibit any analgesic effects [30].
- Menthol and limonene have been considered the most effective transdermal penetration enhancers, significantly increasing the transdermal delivery of certain drugs such as caffeine and hydrocortisone. Additionally, it may also enhance the skin penetration of a variety of drugs. Therefore, a topical formulation, which includes menthol due to its cooling and penetration enhancing effects in combination with anti-inflammatory and analgesic effects such as indomethacin, may contribute to synergistic bioactivities [30].
- A long time traditional medicinal use of *Melissa officinalis* L for the relief of mild symptoms of mental stress, aid in sleep, and for the symptomatic treatment of digestive disorders is well documented. In vitro experiments showed that essential oils have an affinity to binding sites of the GABAA receptor. Isolated components of essential oils exhibited spasmolytic activities on isolated guinea pig ileum, rat duodenum, and on the jejunum and aorta of rabbits. The essential oil also had relaxant effects on guinea pig tracheal muscle and inhibited phasic contractions [26].
- Iridoid glycosides—aucubin and catalposide—strongly suppress the nuclear factor-κB (NF-κB) signaling, a master regulator in the pathogenesis of inflammatory diseases and cancer [31]. Catalposide was also reported to have strong antimicrobial properties, especially on Gram-negative infections [10]. Aucubin and geniposide have shown anticancer activities, as both iridoid glycosides were found to be able to stabilize covalent attachments of the topoisomerase I subunits to DNA at sites of DNA strand breaks, generating cleavage complexes intermediates, hence being active as poisons of topoisomerase I, but not topoisomerase II [32]. Geniposide, a major iridoid glycoside of *Gardenia jasmoides* (Rubiaceae), is also reported to have chemopreventive properties [10].

Antimicrobial agents—mostly by disruption of membrane integrity (especially tymol, carvacrol, eugenol).
Anticancer agents in vitro—mostly by prevention of tumor cell proliferation through necrosis or through induction of apoptosis.

11.4.2 In vivo

- *Anticancer activity*—limonene, one of the most widespread monoterpenes, is found mostly in the essential oils of citrus fruits. Limonene occurs in two optically active forms, S-(−)-limonene and R-(+)-limonene. R-(+)-limonene and its oxygenated derivative, perillyl alcohol, were extensively tested in vivo for their anticancer properties (skin, lung, stomach, neuroblastoma, and leukemia). They altered gene expression, that led to apoptosis, cellular redifferentiation, and, consequently, tumor regression. The basis of the antitumor effects was through inhibition of post-translational isoprenylation of small GTP-binding proteins and thus elevated synthesis of pro-apoptotic proteins. They were effective in blocking both the initiation of cancer in animal models as well as progression and induced regression of existing tumors. Perillyl alcohol administered to mice models showed a 22% reduction in tumor

incidence and a 58% in multiplicity. Phase I clinical trial demonstrated partial response in a patient with breast cancer (oral administration, 0.5−12 g/m^2, 21 days, three cycles) [18,29].

- *Antinociceptive effects* have been investigated in different animal models for the series of essential oils such as *Nigella sativa* (thymoquinone activates) opioid receptors, *Cymbopogon citratus*, *Satureja hortensis*. (−)-Linalool, a naturally occurring enantiomer of lavender essential oil, stimulates the opioidergic, cholinergic, and dopaminergic system, as well as interacts with K^+-channels. Linalool together with linalyl acetate reduced the carrageen-induced rat paw endema thus proving to be an effective potential anti-inflammatory agents [6].
- *Antifoaming and carminative activity*—reductions in gastric and intestinal foam volume were observed. Antifoaming activity associated with the relaxation of the esophageal sphincter possibly released the gastric gas. The antimicrobial activity helps to reduce the intestinal gas. Terpenoids together with flavonoids contributed to the gastrointestinal action of peppermint tea. Antispasmodic activity seemed to be related to the reduction of the calcium influx and the block of noncompetitive contraction induced by 5-hydroxytriptamine [26].
- *Hepatoprotective*—Picroliv is an antioxidant and hepatoprotective product of natural origin, containing iridoid glycoside, a mixture of picroside I, kutkin, and kutoside, obtained from the underground parts of *Picrorhiza kurrooa* (Plantaginaceae). Picroliv (6 and 12.5 mg/kg) has shown efficacy comparable to silymarin (10 and 20 mg/kg) in rodent models. The drug has shown cholerectic and anticholestatic effect in a series of in vivo tests. Phase I and II clinical trials have been conducted and the drug has shown no side effects and is well tolerated [33].
- *Anti-inflammtory*—Harpagoside is an iridoid glycoside that was first isolated from devil's claw (*Harpagophytum procumbens*, Pedaliaceae family), a medicinal plant species, in which it appears to be the major constituent of the iridoid pool. The anti-inflammatory activity of harpagoside has been mainly evaluated in in vivo models of carrageenan-induced edema models, in which intraperitoneal treatment with 10−20 mg/kg body weight (mice or rats) doses significantly abolished paw swelling [21]. Pure harpagoside was also reported to inhibit cyclooxygenases (COX-1/2) expression and nitric oxide (NO) production by peritoneal macrophages [34]. Moreover, harpagoside inhibited the release of the RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), an inflammatory mediator, by stimulated human bronchial epithelial cells, highlighting its potential for treatment of respiratory conditions. It also ameliorated dopaminergic neurodegeneration and movement disorder in a model of Parkinson's disease by elevating the glial cell line-derived neurotrophic factor [21].

Besides pure harpagoside, *Harpagophytum procumbens* has been used since ancient times by the native population of the Kalahari Desert region of Southern Africa for treating a huge number of human ailments, including fever and diabetes, as well as antimicrobial agents. More recently, extracts of the devil's claw tubers have been shown to be effective in the treatment of degenerative rheumatoid arthritis, osteoarthritis, tendonitis, low back pain, kidney inflammation, and heart disease. Hence, *H. procumbens* has been increasingly considered an alternative to the nonsteroidal anti-inflammatory drugs [21]. At present the biggest supplier of devil's claw tubers (the raw material for production of phyto-pharmaceuticals) is Namibia and the majority of harvested dried tubers are exported to Europe (mainly Germany and France). For instance, in 2001, *H. procumbens* became the third most frequently used medicinal plant in Germany. Nowadays, in Germany ca. 60 pharmaceutical products from the Devil's claw species are available. In USA, Devil's claw extracts undergo clinical trials for treatment of hip and knee arthritis [21].

Anticancer agents in vivo—R-(+)-limonene and its oxygenated derivative, perillyl alcohol, altered gene expression, that led to apoptosis, cellular redifferentiation, and, consequently, tumor regression.
Antinociceptive (Linalool), antispasmodic, hepatoprotective (picroside and derivatives), anti-inflammatory (harpagoside), agents.

11.4.3 Clinical Trials

- Artemisinin—is one of the renowned terpene-based drugs with an established medical application. Artemisinin, earlier known as *Qinghaosu*, is a phytoconstituent isolated in 1972 from cold ethereal extracts of *Artemisia annua* L. and is a compound which possesses antimalarial activity. It is a highly oxygenated sesquiterpene, containing a unique 1,2,4-trioxane ring structure, which is responsible for the antimalarial activity of this natural product [35]. This endoperoxide group reacts with the iron in heme giving highly reactive free radicals which react with parasitic molecules (proteins, nucleic acids), leading to death. Clinical studies with patients infected with *Plasmodium vivax*

or *P. falciparum* demonstrated that artemisinin can kill the malarial parasite very quickly as it infects the human red blood cell, side effects were observed (large multicenter trial of 2,099 patients with malaria) [36]. Artemisinin was also completely effective in the treatment of chloroquine-resistant *Falciparum* malaria. Since artemisinin itself has poor bioavailability limiting its effectiveness, several semisynthetic derivatives such as artemether, arteether, and artesunate have been developed. Due to a high rate of recrudescence (about 10−25%) and the need for a 7-day course in order to achieve a radical cure when used as monotherapy, the use of artemisinins has been recommended in combination with partner drugs for the treatment of uncomplicated malaria, in the form of artemisinin combination therapies (the duration of treatment is only 3 days and reduces risk of artemisinin resistance) [35,36].

- Tanshinones—abietane diterpene compounds isolated in 1930 from *Salvia miltiorrhiza* demonstrate anticancer activity on various cancer cells (breast, liver, colon, stomach, prostate, lung, and leukemia), through inhibiting proliferation and promoting apoptosis, in a broad concentration range from sub-μM to high μM. Other important mechanisms to suppress cancer, characteristic for tanshinones, are induction of differentiation, suppression of angiogenesis through inhibiting endothelial proliferation and angiogenic differentiation, inhibition of adhesion, migration and invasion, and thus inhibition of cancer metastasis. Tanshinones may also exert their inhibitory actions through modulation of inflammatory and immune responses [37]. Other cancer-related clinical studies confirm the potential benefit of tanshinone-containing formulas for the treatment of cancer. Also, sodium tanshinone IIA sulfonate, a water-soluble derivative of tanshinone IIA was found to be clinically effective in pulmonary hypertension [37].
- Ingenol 3-angelate—the diterpene isolated from *Euphorbia peplus* L., in series of in vitro studies exhibited strong activity against a variety of tumor cell lines, including strains of malignant melanoma that are resistant to conventional therapeutic agents. As high activity was also confirmed by a series of in vivo studies, the compound was subjected to clinical trials. In 2012 ingenol 3-angelate (Picato), was approved by the FDA for treating actinic keratosis, a precancerous skin condition. Target compound in concentrations of 0.0025%, 0.01%, and 0.05% (as a gel), applied for 1−2 days was found to be safe and efficacious, and depending on the region of the body, showed clearance rates varying from 27.8 to even 88.9%. Ingenol 3-angelate might find wider uses: it is currently undergoing phase II clinical trial for the topical treatment of nonmelanoma skin cancers, such as basal cell carcinoma, squamous cell carcinoma, and intraepidermal carcinoma and is of promise as a new antileukemic agent [38].
- *Ginkgo biloba*—is considered as one of the oldest living tree species. Dry standardized extract contains flavonol glycosides, diterpenes (ginkgolides), and sesquiterpenes (bilobalide) and has been used for a long time for improvement of blood circulation and strengthening of the vessel system. It is also well known for the symptomatic treatment of brain-related impairment of mental performance [26]. *Ginkgo biloba* is recommended for age-related cognitive decline and for slowing the progress of neurodegenerative disorders, such as Alzheimer's disease, and for other forms of dementia (memory deficit, disturbance in concentration, depressive mood, dizziness, tinnitus, and headache). The mechanisms of action includes increasing blood supply by dilating blood vessels, reducing blood viscosity, modification of neurotransmitter systems (inhibition of monoamine oxidase A, uptake of norepinephrine, increasing synaptosomal uptake of 5-hydroxytryptamine, and extracellular levels of dopamine and noradrenaline), protection of neuronal and myocardial cells against ischemia and reperfusion injury, and reducing the density of oxygen free radicals [39]. The ginkgolides A, B, and C also antagonize platelet aggregation factor (PAF) and reduce the release of inflammatory and allergic response mediators. Neuroprotective activity is the effect of improving the blood flow or microcirculation, inhibition of PAF, as well as an antioxidant action as a free radical scavenger [26]. Many clinical trials have been conducted to assess these potential properties; however, obtained results remain controversial. Some of them showed improvement of cognitive function in AD with daily dose ranged from 80 to 600 mg/day (treatment periods ranging from 3 to 52 weeks), while others showed inefficacy as no reduction of incidences of AD were noticed. Meta-analysis, obtaining the treatment effects of *Gingko biloba* standardized extract on 2,561 patients with cognitive impairment and dementia showed the overall benefits for stabilizing or slowing decline in cognition, function, and behavior (duration of 22 to 26 weeks). All these clinical benefits were mainly associated with the 240 mg/day dose [25,26,39].
- *Mentha piperita* is a highly aromatic perennial plant native to Europe and has been used since ancient time as an important flavoring ingredient. In a series of clinical studies peppermint oil demonstrated antispasmodic activity on gastrointestinal tract smooth muscle. This effect is principally due to the activity of menthol, which is a calcium channel antagonist of the intestinal smooth muscle. Enteric-coated peppermint oil (0.2 mL) significantly decreased symptoms of irritable bowel syndrome and seems to be more effective even than hyoscyamine [25].
- Tea tree oil has been used as a traditional medicine for more than 30 years in Europe and around the world, particularly in Australia as it possesses strong antimicrobial properties, especially in treating cutaneous infections and terpinen-4-ol is the main antimicrobial compound. Tea tree oil has been widely investigated in several clinical

studies, which showed its efficacy as an antiseptic in various conditions. In a concentration of 5% the oil was an effective treatment for mild to moderate acne vulgaris. Clinical trials also support its efficacy in the treatment of tinea pedis, onychomycosis (dermatophytic infection of the nails) and oropharyngeal candidiasis—the most common opportunistic infection observed in patients with HIV/AIDS. Treatment with tea tree oil can influence positively wound healing through its antimicrobial activity and is effective in skin lesions or dandruff. Antibacterial activity against *Staphylococcus aureus*, both methicillin-susceptible (MSSA) and -resistant (MRSA) has been demonstrated. The minimum killing tea tree oil concentration was 0.25% and 0.5% for *C. albicans* and nonalbicans *Candida* species, respectively [26].

- A combination of seven naturally occurring terpenes (31% α- and β-pinene, 15% camphene, 10% borneol, 4% anethole, 4% fenchone, and 3% 1,8-cineole) was introduced in Europe in the early 1960s. Due to the antibacterial effects related mostly to pinenes, diuretic, anti-inflammatory, and analgesic properties, the drug has been considered as effective on conservative stone management and reducing symptomatology during spontaneous stone passage. Several trials have been carried out to confirm the efficacy—after 4 weeks of treatment, significantly higher expulsion rates of urinary stones were noticed (81% vs. 59% of control group with placebo). Additionally significantly higher rates of treatment success within 3 weeks of treatment were demonstrated after shock wave lithotripsy, where faster and more efficient stone expulsion was observed. However, large-scale trials are still missing [40].
- Betulinic acid (BA) is a lupane-structured pentacyclic triterpene, which was recognize as effective against HIV through the inhibition of replication. Bevirimat, one of the derivatives, prevents HIV-1 virus maturation and virus release from infected cells and is currently under further investigation in clinical trials [41]. Besides some other interesting activities, such as anti-inflammatory, antimalarial, immunomodulatory, antiangiogenic, antifibrotic, and hepatoprotective, BA showed in vitro cytotoxic activity with interesting IC_{50} values ranging from 0.5 to 4.8 mg/mL for melanoma cell lines, 14−17 mg/mL for human neuroblastoma cell lines, other brain tumors such as 3−13.5 mg/mL for medulloblastoma and 2−17 mg/mL for glioblastoma. Antiproliferative effects in ovarian carcinoma, nonsmall-cell and small cell lung carcinoma, cervix carcinoma, and neuroblastoma cells resistant to other treatment remained sensitive to treatment with BA, whereas the cytotoxicity in normal cells is considerably lower. BA induces apoptosis, generates ROS, thus influences processes of mitochondrial outer membrane permeabilization, inhibits the activation of NF-kB by different stimuli, inhibits the aminopeptidase N—the enzyme taking part in the angioproliferative and metastatic activity of tumors [41,42].

  One of the major drawbacks of BA and its derivatives is their poor solubility due to the lipophilic character, thus, only a few in vivo experiments could be found. Polyvinylpyrrolidone-complexed betulinic acid was injected into nude mice bearing subcutaneous human melanoma xenografts. A dose of 50 mg/kg body weight injected every 4 days was enough to prevent tumor outgrowth and six injections of the same dose induced tumor regression. BA showed antimetastatic activity by itself and in combination with vincristine in a melanoma mouse model (10 mg/kg per day). Oral application was also effective in inhibition of outgrowth of a prostate cancer cell line [41].

  Betulinic acid (20% ointment) is under evaluation in Phase I/II clinical trial for the treatment of dysplastic nevi with the potential to transform into melanoma [42].
- Ursolic acid (UA) is a pentacyclic carboxylic acid present in medicinal herbs, waxlike protective coatings of some fruits like apple peel, and plants such as rosemary. It demonstrates bioactivities ranging from anti-inflammatory, antiproliferative, proapoptotic, antimetastatic to antiangiogenic, reported in both in vitro and in vivo models. Mechanisms of actions include, UA's ability to induce cancer cell apoptosis, prevent tumorigenesis, and inhibit cancer cell proliferation through several signaling pathways such as NF-kB. Similarly, the poor solubility should be noted, which results in low therapeutic potential and restriction to further clinical applications. In vivo experiments showed inhibition of the growth of androgen-independent prostate cancer (200 mg/kg bw; 6 weeks) and significant antitumor activity in a leukemic nude mice model (50 mg/kg, 20 days) [43]. There is scientific evidence of documenting important benefits of UA level in vitro and in vivo on insulin, metabolism of lipids and glucose, as well as on the body weight and metabolic parameters. There are ongoing clinical trials aiming to elucidate the effect of UA administration on insulin sensitivity and metabolic syndrome.
- Lavender essential oil orally administered (capsules of 100, 200 μL) had anxiolytic effect in a series of clinical trials. At a dose of 80 mg it effectively ameliorated generalized anxiety comparable to a common benzodiazepine (lorazepam). The essential oil was also effective in reduction of anxiety when aromatherapy was applied (massage, inhalation). Lavender flower tincture moderately improves the level of depression; however, significant improvement was observed when it was coadministered together with imipramine. A few drops of essential oil on a pillow significantly increased actual sleep time, as well as ease of getting to sleep, and quality of sleep was positively improved.

In in vivo experiments, lavender oil inhibited electroshock-induced convulsions (at 140 mg/kg body weight) and blocked convulsions induced by the lower dose of pentetrazol (inhalation). Its sedative effect was evidenced by its increased effects on the of duration anesthesia. Linalol and linalyl acetate reduced edema at about 30%, showing significant anti-inflammatory properties [26].

- Eucalyptus leaves have been used in medicine mostly due to the presence of essential oils. The main constituent of the volatile oil derived from fresh leaves of eucalyptus species is 1,8-cineole. The content of cineole may contribute to the effects. Application for the treatment of disorders of upper respiratory tract and colds have been well described. It also stimulates the excretion of saliva and gastric juice. It is used as a remedy for fever, influenza, and as antispasmodic stimulant agents in bronchitis and asthma. 1,8-cineole suppressed arachidonic acid metabolism and pro-inflammatory cytokine production, is easily transported into tissues and remains there for a long terminal half-life, therefore it was found to be especially useful in curing chronic ailments of respiratory infections. Beside an oral application of Eucalyptus oil, topical use as an antiseptic agent, repellent, and for the treatment of rheumatic complaints and neuralgias have been reported. Externally used, it has increasing effects on blood flow and skin temperature. Several studies on the effects on bacterial strains, viruses, fungi were conducted, unfortunately effects of the essential oil were recognized in concentrations which are most likely to be too high to get achieved in clinical situations. Recommended dose was established up to 200 mg. Most in vivo effects were investigated only in concentrations above this (expectorant properties, anti-inflammatory effects) [26].

  Clinical data on Eucalyptus leaves or preparations are still not efficient, but the effects of 1,8-cineole after inhalation have been evaluated. A study on 20 healthy humans showed that an inhalation of 100 μL of 1,8-cineole led to an increased feeling of relaxation, acts as mucolytic, and an anti-inflammatory stronger than steroids used in bronchial asthma [26].
- *Valeriana officinalis* L. roots are known to be active on the central nervous system—mostly due to the presence of sesquiteprenoids. Hydroxyvalerenic acid and acetoxyvalerenic acid inhibited the catabolism of GABA at synaptic junctions of the CNS in vitro. Valerenic acid, valerenal, and valeranone demonstrated sedative and muscle relaxant activities. In addition, they prolonged the barbiturate induced sleeping time. Results from a series of trials confirmed a clinical effect in sleep disturbances, especially in elderly patients. The effect increased during treatment over several weeks; however, there is still a need for rigorous trials to determine the efficacy [26].
- *Boswellia serrata* crude extract decreased the knee pain, increased knee flexion, and increased walking distance after 8 weeks of treatment in patients with osteoarthritis of the knee. 300 mg three times daily for a period of 6 weeks improved physical symptoms and signs such as dyspnea and bronchial asthma and decreased number of attacks in 70% of patients. Significant improvements of symptoms were noticed also in Crohn's disease as well as chronic colitis. The pharmacological effects have been attributed to boswellic acids—pentacyclic triterpene, which inhibit cathepsin G and microsomal prostaglandin E synthase, increase the activity of NF-kB, and thus act as anti-inflammatory agents. Among all acids acetyl-11-keto-β-boswellic acid seems to be the most active [44].
- Lutein belongs to the xanthophyll family of carotenoids, which are synthesized within dark green leafy plants. Lutein and its stereoisomer zeaxanthin represent the primary pigment molecule distributed within the macula. These two compounds protect the macula and photoreceptor outer retinal segments from oxidative stress. They also inhibit the expression of inflammation-related genes. There are several clinical studies that link lutein supplementation with decreased risk of age-related macular degeneration (improved visual function and prevented progression of the pathology), increased macular pigment density and improved multifocal electroretinogram responses. Supplementation of 20 mg/day of lutein or 10 mg of both lutein and zeaxanthin improved macular pigment optical density and reduced the risk of cataracts. Although the optimal dose for lutein supplementation has not yet been established, the most common dose in commercial products is 10 mg/day [45]. Another active carotenoid, lycopene, is a potent antioxidant with a singlet-oxygen quenching ability twice that of β-carotene (serves as a prehormone that is converted into retinoic acid) and ten times that of vitamin E. Clinical studies proved suitability for oral lycopene supplementation on patients with cardiovascular disease and prostate cancer patients. Lycopene displayed positive effects on the maintenance of NO levels, contributed to vasodilatation, exhibited anti-inflammatory properties, and improved lipid homeostasis. In a Phase II clinical trials, the efficacy of lycopene and soy isoflavones delayed progression of both hormone-refractory and hormone-sensitive prostate cancer [18].
- β-Sitosterol has been used for thousands of years to prevent and relieve prostate symptoms. At the dose of 60−110 mg/day, β-Sitosterol significantly improved urinary symptoms by increasing the maximum urinary flow and decreasing the volume of urine left in the bladder. The mechanism of action is believed to be associated inhibition of 5-α-reductase, thus blocking the conversion of testosterone into dihydrotestosterone responsible for the

enlargement of prostate. β-Sitosterol also displayed antioxidant and anti-inflammatory properties in addition to strengthening the immune system [46].

Terpenoids under clinical trials: artemisinin as antimalarial; tanshinones, ingenol 3-angelate, betulinic acid, ursolic acid as anticancer; standardized extract of *Gingko biloba* as antiplatelet and improving of cognitive function; *Mentha piperita* as antispasmodic; tea tree oil as antimicrobial; mixtures of monoterpenes as antiurolithiasis; lavender essential oil as anxiolytic; *Valeriana officinalis* as sedative; *Boswellia serrata* as antiosteoarthritis; lutein as improving visual function; β-Sitosterol as antiprostate.

11.5 EXTRACTION AND CHEMICAL TESTS

Chemically, terpenoids are generally lipid-soluble compounds. Most of them are colorless liquids which are lighter than water and boil between 150 and 180 °C. These are volatile in steam, usually highly refractive, and optically active. Terpenoids are unsaturated compounds having one or more double bounds. Consequently, they undergo addition reactions with hydrogen, halogens, halogen acids, etc. Some of them form hydrates. They also form characteristic addition products with NO_2, NOCl, and NOBr, which crystallize easily, and are found to be useful in the identification of terpenoids. However, there is no sensitive universal reagent for this group of compounds [16,47]. The compounds containing lactone group, like sesquiterpene lactones, give a positive reaction with the following tests [48]:

1. Kedde reagent—*Solution I*: dissolve 2% of 3,5-dinitrobenzoic acid in MeOH. *Solution II*: 5.7% aqueous KOH. *Procedure*: add one drop of each solution to 0.2−0.4 mL of the sample solution, and a bluish to purple color solution will appear within 5 min. The solution should not contain acetone, which gives a deep bluish color.
2. Baljet reagent—*Solution I*: dissolve 1 g picric acid in 100 mL EtOH. *Solution II*: 10 g MaOH in 100 mL water. *Procedure*: combine solutions I and II (1:1) before use and add two to three drops to 2−3 mg of sample; a positive reaction is indicated by an orange to deep red color solution.

For isolation of mono- as well as sesquiterpenoids the classic procedure is to obtain essential oils by steam-, hydro-, or dry distillation, or by means of mechanical treatment [6]. However, extraction with nonpolar solvents such as petroleum ether, ether, and hexane can be preferred due to artifact formation at the raised temperatures [16]. Extractions of sesquiterpene lactones, diterpenes, sterols, and less polar triterpenoids can also be performed by using ether and chloroform. Ethyl acetate and acetone extracts contain oxygenated diterpenoids, sterols, and triterpenoids. Ethanol, methanol, and water lead to the extraction of highly oxygenated triterpenes, namely polar in nature, as well as triterpenoid and sterol glycosides. Total extraction of the material carried out by any polar solvents such as acetone, aqueous methanol (80%) and aqueous ethanol followed by reextraction with hexane, chloroform, and ethyl acetate also leads to successive extraction of terpenoids and sterols [16,49].

Supercritical fluid extraction (SFE) using carbon dioxide as the extraction solvent shows great promise as a "green alternative" to conventional extraction methods, because it uses an essentially nontoxic solvent, exhibits minimal potential for artifact formation, and CO_2 can be obtained in high purity suitable for production of food-grade extracts. The addition of polarity modifiers, such as EtOH, and the development of SFE equipment capable of producing pressures in excess of 600 bar, have made possible the extraction of some compounds of intermediate polarity [48].

Gas chromatography (GC) is known as the best method for analyses of terpenoids, especially mono- and sesquiterpenoids present in essential oils. Isolation of the mono- and sesquiterpenoids is also achieved by preparative GC. For identification of individual components, gas chromatography is usually coupled to mass spectrometry (GC-MS), which provide structural information. The most frequent and simple identification method in GC-MS, which however does not always furnish unambiguous results, consists of the comparison of the acquired unknown mass spectra with those contained in a reference MS library [6].

Thin layer chromatography (TLC) can be used as another rapid, useful method for terpenoids detection with concentrated H_2SO_4 and heating, due to all terpenoids (except carotenoids) being colorless compounds [14]. There are some more TLC spray reagents for the detection of terpenoids. These are [50]:

1. Godin reagent I: *Solution I*: prepare 1% solution of vanillin in EtOH and mix in 1:1 ratio with 3% solution of perchloric acid in water. *Solution II*: prepare 10% solution of H_2SO_4 in ethanol. *Procedure*: Spray solution I onto previously dried TLC plate, and then continue with solution II. This is followed by heating at 105 °C for 3 min. Many terpenes give red and blue colors.

2. Godin reagent II: *Solution I*: prepare 1% vanillin in EtOH. *Solution II*: prepare 5% H_2SO_4 in EtOH. *Procedure*: Spray solution I onto previously dried TLC plate, and then continue with solution II. This is followed by heating at 105 ^oC for 3 min. Many terpenes give red and blue colors.

TLC also allows for the isolation of various classes of terpenoids on silica gel and silver nitrate impregnated silica gel coated plates. For isolation of various terpenoids, especially sesqui-, di-, tri-, and tetraterpenoids, column chromatography is a convenient method. As stationary phase silica gel, alumina, cellulose, sephadex, polyamide are used for the separation of different types of secondary metabolites, but of these silica gel is the most extensively used adsorbent for particularly nonpolar and medium polar compounds. Silver nitrate impregnated silica gel can also provide separation of unsaturated terpenoids [16,49].

Most of the terpenoids are hydrophobic, readily soluble in nonpolar organic solvents and insoluble in water, they are readily oxidized, polymerized, hydrogenated, halogenated, and isomerized.
Gas chromatography (GC) is the best method for analysis of volatile terpenoids.

11.6 PHARMACEUTICAL APPLICATION

As plants containing terpenoids have a broad array of pharmacological activities and are widespread all over the world, they have been used for a long time in traditional medicines. Many formulations can be found in the market place, mostly as dietary supplements, however, some of them are registered as drugs. Examples of the most common formulations together with indications can be found in Table 11.14 [25,26].

11.7 NUTRACEUTICAL APPLICATIONS

The terpenoids are the largest class of phytonutrients in green foods, soy plants, and grains. The importance of terpenoids to plants relates to their necessity to fix carbon through photosynthetic reactions using photosensitizing pigments. This dependence on photoreactive chemistry, in addition to the inability of plants to move to avoid irradiation, places a strong reliance on a spectrum of phytochemical protectants on oxidative reactions [51].

Terpenoids, especially carotenoids, have a unique antioxidant activity in their interaction with free radicals. β-Carotene, along with γ-carotene, lycopene, and lutein, seem to offer protection against lung, colorectal, breast, uterine, and prostate cancers. Carotenes are tissue-specific in their protection. Overall protective effects are therefore greater when all carotenes are taken together. Carotenes also enhance immune response and protect skin cells against UV radiation [52].

Phytosterols compete with dietary cholesterol for uptake in the intestines. They have demonstrated the ability to block the uptake of cholesterol (to which they are structurally related) and facilitate its excretion from the body [51].

The value of our nutrition, in terms of nutritional physiology, is not only conditioned by nutrient and calorie contents but adequate meal preparation and presentation. Thus fragrances play an important role to nutrition and can be associated to the terminology "soul food," as the information of scents transcend our entire being, both physically and intellectually contributing to a holistic point of view. Adding spice with essential oils according to the Aroma-Vital cuisine combines sensuality with therapeutic potential. Essential oils extracted from aromatic plants and spices are not supposed to supersede fresh herbs but complement them. For cooking, solely, 100% pure essential oils from controlled organic cultivation should be used. Oils that are not sourced from a controlled organic source should be checked for pesticide content [53].

11.8 CONCLUSIONS

Among plant secondary metabolites, terpenoids are the most abundant and diverse class of natural compounds. Terpenoids are commonly present in higher plants, and normally produced in vegetative tissues, flowers, and, occasionally, roots. The diversity of terpenoids is probably a reflection of their many biological activities in nature, which have made them a widely used resource for traditional and modern human exploitation. Naturally occurring terpenoids provide new opportunities to discover new drugs with minimum side effects. They are usually the constituents of essential oils of economic importance as flavors and perfumes. These are also commonly used as natural flavoring compounds in food industries.

TABLE 11.14 Examples of the Most Common Terpenoids Formulation and Their Application in Pharmacy [25,6]

Herbal Substance	Pharmaceutical Form	Indications
Achillea millefolium L.	Powdered herbal substance, tinctures, expressed juice, liquid extract	– Topically as soothing and antipruriginous application for dermatological ailments – gastrointestinal disorders – in problems of menstruation, in bleeding hemorrhoids
Artemisia absinthium L.	Powdered herbal substance, tinctures, expressed juice	– Dyspeptic disorders (gastrointestinal spasms, repletion and flatulence) – lack of appetite
Boswellia serrate Roxb. ex Colebr.	Gum resine extract (capsules)	– Rheumatism, arthritis – bronchial asthma – inflammatory bowel diseases (Crohn's disease, colitis ulcers)
Carum carvi L.	Herbal tea, essential oil	– Relief of digestive disorders (flatulence and indigestion)
Chamaemelum nobile (L.) All. (=*Anthemis nobilis* L. (Asteraceae)	Dried flowerheads, liquid extract	– Dyspepsia, nausea and vomiting, flatulent dyspepsia associated with mental stress – analgesic in diseases of the oral cavity
Chamomilla recutita L. Rauschert	Herbal substances, liquid and dry extracts, essential oil	– Treatment of minor gastrointestinal complaints – minor ulcers – inflammations of the mouth and throat – symptoms of common cold – inflammation of the skin
Cinnamomum camphora (L.) J. Presl	Essential oil	– Treatment of minor muscle aches and pains – for reducing of cough frequency – stimulation of heart and peripheral circulation
Cinnamomum verum J. S. Presl. (=*Cinnamomum zeylanicum* Nees)	Powdered herbal substance, tinctures, essential oil	– Dyspeptic disorders (gastrointestinal spasms, bloating, flatulence, loss of appetite, and diarrhea)
Eucalyptus globulus Labill.	Herbal substances, tinctures, essential oil	Common infections such as flu, whooping cough, loss of appetite, dyspeptic complaints, inflammatory and infectious diseases of kidneys and bladder, diabetes, rheumatic complaints
Foeniculum vulgarae L.	Herbal tea, essential oil, powder	– Dyspeptic complaints (bloating and flatulence) – infantile colic – as expectorant and anti-inflammatory in respiratory tract infections
Ginkgo biloba L.	Dry extract	– Treatment of brain-related impairment of mental performance, memory impairment, impaired concentration – symptoms typical of mild to moderate Alzheimer-type dementia – depressive mood, dizziness, and headache – lack of concentration and weakness of memory – anxiety, depressive mood – cold hands and feet with numbness, prickle, and calf pain at walking – cerebral insufficient blood supply, dizziness – peripheral vascular insufficiency
Humulus lupulus L.	Herbal tea, infusions, powdered herbal substance or alcoholic extracts	– Relief of insomnia – excitability, restlessness, and anxiety – sleep disturbances

(*Continued*)

TABLE 11.14 (Continued)

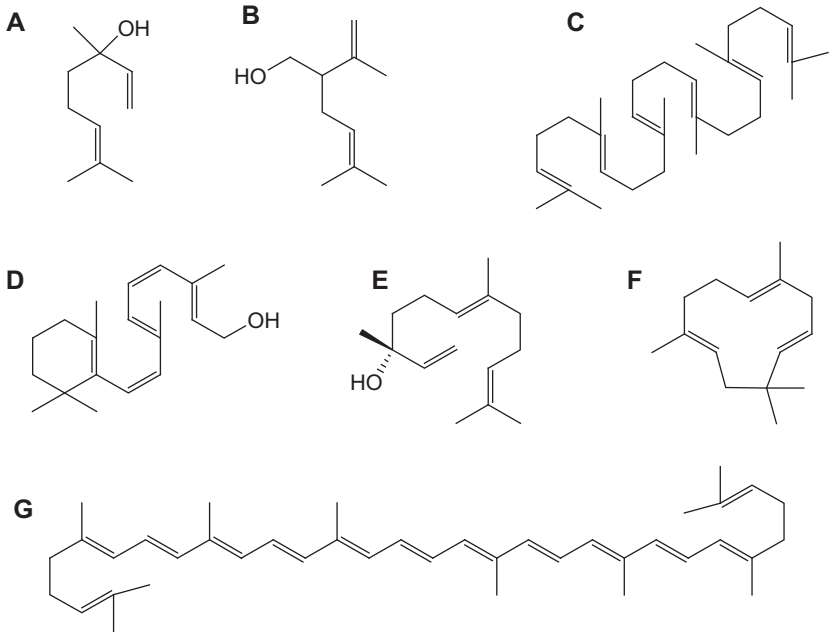
Herbal Substance	Pharmaceutical Form	Indications
Juniperus communis L.	Liquid extracts and tincture, essential oil	– Diuretic – gastrointestinal disorders
Lavandula angustifolia Miller	Herbal substances, tinctures, essential oil	– Mood disturbances such as anxious, restlessness, anxiety, insomnia, neurasthenia – gastrointestinal complaints – musculoskeletal disorders, rheumatism, neuralgia – respiratory diseases: asthma, whooping cough, influenza, bronchitis due to whooping cough – skin infections
Melaleuca alternifolia (Maiden and Betch) Cheel	Essential oil	– Cutaneous and vaginal infections – surgical and dental practice – wound healing
Melissa officinalis L.	Dry and alcohol extracts, liquid extract, tincture, powdered substances, essential oil	– Treatment of gastrointestinal disorders (bloating and flatulence) – relief of mild symptoms of mental stress and to aid sleep
Mentha piperita L.	Infusions, tinctures, essential oil	– digestive disorders (dyspepsia), flatulence, gastritis, enteritis – as cholagogue – symptomatic relief of the irritable bowel syndrome – treatment of neuralgic pain, headache, cold, and cough
Pimpinella anisum L.	Herbal tea, essential oil	– Dyspeptic complaints (bloating and flatulence) – as expectorant and anti-inflammatory in respiratory tract infections
Pinus silvestris L.	Turpentine	– Neuropathy, neuralgia, headaches – joint, muscle pains – inhalation in respiratory track disorders
Plantago lanceaolata L.	Herbal substance, dry and liquid extracts, expressed juice, sirup	– Colds of the respiratory tract – inflammation in the mouth or the throat – skin inflammation
Salvia officinalis L.	Dry extract, liquid extract, herbal substance, tincture, essential oil	– Mild dispeptic disorders – inflammations – relief of excessive sweating
Syzygium aromaticum (L.) Merill et L.M. Perry	Essential oil	– Treatment of toothache and minor infections of the mouth and skin – inflammations of the oral and pharyngeal mucosa—in dentistry for topical anesthesia – sore throats and coughs associated with the common cold – rheumatic complaints – dyspeptic disorders and flatulence
Thymus vulgaris L., *Thymus zygis* L.	Dry extracts, liquid extract, tincture, powdered substances, essential oil	– Coughs and colds with viscous mucilage – treatment of bronchitis
Verbascum thapsus L. *Verbascum phlomoides* L.	Herbal substance, dry and liquid extracts, sirup	– Treatment of respiratory disorders, asthma, spasmodic coughs, influenza – eczema, rheumatism, wounds
Zingiber officinale Roscoe	Powdered substance	– Dyspepsia – motion sickness (to avoid nausea, dizziness, and vomiting) – rheumatic complaints

Besides diverse biological activities, terpenoids pay an important role in plant−insect, plant−pathogen, and plant−plant interactions. Volatile terpenoids from flowering plants can serve as important environmental cues to attract or deter pollinators. Mono- and sesquiterpenoids are the majority of volatile compounds released from plants after herbivore damage, attracting arthropods that prey on or parasitize herbivores, in order to avoid further damage. In addition to volatile terpenoids, certain di- and sesquiterpenoids are phytoalexins involved in the direct defense of plants against herbivores, and microbial pathogens.

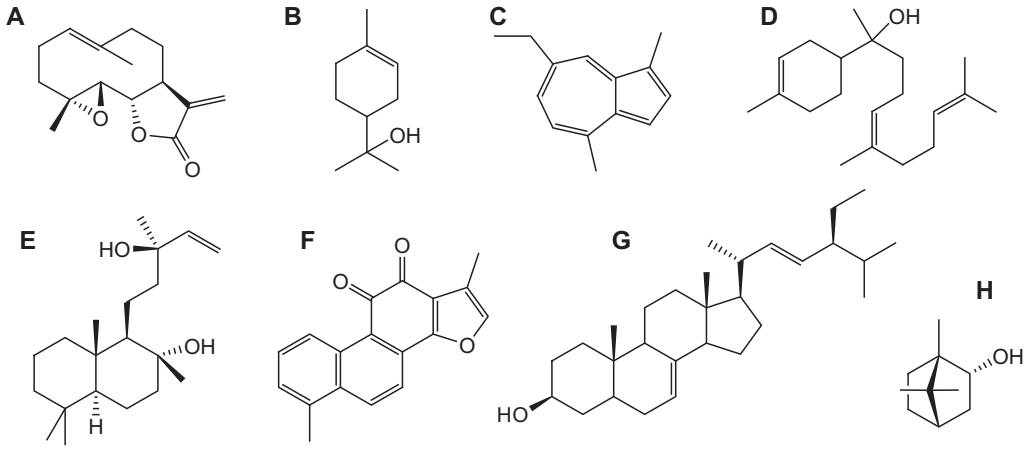
Thus, society has benefited tremendously from terpenoids, although the biological and ecochemical functions of terpenoids have not yet been fully investigated.

11.9 SELF-EVALUATION QUESTIONS

1. Please classify terpenoids according to number of isoprene units or carbon atoms?
2. Please describe modes of linking of isoprene units?
3. Which acyclic terpenoids are not linked head-to-tail?



4. What is an essential oil?
5. How would you classify these compounds? Identify the corresponding structures with the following: mono-, sesqui-, di-, and triterpenoids.



6. Describe how to extract and analyze plant terpenoids?

7. In what plant families can terpenoids be found?
8. Explain the mechanisms of anticancer activities of terpenoids?
9. Describe the relationship between chemical structure and antimicrobial activity of terpenoids?
10. What are the typical iridoids acting as anti-inflammatory agents?
11. Explain the activity of *Gingko biloba* and its clinical application?

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Chapter 12

Other Plant Metabolites

D. Simpson and S. Amos
Cedarville University, Cedarville, OH, United States

Chapter Outline

12.1 LIGNINS

Lignins are polymers that are responsible for the structural integrity of plant cell walls. They are obtained via the shikimate pathway. They contain repeating units of one or a combination of coniferyl alcohol, 4-hydroxycinnamyl alcohol, and sinapyl alcohol (Fig. 12.1). There has been considerable interest in the development of methods to isolate intact lignins from plant material. Some of the methods employed in the literature include enzymatic processes, acid hydrolysis, base hydrolysis, and a combination of enzymatic and acid hydrolyses [1−3].

12.1.1 Lignans

Lignans are natural products that occur widely in the plant kingdom. They contain the same monomers as lignins (Fig. 12.1) but are dimeric instead of polymeric. They are characterized by a phenylpropanoid core. The International Union of Pure and Applied Chemistry (IUPAC) identifies lignans as dimeric C_6C_3 coupled motifs linked at carbons 8 and 8′ (Fig. 12.2) [4]. The IUPAC identifies compounds with the coupling of the two C_6C_3 units at positions different from C8−C8′ as neolignans (Fig. 12.2) [4].

Lignans are biosynthesized via the phenylpropanoid pathway. Lignans are divided into several categories based on their molecular architecture. Categories of lignans include: arylnaphthalene, aryltetralin, dibenzylbutane, dibenzylbutyrolactone, tetrahydrofuran, and furofuran (Fig. 12.3).

Lignans have been attributed with a range of biological activities including anticancer, antioxidant, antihypertensive, antiviral, estrogenic, and insecticidal properties. Podophyllotoxin (Fig. 12.4) is the most prominent lignan due to the significant pharmacological activities of its derivatives. Semisynthetic derivatives of podophyllotoxin include the antineoplastic drugs etoposide and teniposide (Fig. 12.4). Etoposide is employed in the treatment of testicular and small cell lung cancers along with other tumors. Teniposide is approved for the treatment of acute lymphoblastic leukemia. The dietary lignans matairesinol and secoisolariciresinol (Fig. 12.4) are converted by intestinal flora to enterolactone and enterodiol (Fig. 12.4) [5]. Enterolactone and enterodiol possess estrogenic activity and have been attributed with lowered incidences of breast cancer mortality in patients with diets rich in matairesinol and secoisolariciresinol [6]. Several novel lignans have been reported in the recent literature, some of those will be discussed below.

Pharmacognosy. DOI: http://dx.doi.org/10.1016/B978-0-12-802104-0.00012-3

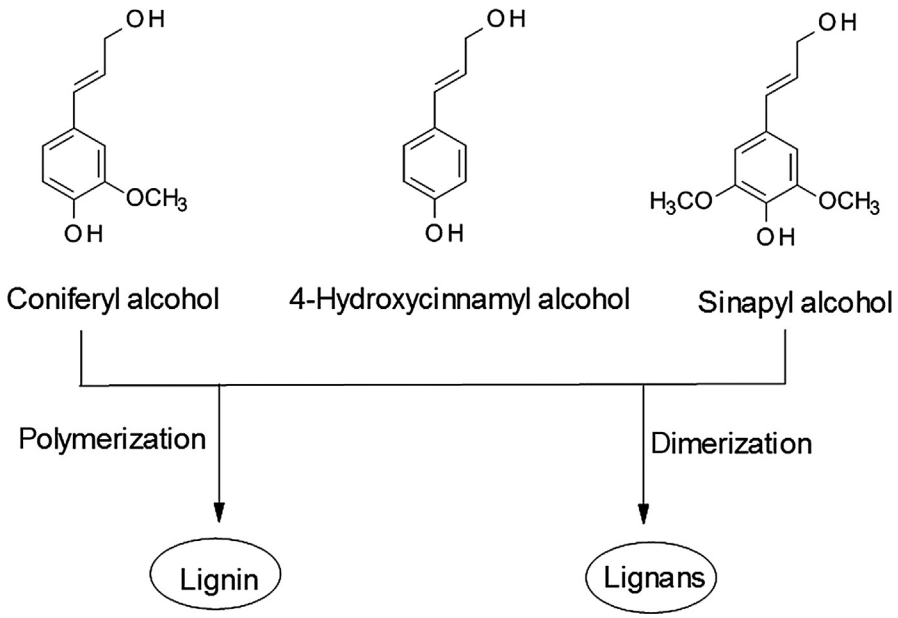


FIGURE 12.1 Structures of lignin and lignan monomers.

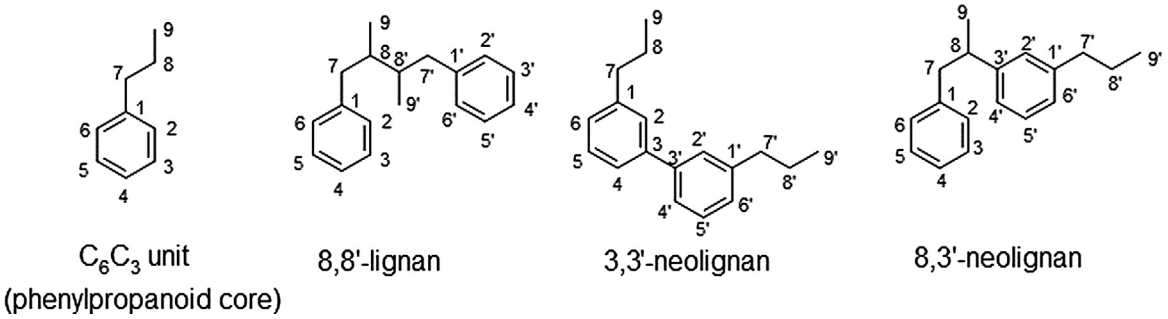


FIGURE 12.2 Lignan and neolignan core structures.

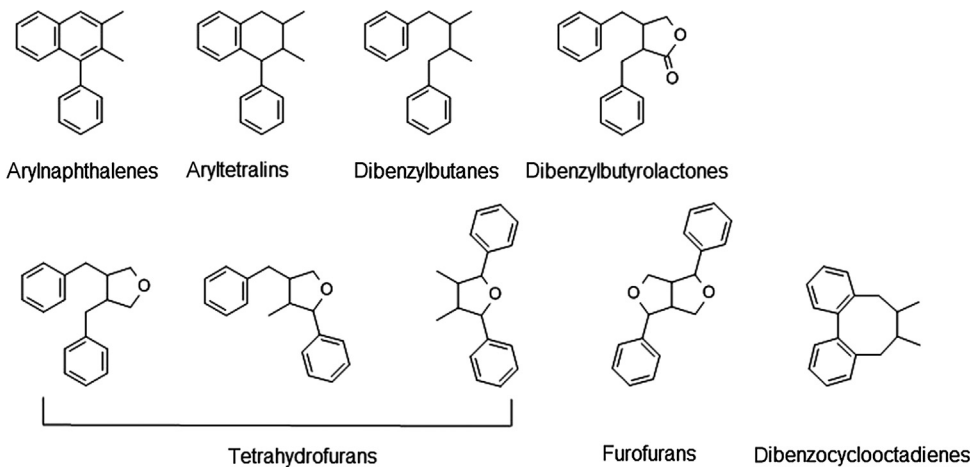


FIGURE 12.3 Structural categories of lignans.

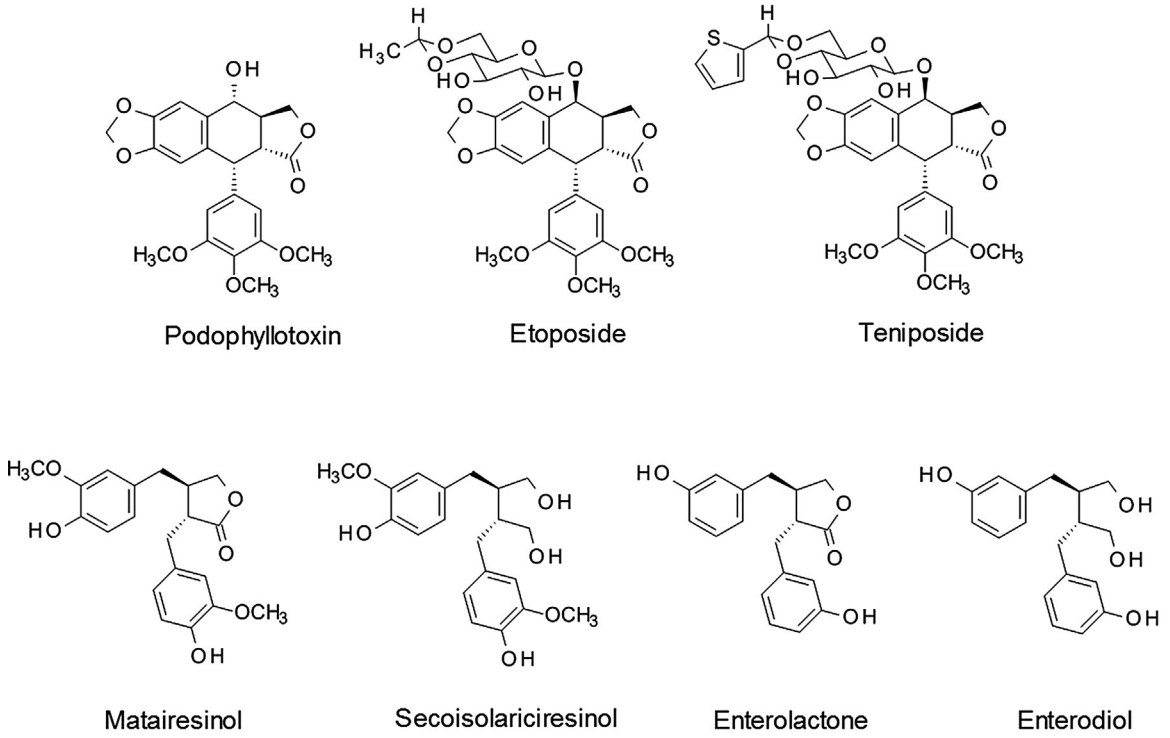


FIGURE 12.4 Examples of prominent lignans.

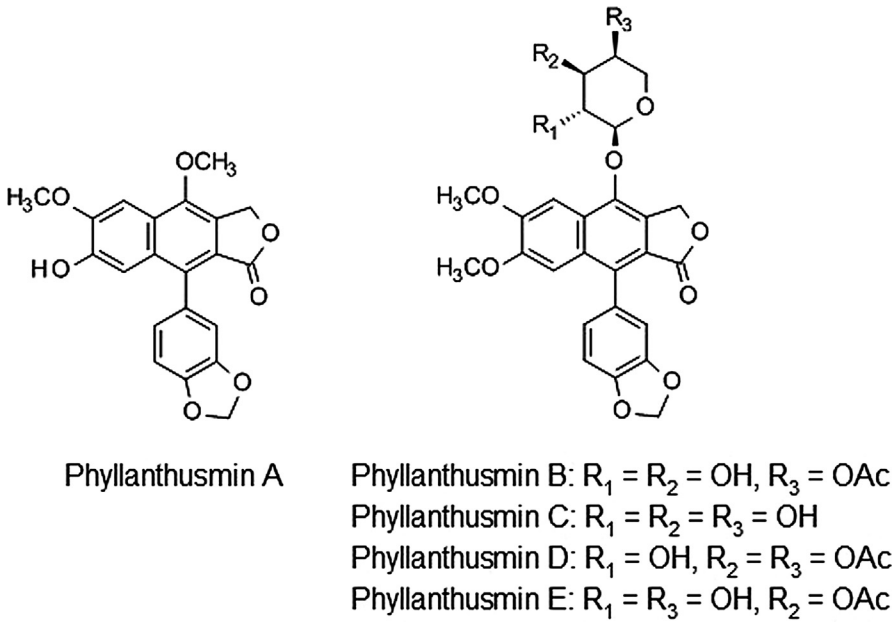


FIGURE 12.5 Examples of arylnaphthalene lignans.

12.1.2 Arylnaphthalene Lignans

These lignans possess a naphthalene core (Fig. 12.3). Phyllanthusmins A–C (Fig. 12.5) were reported from the methanol extract of the stems and roots of *Phyllanthus oligospermus* (Phyllanthaceae) by Wu and Wu [7]. Phyllanthusmin A was determined to be cytotoxic toward human epidermal carcinoma (KB) and murine leukemia (P-388) cell lines [7]. Phyllanthusmins D and E (Fig. 12.5) were isolated from the methanol extract of various parts of

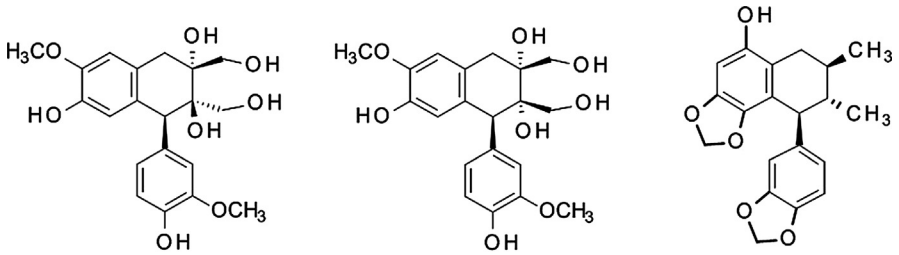


FIGURE 12.6 Examples of aryltetralin lignans.

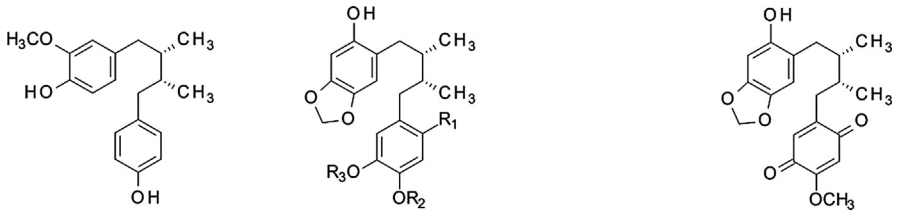


FIGURE 12.7 Examples of dibenzylbutane lignans.

Phyllanthus poilanei by Ren et al. [8]. Phyllanthusmin D displayed significant activity against the human colon carcinoma (HT-29) cell line [8].

12.1.3 Aryltetralin Lignans

These lignans have a tetrahydronaphthalene (tetralin) core (Fig. 12.3). Dolomiaeasins A and B (Fig. 12.6) were reported from the ethanol extract of the roots of *Dolomiaea souliei* (Franch.) Shih (Compositae) by Wei et al. [9]. Saurunarin (Fig. 12.6) was isolated from the ethanol extract of the aerial parts of the traditional Chinese medicinal plant *Saururus chinensis* Baill (Saururaceae) [10].

12.1.4 Dibenzylbutane Lignans

Pycnantolol (Fig. 12.7) was isolated from the dichloromethane extract of the stem bark of the African medicinal plant *Pycnanthus angolensis* (Myristaceae) by Abrantes et al. [11]. Saurulignans A–E (Fig. 12.7) were reported from the ethanol extract of the aerial parts of *S. chinensis* Baill (Saururaceae) [10]. Saurulignan E was attributed with platelet aggregation inhibitory activity [10].

12.1.5 Dibenzylbutyrolactone Lignans

Arctium lappa L. (Asteraceae), a traditional Chinese medicinal plant, was reported to be a prolific producer of a variety of structurally dissimilar lignans [12]. The aqueous ethanolic extract of the fruits of *A. lappa* yielded two novel compounds (Fig. 12.8) belonging to the dibenzylbutyrolactone group of lignans [12]. Tupichilignan A (Fig. 12.8) was identified from the methanol extract of the rhizomes of *Tupistra chinensis* (Liliaceae) [13]. The ethanol extract of *Saussurea conica* (Asteraceae) was found to produce the dimeric conicaol A and the monomeric conicaol B (Fig. 12.8), both dibenzylbutyrolactone lignans [14]. Cyclooxygenase-2 (COX-2) bioassay-guided fractionation of the methanol extract of the seeds of *Hernandia ovigera* (Hernandiaceae) resulted in the identification of (2R,3R)-5′-methoxyguayarol (Fig. 12.8) [15].

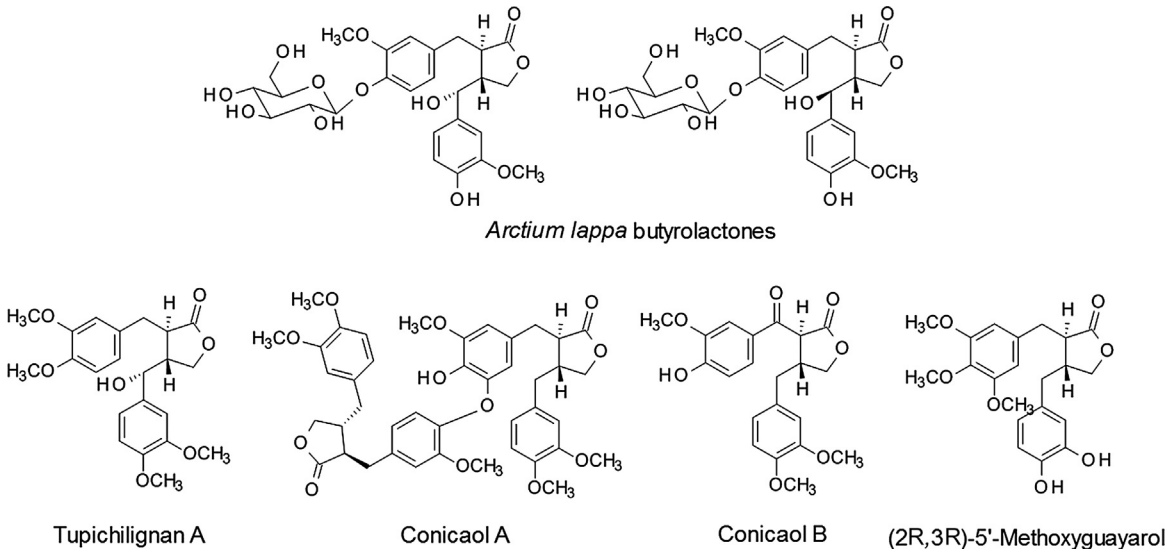


FIGURE 12.8 Examples of dibenzylbutyrolactone lignans.

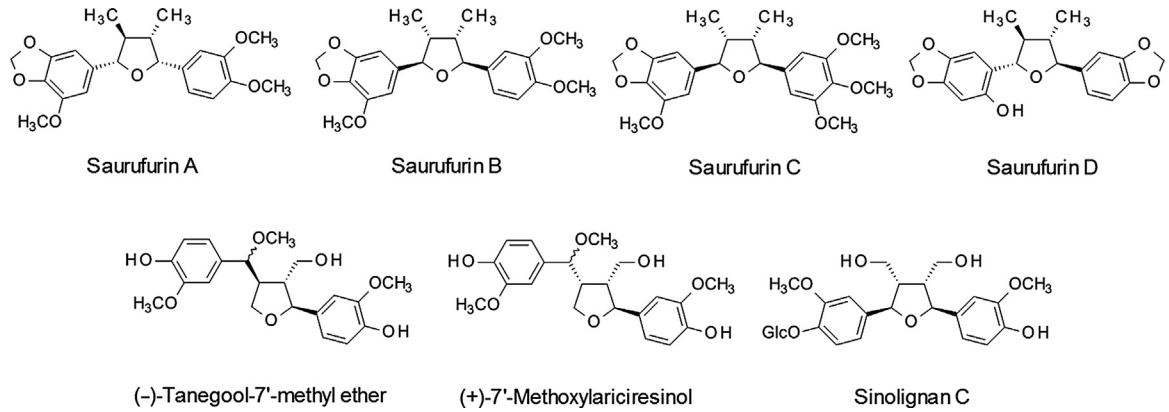


FIGURE 12.9 Examples of tetrahydrofuran lignans.

12.1.6 Tetrahydrofuran Lignans

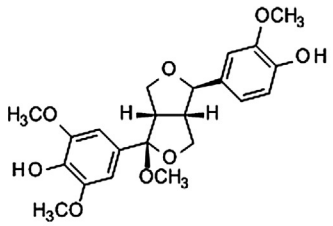
Saurufurins A–D (Fig. 12.9) are tetrahydrofuran lignans that were obtained from the ethanol extract of the aerial parts of *S. chinensis* Baill (Saururaceae) [10]. The tetrahydrofuran lignans (−)-tanegool-7′-methyl ether, (+)-7′-methoxylariciresinol, and sinolignan C (Fig. 12.9) were reported from the roots and rhizomes of *Sinopodophyllum emodi* Wall. (Berberidaceae) [16]. (−)-Tanegool-7′-methyl ether was reported to display good cytotoxic activity against human cervical adenocarcinoma (HeLa) and KB cells [16]. In fact, (−)-tanegool-7′-methyl ether was described as being more potent than etoposide against KB cells under the conditions tested [16].

12.1.7 Furofuran Lignans

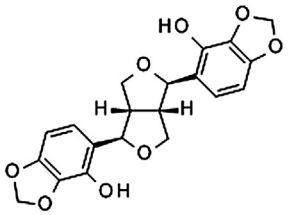
Separation of constituents of the ethanol extract of the whole medicinal herb *Chromolaena odorata* (L.) (Asteraceae) resulted in the identification of 7-methoxy-7-epi-medioresinol (Fig. 12.10) [17].

12.1.7.1 Neolignans

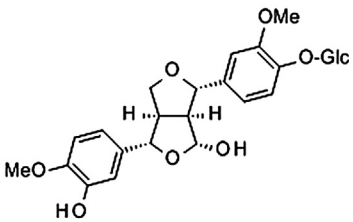
Neolignans are structurally diverse and fall under several subgroups based on their molecular architecture. Burseneolignan (Fig. 12.11) was identified from the methanol extract of the roots of *Bursera tonkinensis* Guillaum (Burseraceae) [18].



7-Methoxy-7-*epi*-medioresinol

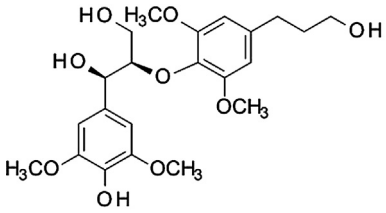


(–)-Sesamin-2,2'-diol



Petaslignolide A

FIGURE 12.10 Examples of furofuran lignans.



Burseneolignan

FIGURE 12.11 Examples of neolignans.

Cu and coworkers reported that bioassay-guided fractionation of the ethanol extract of the roots of *S. chinensis* Baill (Saururaceae) resulted in the identification of 19 new neolignans (Fig. 12.12), 15of which displayed activity against the replication of Epstein−Barr virus (EBV) [19]. Structure−activity relationship studies indicated that the dineolignan or sesquineolignan core is required for appreciable activity [19]. The tetrahydrofuran ring was also deemed important for anti-EBV activity [19]. The stereochemistry of the substituents on the tetrahydrofuran ring did not significantly influence anti-EBV activity [19].

12.1.8 Polyketides

Polyketides are widely distributed in nature and are found in plants, microbes, insects, and marine organisms. Polyketide metabolites are those compounds whose assembly is mediated by polyketide synthase enzymes [20]. There are three broad types of polyketide synthases: type I, type II, and type III [20]. Types I and II polyketide synthases mostly operate in microbes [20]. Type III polyketide synthases function in plants, bacteria, and fungi [20]. Type III polyketide synthases are homodimeric proteins possessing a single active site and utilize coenzyme A (CoA) esters [20]. Aromatization, condensation, cyclization, and decarboxylation reactions are mediated by type III polyketide synthases [20]. The type III family has plant-specific subtypes that are responsible for polyketide synthesis in plants [20]. The focus of this section will be on the metabolites produced via plant-specific type III synthases.

Polyketides share a common acetate biosynthetic pathway (Fig. 12.13). They are assembled from acetic acid via malonyl CoA and polyketide synthase and undergo a series of Claisen condensations followed by various types of complex functionalizations [20].

Structural diversification arise from the starter and extender units [20]. Starter units include malonate, acetate, and unsaturated fatty acids [20]. The extender units are typically linked to CoA and include malonyl CoA, (2*S*)-methylmalonyl CoA, and (2*S*)-ethylmalonyl CoA (Fig. 12.14) [20]. (2*S*)-methylmalonyl CoA and (2*S*)-ethylmalonyl CoA have only been observed in a few cases [20].

Polyketides possess as diverse a spectrum of pharmacological activities as their varied chemical architectures. Some properties of polyketides include antibacterial, antiparasitic, antitumor, cholesterol-lowering, and immune-suppressing activities. Plant polyketides are believed to act as allelochemicals, pigments, and virulence factors. Structural classes of compounds arising from the polyketide pathway include acetogenins, anthraquinones, flavonoids, and stilbenes. Phenyl-containing compounds obtained from the polyketide pathway contain hydroxyl and functionalized hydroxyl groups which bear a meta relationship to each other.

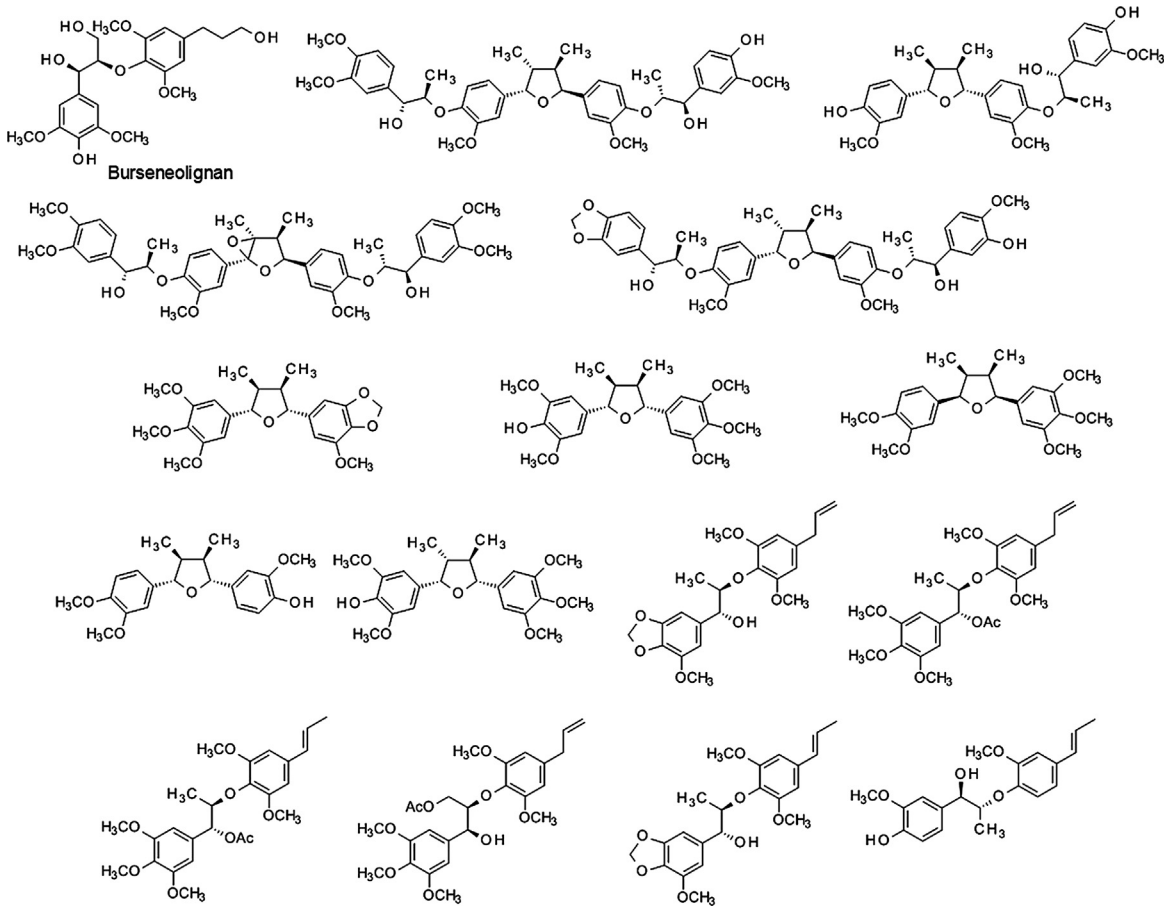


FIGURE 12.12 More examples of neolignans.

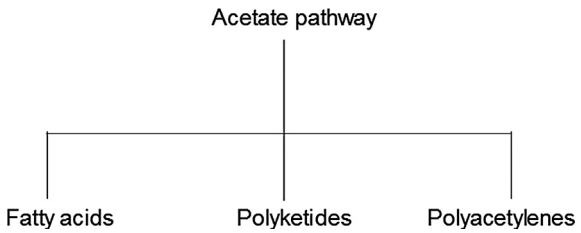


FIGURE 12.13 Branching of acetate pathway.

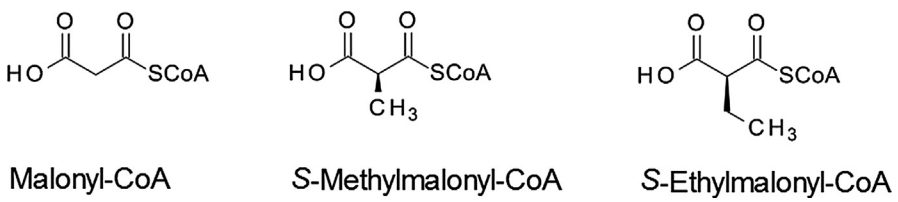


FIGURE 12.14 Polyketide extender units.

12.1.8.1 Acetogenins

The acetogenin class of polyethers is found exclusively in the Annonaceae family of plants [21]. They are typified by a C32 or C34 fatty acid chain with a terminal γ-lactone (Fig. 12.15) [22]. These molecules may also feature epoxide, hydroxyl, ketone, tetrahydrofuran, and tetrahydropyran groups [21].

Annonaceous acetogenins have been attributed with a range of pharmacological activities including antifeedant, antimicrobial, antiparasitic, antitumor, immunosuppressant, and pesticidal activities [22–27]. The stereochemical and pharmacological diversity of the acetogenins have made them attractive targets for synthetic organic and medicinal chemistry efforts worldwide. Some recent examples of acetogenins are given in Fig. 12.16. The medicinal plant *Annona muricata* is commonly known as graviola in South and North America. This plant was reported to produce muricins J–L (Fig. 12.16), compounds with inhibitory activity against human prostate cancer (PC-3) cells [28]. The seeds of custard apple (*Annona squamosa*), a succulent tropical fruit, were reported to provide annosquacins A–D, annosquatin A, and annosquatin B (Fig. 12.16) [29]. These compounds exhibited activity against human lung small cell carcinoma (A-549), human cervical adenocarcinoma (HeLa), human breast adenocarcinoma (MCF-7), human hepatocellular carcinoma (Hep G2 and SMMC-7721), and human gastric adenocarcinoma (MKN-45) cells [29].

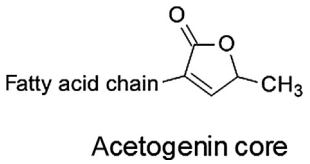


FIGURE 12.15 Acetogenin core structure.

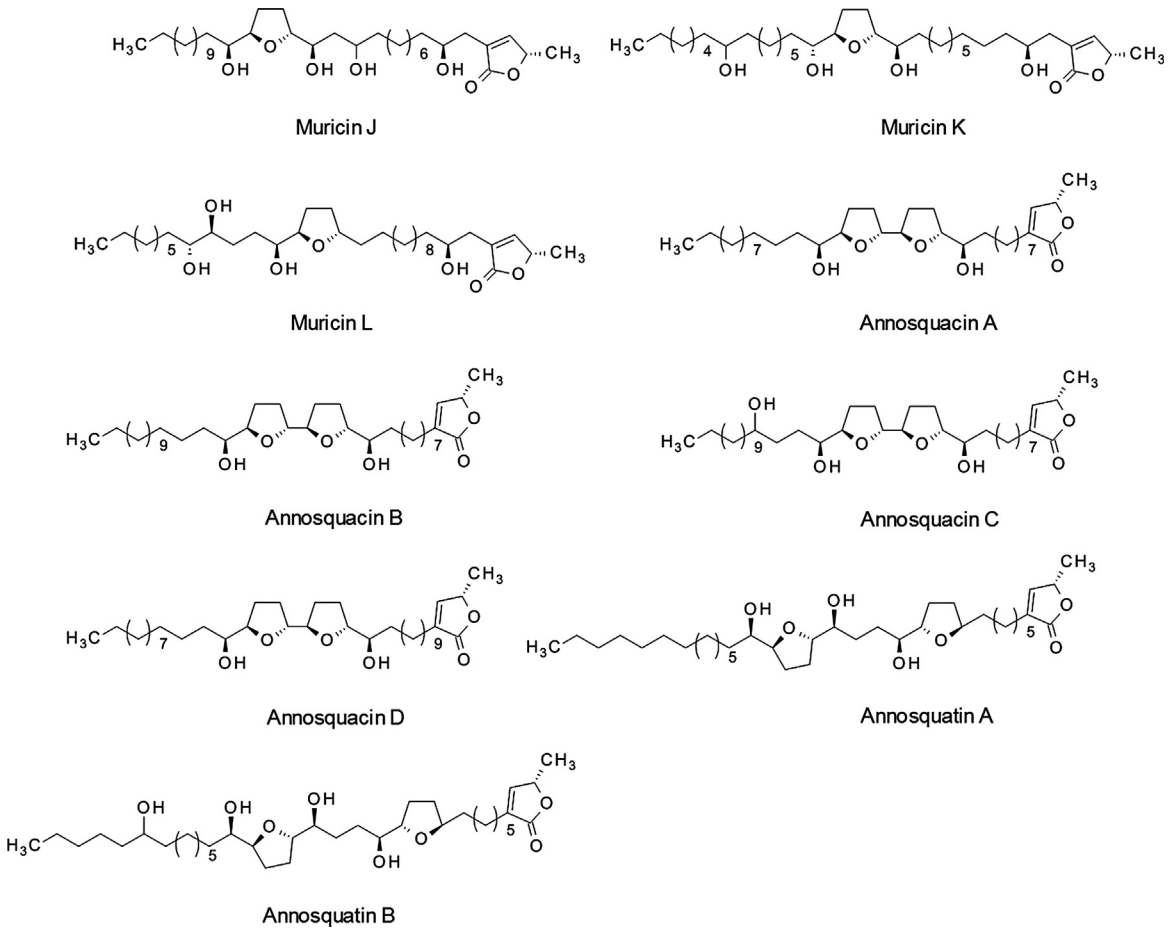


FIGURE 12.16 Examples of acetogenins.

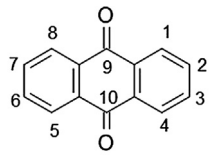
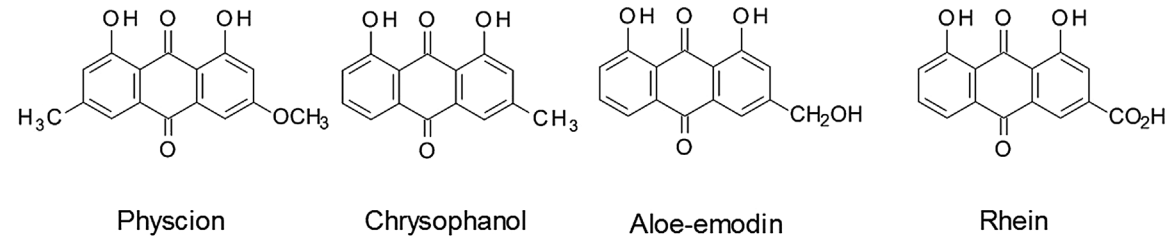


FIGURE 12.17 Anthraquinone core.



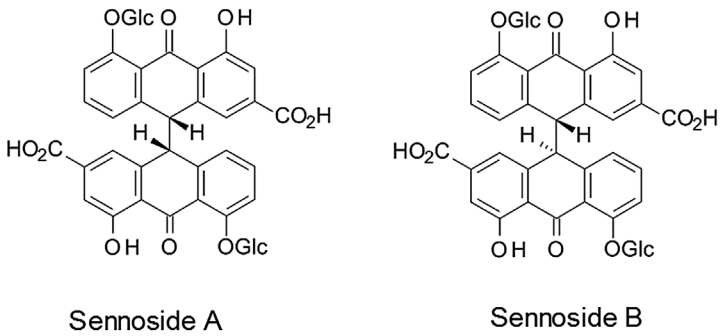


FIGURE 12.18 Notable anthraquinones.

12.1.8.2 *Anthraquinones*

Anthraquinones are structurally related to anthracene and possess the 9,10-anthracenedione core (Fig. 12.17). They are sometimes referred to as 9,10-dioxoanthracene. Anthraquinones typically occur in their glycosidic forms.

These compounds impart color to plants and have been widely utilized as natural dyes. In addition, they are also used as laxatives and possess antifungal and antiviral activities. Laxative anthraquinones include physcion, chrysophanol, aloe-emodin, rhein, and sennosides (Fig. 12.18). Sennosides are the active components of over-the-counter constipation aids prepared from plants belonging to genus *Senna*.

12.1.8.3 *Flavonoids*

Flavonoids are ubiquitous polyphenolic secondary compounds that have been reported to possess a wide range of biological activities. Flavonoids are important dietary compounds which can be found in fruits, vegetables, and other foods. Some of the reported biological activities include anticancer, antimicrobial, antioxidant, antiviral, cardioprotective, and neuroprotective properties [30,31]. Flavonoids may occur in nature in glycosidic form. Flavonoids typically contain a flavan nucleus and may be divided into anthocyanidins, catechins, chalcones, flavanones, flavones, flavonols, and isoflavones (Fig. 12.19). The substitution pattern on the central heterocyclic ring is a defining feature for the different subclasses of flavonoids. A hydroxyl group is featured at C-3 in anthocyanidins, catechins, and flavonols but is absent in flavones and flavanones [32]. A carbonyl group is present at C-4 of flavanones, flavones, flavonols, and isoflavones but is not featured in anthocyanidins and catechins [32]. Isoflavones bear the B-ring at C-3 instead of at C-2 as seen in the other flavonoid subclasses [32].

Anthocyanidins are responsible for the red, blue, and violet colors seen in some fruits and vegetables. Some of the more common anthocyanidins include cyanidin and delphinidin (Fig. 12.20).

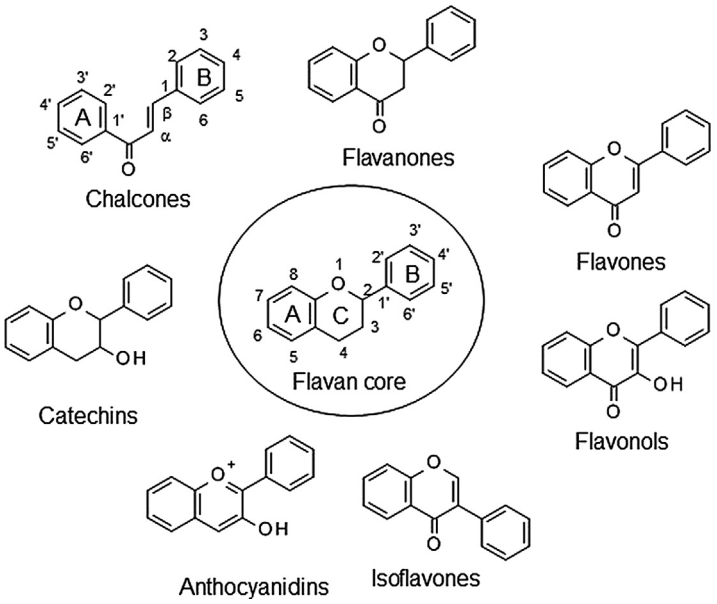


FIGURE 12.19 Structures of the flavan nucleus and main flavonoid classes.

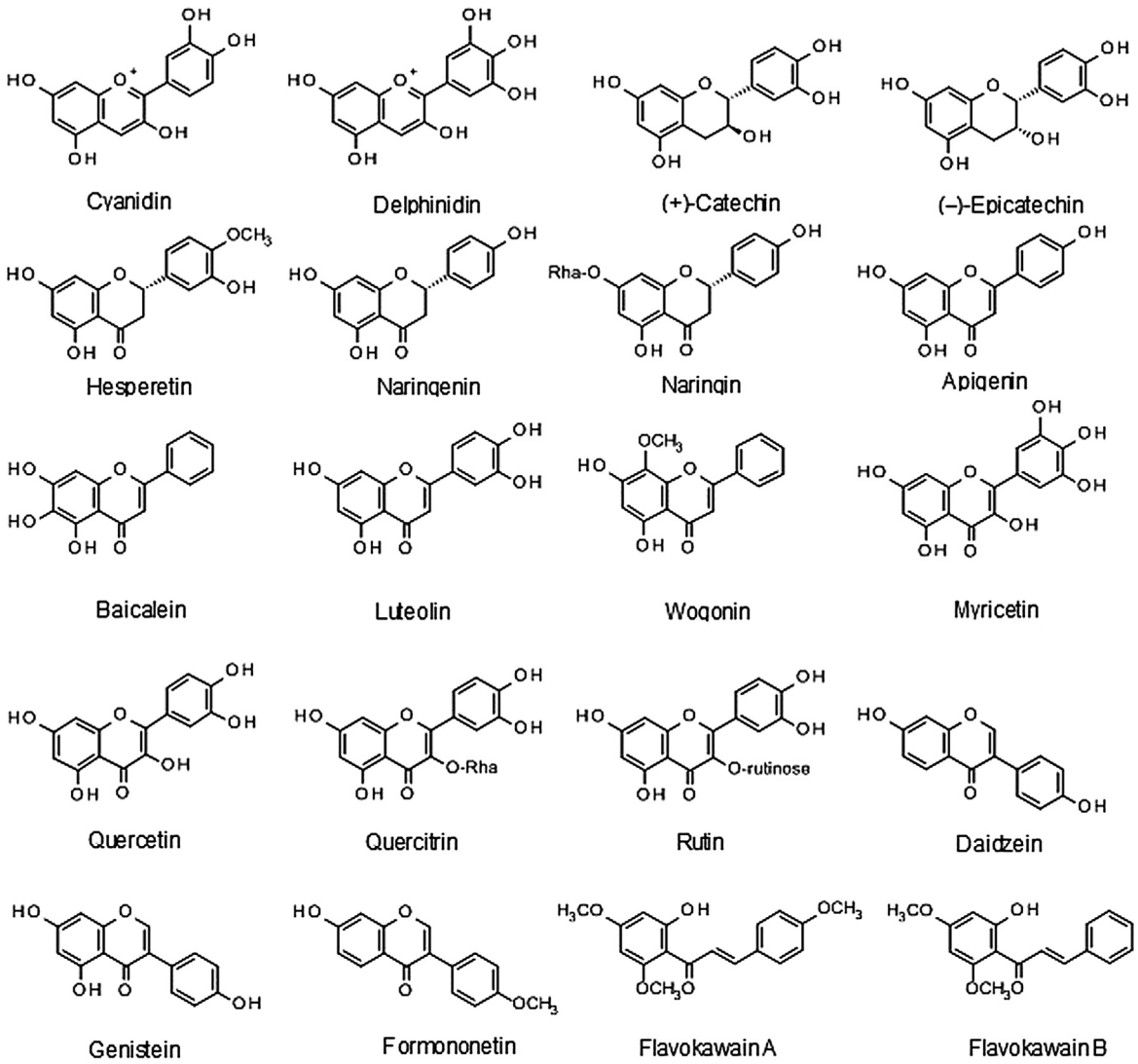


FIGURE 12.20 Examples of flavonoids.

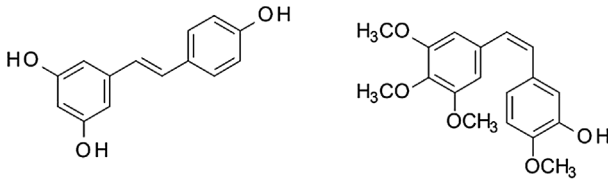


FIGURE 12.21 Examples of stilbenes.

In chalcones ring C is not cyclized. Examples of biologically active chalcones include the flavakawains which occur in *Piper methysticum* (Piperaceae) [33]. Flavakawains A and B (Fig. 12.20) have been reported to display anticancer activity [33].

Catechins occur widely in plants. (+)-Catechin and (−)-epicatechin (Fig. 12.20) as well as their gallic acid derivatives are found in tea and cocoa.

The flavanones hesperetin, naringenin, and naringin (Fig. 12.20) are commonly found in the *Citrus* genus. The flavones apigenin, baicalein, luteolin, and wogonin (Fig. 12.20) have been demonstrated to possess activity against a variety of cancers [34].

Common flavonols include myricetin, quercetin, quercitrin, and rutin (Fig. 12.20). Quercetin is the principal plant flavonoid and has been the subject of many research studies aimed at elucidating its therapeutic potential. Quercetin has a range of biological activities including anticancer, antioxidant, antiviral, and immunoprotective properties [35].

Isoflavones abound in the *Soy* genus. Isoflavones have phytoestrogenic activity and include daidzein, genistein, and formononetin (Fig. 12.20).

12.1.8.4 Stilbenes

Stilbenes are believed to act as phytoalexins. They possess a $C_6-C_2-C_3$ core. Resveratrol (Fig. 12.21) is the most popular stilbene. It is found in grapes (*Vitis* species, Vitaceae) and grape products. It possesses anticancer, antifungal, antiinflammatory, antioxidant, platelet antiaggregation, and cardiovascular benefits [36,37]. Combretastatin A-4 (Fig. 12.21), a well-known anticancer stilbenoid, occurs in *Combretum caffrum* (Combretaceae) [38].

12.2 POLYACETYLENES

Polyacetylenes are also referred to as acetylenic natural products [39]. They contain alkyne functional groups. They are biosynthesized from fatty acid and polyketide precursors [40]. They are derived from crepenynic acid, stearolic acid, or tariric acid (Fig. 12.22) [39]. They are chemically reactive compounds with a range of biological activities. Pharmacological actions of polyacetylenes include antibacterial, antifungal, antitumor, antiviral, cytotoxic, immunosuppressant, neurotoxic, and piscicidal activities [40]. Contact dermatitis and skin reactions have been correlated with C_{17} polyacetylenes [40].

Some of the most well-known compounds in this class are polyacetylenic alcohols and include falcarinol (panaxynol), panaxydol, and panaxytriol (Fig. 12.23). Falcarinol (panaxynol), panaxydol, and panaxytriol are secondary compounds obtained from *Panax ginseng* (Araliaceae) [40]. Falcarinol and falcarindiol are characteristic of the Araliaceae plant family [40]. Falcarinol and falcarindiol are aliphatic C_{17} acetylenes possessing antiinflammatory and antiplatelet properties [40]. These two compounds may cause dermatitis if they come in contact with exposed skin [40]. Other alcohol-containing polyacetylene compounds include oenanthotoxin (Fig. 12.23), which occurs in hemlock water dropwort (*Oenanthe crocata*, Umbelliferae/Apiaceae), and cicutoxin (Fig. 12.23), which is found in water hemlock (*Cicuta virosa*, Umbelliferae/Apiaceae). Ingestion of oenanthotoxin and cicutoxin may result in violent convulsions and death [40].

Wyerone (Fig. 12.24) is a furan-containing antifungal agent which occurs in broad beans (*Vicia faba*, Leguminosae/Fabaceae) [41]. Nakano and coworkers reported the novel thiophene-containing polyacetylene compounds echinopsacetylenes A and B (Fig. 12.24) from the dichloromethane extract of the roots of *Echinops transiliensis* (Asteraceae) [42]. Echinopsacetylene A was found to display toxicity against Formosan subterranean termites [42].

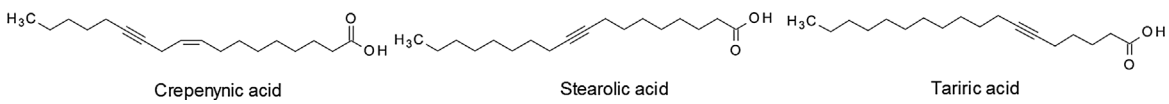


FIGURE 12.22 Polyacetylene precursors.

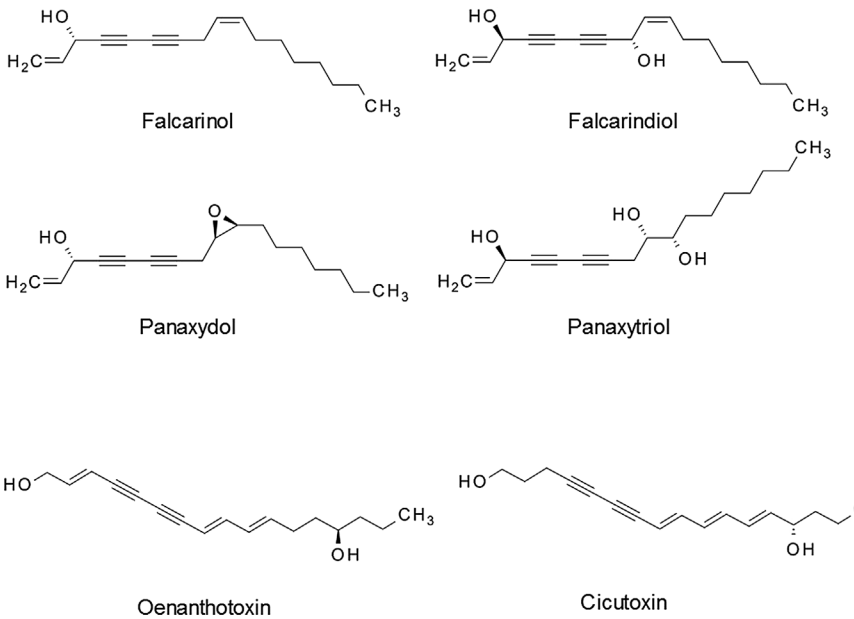


FIGURE 12.23 Examples of polyacetylenic alcohols.

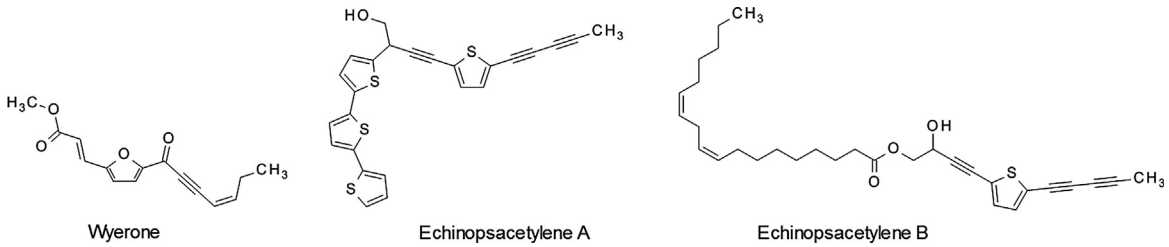


FIGURE 12.24 Examples of furan- and thiophene-containing acetylenes.

12.3 CONCLUSIONS

The metabolites produced by plants are structurally and pharmacologically diverse. Lignins and lignans, polyketide-derived secondary metabolites, and polyacetylene compounds are found in a variety of terrestrial plants and possess intriguing molecular architectures. Flavonoids and related polyketides are important dietary compounds and have been attributed with significant therapeutic functions.

12.4 SELF-EVALUATION QUESTIONS

1. What is the currently accepted biosynthetic route to lignans?
2. Name the biosynthetic precursors of the polyacetylenes.
3. Discuss the similarities and differences in the core structures of polyketide secondary metabolites.

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Chapter 13

Vitamins

T. Baj and E. Sieniawska
Medical University of Lublin, Lublin, Poland

Chapter Outline

To understand the role of vitamins to human health

To differentiate between water-soluble vitamins—thiamine, riboflavin, niacin, B_6 (pyridoxine), vitamin B_{12} (cobalamin), folate, pantothenic acid, biotin and vitamin C—and fat-soluble vitamins—A, D, E, K

To know the various accepted levels of vitamin intake and their derivation.

13.1 INTRODUCTION

The history of vitamins begins in 1912, when the Polish biochemist, Casimir Funk, isolated a bioactive substance from rice bran which was at first given the name "vita-amine" (later "aneurin" for "antineuritic vitamin" and eventually "thiamine"). Funk realized that this substance could cure chickens and humans of beriberi. He published a landmark paper, "The etiology of the deficiency diseases" [1], and stated that all "deficiency diseases can be prevented and cured by the addition of certain preventive substances, the deficient substances," for which he proposed the name "vitamins." Next, in 1916, the American biochemist, Elmer McCollum, introduced the capital letters A−D to differentiate between vitamins. Later, vitamins E and K were added, and it was realized that vitamin B can contain more than one factor, so a further differentiation into vitamins B_1, B_2, and so on, was made [2,3]. The current principles of nomenclature of vitamins were introduced by the IUPAC−IUB, in 1967 [4].

Vitamins are a chemically heterogeneous group of organic compounds. Furthermore, they are grouped according to their solubility: the water-soluble vitamins: thiamine, riboflavin, niacin, B_6 (pyridoxine), vitamin B_{12} (cobalamin), folate, pantothenic acid, biotin, and vitamin C; and the fat-soluble vitamins: A, D, E, K [5]. For the most part, they are delivered into the human body by way of the intake of food, but some of them (in small quantities) can be produced endogenously. The need for this group of compounds is not the same for each organism and depends on many factors including age, sex, physical condition, as well as dietary habits. A lack of adequate amount of vitamins in the body may lead to hypovitaminosis or vitamin deficiency (lack of), while an excessive amount of vitamins can lead to hypervitaminosis (this applies mainly to fat-soluble vitamins). The avitaminosis condition is now very rare and may be due

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to a hypovitaminosis that can be brought about by insufficient amount of vitamins in the diet, inadequate absorption in the gastrointestinal tract, or increased demand of the body.

Systematic research into the causes and effects of many diseases (including diseases of civilization) have allowed the observation of the correlation between nutrition and the body's homeostasis. Those researching in this field of study have attempted to develop optimal health and nutritional recommendations, a challenging task given the fluctuations in vitamins intake by way of the foods typical to particular diets among individuals and national groups. In order to harmonize the need for vitamins, researchers have implemented appropriate recommendation levels of vitamins and minerals within processed food items. Hence, food and dietary specialists in Canada and the United States have been working together to develop nutrient recommendations based on the latest information. These recommendations are called dietary reference intakes (DRIs) [6]. The DRIs encompass types of nutrient reference values, each with different uses. These are highlighted below [6]:

- *Estimated Average Requirement (EAR)*—the amount of a nutrient that is estimated to meet the requirement of half of all healthy individuals in a given age and gender group. This value is based on a thorough review of current scientific literature.
- *Recommended Dietary Allowance (RDA)*—the average daily dietary intake of a nutrient that is sufficient to meet the requirement of nearly all (97–98%) healthy persons. This is the number to be used as a goal for individuals. It is calculated from the EAR. The equations used to calculate the RDA are as follows: RDA = EAR + 2 SD (EAR).
- *Adequate Intake (AI)*—only established when an EAR (and thus an RDA) cannot be determined because the data are not clear-cut enough; a nutrient has either an RDA or an AI. The AI is based on experimental data or determined by estimating the amount of a nutrient eaten by a group of healthy people, and then assuming that the amount they consume is adequate to promote health.
- *Tolerable Upper Intake Level (UL)*—the highest continuing daily intake of a nutrient that is likely to pose no risks of adverse health effects for almost all individuals. As intake increases above the UL, the risk of adverse effects increases.

13.2 WATER-SOLUBLE VITAMINS

13.2.1 Vitamin B Group

B vitamins are important cofactors of enzymatic reactions. They are soluble in water, and the excess is eliminated in the urine. For humans, the most important vitamins of this group include thiamine (vitamin B_1), riboflavin (vitamin B_2), niacin (nicotinic acid, nicotinic acid amide, vitamin B_3), pantothenic acid (vitamin B_5), vitamin B_6 (pyridoxine, pyridoxal, pyridoxamine), biotin (vitamin B_7), folic acid (pteroylglutamic acid, vitamin B_9), cobalamin (vitamin B_{12}) [5].

Elevated levels of B vitamins are needed alongside increased physical activity, as B vitamins are involved in the processing of carbohydrates and fats for energy production. As a result, their significance is heightened in athletes and important to their performance levels. It should be also be noted that some of the B vitamins are needed to help form hemoglobin in red blood cells, a major determinant of oxygen delivery to the muscles during aerobic endurance exercise [7].

13.2.2 Vitamin B_1 (Thiamine)

Sources of vitamin B_1: spinach, bottle gourd, potato, tomato, cauliflower, lettuce, cabbage, carrot, bananas, okra.

Thiamine (Vit B_1) is composed of a thiazole ring and a pyrimidine group, which together, make up the sulfur-containing structure of two rings joined by a methylene group [8]. The structure of thiamine is shown in Fig. 13.1.

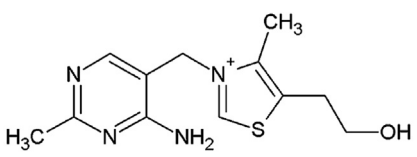


FIGURE 13.1 The structure of thiamine.

The active form of vitamin B_1 is thiamine pyrophosphate. The synthesis of this in eukaryotes requires thiamine pyrophosphokinase, which catalyzes the transfer of a pyrophosphate group from ATP to thiamine [9].

Thiamine deficiency (TD) can be caused by chronic alcohol consumption. This can lead to symptoms of TD and cause damage to the brain (the Wernicke−Korsakoff syndrome) [10]. Particularly sensitive to TD and most damaged due to chronic alcohol consumption are areas of the cerebellum [11]. The lack of thiamine brings about the deficiency disease called beriberi [12], which has been known since antiquity. TD is rare in people who follow a normal diet, but it can occur in people suffering from malnutrition. TD occurs where the diet consists mainly of milled white cereals, including polished rice and refined wheat flour—all very poor sources of thiamine [13].

Vitamin B_1 deficiency produces beriberi, and 70% of patients with beriberi have ocular abnormalities such as dry eye, optic atrophy, and epithelial changes in the conjunctiva [14].

Vitamin B_1 deficiency is often due to inadequate nutrition. This, in turn, can induce dysfunction of the nervous system, the cardiac muscle, and the skeletal muscle. This may result in lack of appetite, muscle weakness, paresthesias, reduced blood pressure, and hypothermia [15].

Thiamine is widely distributed in common food items. Analysis of vitamin B_1 in some vegetables carried out by Hanif et al. [16] showed that the highest level was found in spinach (*Spinacia oleracea*), and the lowest for the bottle gourd (*Legenaria vulgaris*). Vitamin B_1 is also present in potato (*Solanum tuberosum*), tomato (*Lycopersicum esculentum*), cauliflower (*Brassica oleracea*), lettuce (*Lacluca sativum*), cabbage (*Brassica oleracea capitata*), and carrot (*Daucus carota*). Additionally, in the work of Ismail et al., an analysis of thiamine content in vegetables and fruits purchased from local markets in Pakistan showed that the highest content of vitamin B_1 was evident in bananas (*Musa paradisiaca*) and okra (*Hibiscus esculentum*) [17].

13.2.3 Vitamin B_2 (Riboflavin)

Sources of vitamin B_2: spinach, brinjal.

Riboflavin (Fig. 13.2) is a substrate for the synthesis of flavin mononucleotide and flavin adenine dinucleotide.

Free riboflavin and reduced flavin coenzymes are characterized by their faint yellowish coloring [18]. Indications of a lack of riboflavin are anemia and migraine prophylaxis [19]. Riboflavin deficiency is evident in populaces with a low intake of milk and meat products. Current research on the importance of vitamin B_2 focus on the preventive action of an adequate supply of the vitamin with regard to cardiovascular disease, cancer, and vision problems [20]. Supplemental administration of drug vitamin B_2 is undertaken in an alternative therapy with diuretics, after antibiotic therapy, before and after surgery, in remedying dysfunctions of the liver, stomach, and intestines, in treating persistent diarrhea, and in treating chronic and debilitating diseases (diabetes, infections of different origins, skin inflammation, hyperthyroidism, psychosomatic illness, alcoholism) [21]. According to Ismail et al., the riboflavin can be found in natural food items, such as [17] brinjal (*Solanum melongena*) and spinach (*S. oleracea*).

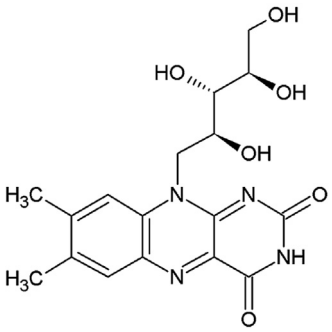


FIGURE 13.2 The structure of riboflavin.

13.2.4 Vitamin B_3 (Niacin, Nicotinic Acids, Nicotinamide)

Sources of vitamin B_3: spinach, bottle gourd, potato, tomato, cauliflower, lettuce, cabbage, carrot.

Vitamin B_3 (Fig. 13.3) is biosynthetically converted to nicotinamide adenine dinucleotide (NAD^+), a versatile acceptor of hydride equivalents, to form the reduced dinucleotide, NADH. The phosphorylated forms of the nicotinamide dinucleotides (NADP/NADPH) perform similar chemical functions within cells, although these are generally used in biosynthetic pathways and in cell protection mechanisms against reactive oxygen species [22].

Niacin deficiency creates symptoms such as anorexia, anxiety, depression, irritability, and weakness [23]. Moreover, it induces pellagra, which is found mostly among people eating corn-based diets in parts of China, Africa, and India. Pellagra, in North America, is found mainly among alcoholics; in patients with congenital defects of the intestine and kidneys in the absorption of tryptophan, and in patients with carcinoid syndromes in which there is increased conversion of tryptophan to serotonin [24]. The primary cause of niacin deficiency is a diet low in tryptophan and nicotinamide. Secondary deficiency can be brought about by chronic diarrhea, cirrhosis, alcoholism, as well as through administration of intensive parenteral nutrition fluids containing no vitamins. After oral administration, niacin is absorbed to a percentage of 60–80% [25].

Toward human sustenance, niacin is sourced primarily from animal products (meat, liver, fish), nuts, and grains. Niacin is also present within commonly consumed vegetables: potato, spinach, cauliflower, tomato, carrot, and lettuce, and in lower amounts in cabbage and bottle gourd [16]. According to current data [26], the highest content of niacin is in spirulina. Among the spices and herbs, the greater vitamin B_2 level is in paprika, ginger (ground), and fennel seed [26].

13.2.5 Vitamin B_5 (Pantothenic Acid)

Sources of vitamin B_5: potatoes, spinach, cauliflower.

In the human body, pantothenic acid (Fig. 13.4) occurs mainly as coenzyme A. This vitamin is essential for many metabolic processes, in particular, the metabolism of carbohydrates, fats, and proteins. Pantothenic acid is involved in the process of growth of the organism and has an effect on the regeneration of skin, as well as hair and nail growth [27].

The daily requirement of pantothenic acid is fully covered by the typical Western diet. Particularly rich sources of this vitamin are liver, yeast, egg yolks, whole grains, and vegetables. Of note, human pantothenic acid deficiency has only been demonstrated by experimental feeding of diets low in pantothenic acid or by administration of a specific antagonist of pantothenic acid [24].

Common vegetable sources of vitamin B_5 are potatoes, spinach, and cauliflower [16].

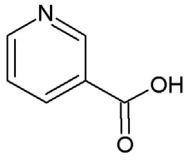


FIGURE 13.3 The structure of niacin.

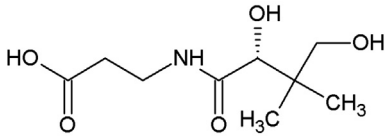


FIGURE 13.4 The structure of pantothenic acid.

13.2.6 Vitamin B_6

The presence of vitamin B_6: spinach, okra, brinjal, guava.

Vitamin B_6 comprises a group of three derivatives: pyridoxine, pyridoxal, and pyridoxamine (Fig. 13.5).

Vitamin B_6 is commonly present in the body as pyridoxine, pyridoxal phosphate, and pyridoxamine phosphate. Pyridoxyne, after conversion to the active form (pyridoxal phosphate), is involved in a number of metabolic reactions in the body. Furthermore, pyridoxal is a cofactor for transaminases, decarboxylases, and other enzymes used in the metabolic transformations of amino acids and nitrogen-containing compounds [28].

Vitamin B_6 deficiency in infants may lead to growth retardation, weight loss, hyper-irritability, convulsions, and anemia. Deficiency of this vitamin in adults may result in depression, convulsions, seborrheic dermatitis, and cheilosis [23].

Vitamin B_6 supplementation is undertaken for the treatment and prevention of hypovitaminosis in acrodynia (a disease caused by the toxic effects of mercury), radiation disease, inflammation of the skin and mucous membranes, polyneuritis as caused by isoniazid, as well as for alternative treatment of microcytic anemia, leukopenia, nausea, and vomiting in pregnancy [29]. According to the analysis carried out by Ismail et al. [17], vitamin B_6 was found in okra, spinach, and brinjal (*S. melongena*), as well as guava (*Psidium guajava*).

13.2.7 Vitamin B_7 (Biotin)

Biotin (Fig. 13.6) is produced in the intestines by bacterial action and is a cofactor in fatty acid synthesis. Deficiencies in biotin result in baldness and dry skin, lassitude, anorexia, depression, and hypercholesterolemia [23].

Biotin deficiency in humans induces localized inflammation of the skin around the eyes, nose, mouth, conjunctivitis, abnormal nails, and hair growth (and its excessive fragility), in addition to abnormal fat metabolism. Biotin supports the processes of formation of keratin and the differentiation of epidermal cells and hair and nails, improving their condition [30]. A deficiency of biotin is a disorder rarely described in the literature due to the low incidence.

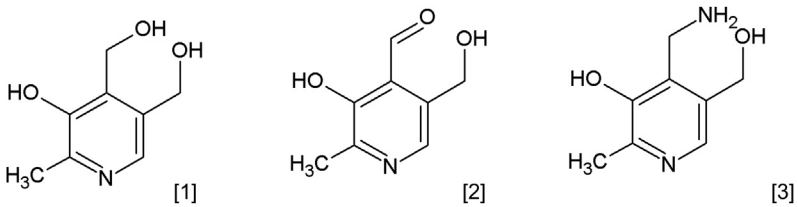


FIGURE 13.5 The structures of pyridoxine [1], pyridoxal [2], and pyridoxamine [3].

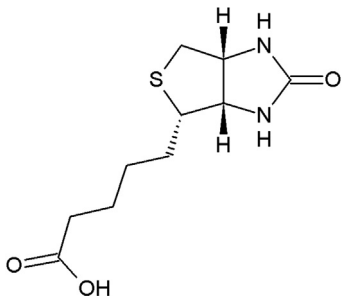


FIGURE 13.6 The structure of biotin.

13.2.8 Vitamin B_9 (Folate, Folic Acid)

Sources of vitamin B_9: banana, carrot, brinjal.

Folate functions as a coenzyme in single-carbon transfers in the metabolism of nucleic and amino acids. In addition, folic acid (Fig. 13.7) and other forms (containing more glutamic acid residues) serve as coenzymes in the reactions of transferring one-carbon groups, as well as the biosynthesis of purine and pyrimidine bases necessary for DNA and RNA synthesis. Folic acid is converted in the body to folinic acid (FH4) [30].

Folinic acid, as a specific active substance, is in clinical use for inhibition of dihydrofolate reductase in the treatment of folic acid antimetabolites (methotrexate). Folic acid, cobalamin, and pyridoxal phosphate are involved in the metabolism of homocysteine [30].

As indicated by a study of the level of vitamin B_9 in selected fruits and vegetables described by Ismail et al. [17], the highest folate was found for banana, carrot (*D. carota*), and brinjal (*S. melongena*).

13.2.9 Vitamin B_{12} (Cyanocobalamin)

Cyanocobalamin (Fig. 13.8), essential for human health, affects the formation of blood cells, and the proper operation of the nervous system. Vitamin B_{12} deficiency can cause anemia, as well as malignant and irreversible neurological complications.

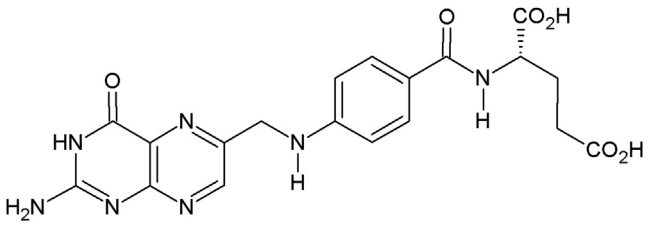


FIGURE 13.7 The structure of folic acid.

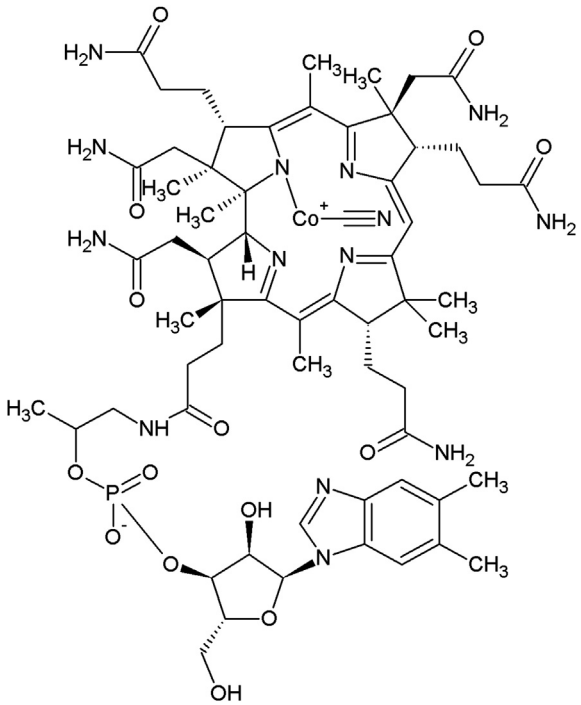


FIGURE 13.8 The structure of cyanocobalamin.

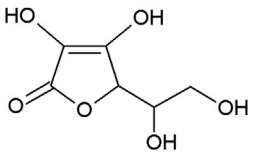


FIGURE 13.9 The structure of ascorbinic acid.

The injectable form of vitamin B_{12} is crucial to the treatment of many conditions such as: malignant Addison−Biermer (a specific type of anemia that results from a lack of vitamin B_{12}); megaloblastic anemia; vitamin B_{12} deficiency that results from a diet without animal products; the loss of castle intrinsic factor (a glycoprotein that is secreted from the gastric mucous membrane and enables the absorption of vitamin B_{12} in the intestines) caused by stomach resections; chronic atrophic gastritis; malabsorption syndromes after resection of the ileum; celiac disease (caused by gluten intolerance); tropical sprue (called sprue—a disease of the gastrointestinal tract leading to the malabsorption of nutrients); Crohn's disease (an inflammatory disease of the gastrointestinal tract); and blind loop syndrome. It is also used in the Schilling Test (a means of studying the absorption of vitamin B_{12}) [31].

Rich sources of vitamin B_{12} are commonly available and consumed in meat and dairy products. The typical diet, in most cases, provides full coverage of vitamin B_{12} needs, and the human body when functioning normally, has a large reserve.

13.2.10 Vitamin C (Ascorbic Acid)

Sources of vitamin C: acerola, rose hip, strawberries, cantaloupe, tomatoes, cabbage, peppers, thyme.

Ascorbic acid (Fig. 13.9) is involved in many functions of the body. In particular, it is necessary for collagen synthesis (the protein that serves so many connective functions in the body). The production of certain hormones and neurotransmitters, as well as the metabolism of some amino acids and vitamins that also require vitamin C. Moreover, ascorbic acid has antioxidant properties, and its reaction with compounds, histamine, and peroxides reduces symptoms of inflammation [32].

Long-term deficiency of vitamin C can accelerate the development of oxidative stress in the brain during normal aging, and such a deficiency also contributes to the production of amyloid oligomerization and/or its storage. According to Dixit et al. [33], vitamin C may play a key role in protection against both Alzheimer's neuropathology and normal aging, but that more attention should be paid to the nutritional intake of this vitamin in early middle age, rather than waiting for later life interventions [33]. Vitamin C deficiency comes about due to incorrect diet. Patients with chronic diseases such as cancer, chronic kidney disease, and individuals who smoke are also exposed to the deficiency of this vitamin [5]. In vitamin C deficiency (scurvy), hemorrhages may develop in a variety of sites, e.g., in the skin, in the mucous membranes, in the body cavities, the orbits, and, subperiosteally, in the joints. Hemorrhages may also occur in the eye lids, the subconjunctival spaces, the anterior chamber, the vitreous cavity, and the retina [34].

The sources of vitamin C are citrus fruits, strawberries, cantaloupe, tomatoes, cabbage, and green leafy vegetables, as well as walnuts, but it must be kept in mind that it is best to consume raw foods or supplements, because cooking destroys vitamin C [35]. One of the richest sources of vitamin C is acerola (*Malpighia glabra*). A high amount of vitamin C can also be found in, e.g., (wild) rose hip; peppers—hot chili, green, and sweet, yellow; as well as thyme (fresh) [26].

13.3 FAT-SOLUBLE VITAMINS

Fat-soluble vitamins are apolar molecules that are hydrophobic derivatives of isoprene. In the body, they are not synthesized in sufficient quantities, hence, they have to be taken in as food. Indeed, the smooth absorption of these vitamins comes about through proper absorption of fats. Transport of these vitamins in the blood is mediated by specific lipoproteins or by carrier proteins.

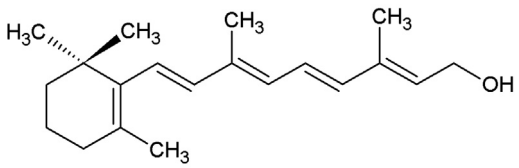


FIGURE 13.10 The structure of retinol.

13.3.1 Vitamin A

Sources of vitamin A: carrots, spinach, waterleaf, tomato.

Vitamin A, as a group, incorporates retinol (vitamin A_1) (Fig. 13.10), 3,4-didehydroretinol (vitamin A_2), as well as their retinyl esters, and aldehyde (retinal, 3,4-didehydroretinal). The biological functions of vitamin A are manifold and involve sensory performances like vision, embryonic development, cell differentiation, hematopoesis, growth, reproduction, immune functions, and development of tumor resistances [36].

Retinol is formed inside humans and other animals from carotene (provitamin A). This, in the vast majority, is manufactured by plants, and to a lesser extent, by microorganisms. Of note, some of the most widely represented dyes are natural carotenoids. Some of the carotenoids also serve as precursors of vitamin A, thus allowing their classification as either provitamin A or nonprovitamin A carotenoids. Provitamin A carotenoids yield vitamin A and its metabolites (retinoids) upon enzymatic and nonenzymatic cleavage, with β-carotene being the most abundant and well-characterized precursor of vitamin A in the human diet [37].

Vitamin A deficiency (VAD) is most common in children of preschool age. VAD is among the most common and serious of all nutritional deficiency diseases and also probably the one for which there is the greatest need for remedial action [38]. VAD is undoubtedly the leading cause of childhood blindness in developing countries.

Deficiencies of vitamin A are evident, indications of these include, peeling and dry skin, in the hyperkeratosis of skin cells and the atrophy of the sebaceous glands. Retinoid deficiency contributes to changes in bone tissue (herein, excessive growth of bones of the skull leads to cranial nerve compression), impaired fertility, keratinization of the epithelium of the mucous membranes of the gastrointestinal tract, respiratory problems, increased susceptibility to infections, as well as difficulty in wound healing [39].

The need for supplementation is seen in conditions such as diarrhea, gastrectomy, hypothyroidism, chronic infections, gastrointestinal disease (celiac disease, Crohn's disease), further malabsorption associated with pancreatic insufficiency, measles, severe protein deficiency, and adjunctive therapy in skin diseases associated with a deficiency of vitamin A [40].

Excessive intake of vitamin A (Hypervitaminosis A) is characterized by anorexia, headache, hepatosplenomegaly, irritability, scaly dermatitis, patchy loss of hair, bone pain, and hyperostosis [41].

Among vegetables with relatively high levels of total carotenoid and β-carotene contents are carrots, spinach, waterleaf (*Talinum triangulare*), tomato (*L. esculentum*) [42].

13.3.2 Vitamin D

Sources of vitamin D: Solanaceae family.

Vitamin D is produced under the influence of daily sunlight as a result of the photolysis of ergosterol (found in plants). In this manner, ergosterol (provitamin D_2) is converted into ergocalciferol (vitamin D_2), and 7-dehydrocholesterol (provitamin D_3) is converted into cholecalciferol (vitamin D_3) (Fig. 13.11).

The formation of active forms of vitamin D occurs in two stages. The first step occurs in the liver, this is then followed by the hydroxylation of vitamin D. This results in 25-hydroxyvitamin D [25(OH)D], which is the major form

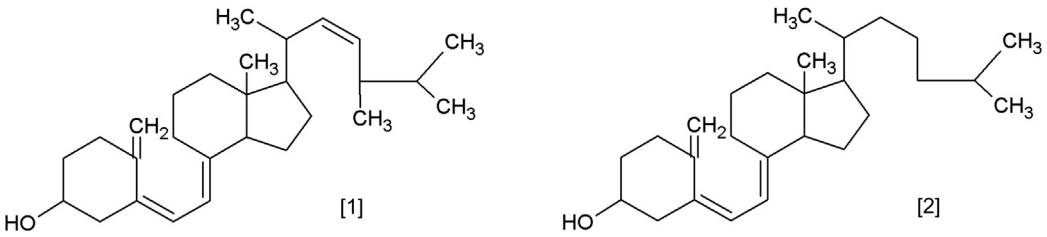


FIGURE 13.11 The structures of ergocalciferol (vitamin D_2) [1] and cholecalciferol (vitamin D_3) [2].

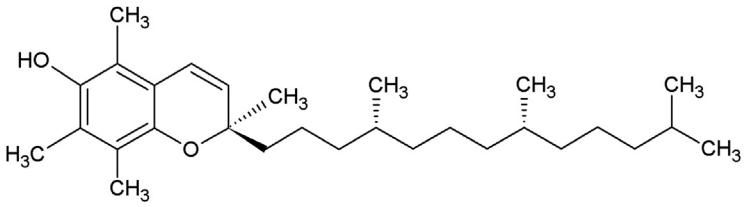


FIGURE. 13.12 The structure of alpha-tocopherol.

of the vitamin in the bloodstream. The second phase change takes place in the kidneys, where there is a 1α hydroxylation leading to the synthesis of 1,25-dihydroxyvitamin D [1,25 (OH) 2D]. This is widely considered to be the most active form of vitamin D. In addition, inside the kidney, 1,25(OH) 2D may produce other dihydroxy derivatives of vitamin D, such as 24,25-dihydroxyvitamin D (24,25$(OH)_2$D) and 25,26 $(OH)_2$D [43].

It is reported that vitamin D deficiency occurs in approximately 30−50% of the global population [44] and can lead to disorders of the musculoskeletal skeletal and cardiovascular systems [45]. One of the main reasons for vitamin D deficiency is insufficient exposure to the sun [46]. On the other hand, hypervitaminosis D is associated with weight loss, calcification of many soft tissues, and eventual renal failure [41].

Animal products have been recognized as the primary source of vitamin D_3; however, numerous studies have demonstrated the presence of vitamin D_3 (and its metabolites) in plants, mainly from the Solanaceae family. According to the data collected by Japelt and Jakobsen [47], depending on the analytical method used, vitamin D_3 content of the respective sources are as follows: *Solanum lycopersicum*; *Solanum tuberosum*; *Cucurbita pepo*; *Solanum glaucophyllum*; *Cestrum diurnum*; *Madicago sativa*; *Trisetum flavescens*; and *Capsicum annuum*. In *Nicotiana glauca*, vitamin D_3 content was identified but not quantified [47].

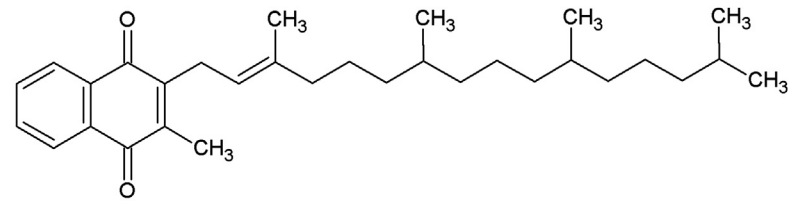
13.3.3 Vitamin E

Sources of vitamin E: grains and nut oils.

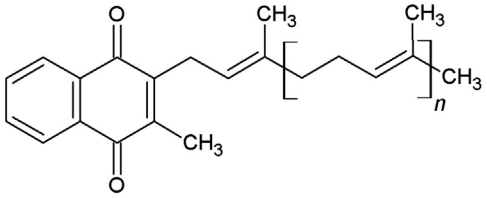
Structural analyses have revealed that molecules having vitamin E antioxidant activity include four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ) (Fig. 13.12). Their confirmed antioxidant activity gave direction toward examining their potential effect on diseases associated with oxidative stress (such as cardiovascular diseases, atherosclerosis, and cancer) [48]. Indeed, research conducted by Yoshida et al. [49] show the effectiveness of vitamin E molecules in reacting with radical groups known to cause cell damage.

Vitamin E is located primarily within the phospholipid bilayer of cell membranes. The major biologic role of vitamin E is to protect PUFAs (polyunsaturated fatty acids) and other components of cell membranes and low-density lipoproteins from oxidation by free radicals. It is particularly effective in preventing lipid peroxidation, a series of chemical reactions involving the oxidative deterioration of PUFAs [50].

A normal diet contains sufficient levels of vitamin E. There is very little clinical evidence of deficiency disease in humans except in certain inherited conditions where the metabolism of vitamin E is disturbed. Indeed, even biochemical evidence of poor vitamin E status in both adults and children is minimal [50]. Among the main source of alpha-tocopherol are grains and nut oils [26].



Phylloquinone



Menaquinones ($n = 3 - 9$)

FIGURE. 13.13 The structure of phylloquinone (vitamin K_1) [1] and menaquinones (vitamin K_2) [2].

13.3.4 Vitamin K

Sources of vitamin K: kale, basil, sage, thyme, coriander leaf, parsley, broccoli, cauliflower, carrot.

Vitamins K are widely distributed in plants and animals. In plants, the only important molecular form is phylloquinone (vitamin K_1), whereas bacteria synthesize a family of compounds called menaquinones (vitamin K_2) [50] (Fig. 13.13).

Vitamin K is an essential fat-soluble micronutrient which is needed for a unique posttranslational chemical modification in a small group of proteins with calcium-binding properties, collectively known as vitamin K-dependent proteins or Gla-proteins. Thus far, the only unequivocal role of vitamin K in health is in the maintenance of normal coagulation [50]. Hypervitaminosis K is characterized by gastrointestinal disturbances and anemia [42].

The content of vitamin K_1 substantially depends on the place of cultivation; different levels of vitamins may vary several times. For example, kale growing in Boston contain vitamin K_1 6.21 μg/g while growing in Montreal, 16.57 μg/g [51]. Phylloquinone is found in high levels in: basil (dried); sage (ground), thyme, coriander leaf, parsley, amaranth leaves; chard (swiss) dandellion greens, broccoli, cauliflower, and carrot [28,52].

13.4 CONCLUSION

This chapter discussed the most important functions of vitamins and the effects of their deficiencies. Almost all vitamins are essential for normal growth and development of the human body. Moreover, the recommended vitamin intake depends on many factors; age, sex and health. However, the different eating habits that occur in different countries make it difficult to establish strict standard supplementation. This chapter also outlined the vitamin content in different fruits and vegetables. These are only examples, as their levels may vary depending on, among others, species, growing conditions, time of harvest, and processing before consumption. A diet rich in natural ingredients that are sources of vitamins can protect us from serious health problems.

13.5 SAMPLE QUESTIONS

1. What are vitamins?
2. Discuss the water-soluble vitamins.
3. Discuss the fat-soluble vitamins.
4. Discuss the importance of vitamin A toward human health and their occurrence in plants.
5. Discuss the importance of vitamin D toward human health and their occurrence in plants.

6. Discuss the importance of vitamin E toward human health and their occurrence in plants.
7. Discuss the importance of vitamin K toward human health and their occurrence in plants.
8. Discuss the importance of B vitamins toward human and their occurrence in plants.
9. Discuss the importance of vitamin C toward human health and their occurrence in plants.

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Chapter 14

Chemotherapeutics

M. Shields
Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, United States

Chapter Outline

Learning Objectives

- Chemotherapy is classified into different classes based on the mechanism of action.
- DNA and cell division are major targets of chemotherapy.
- Cancer is a major cause of death worldwide.
- Lung, breast, and prostate cancers make up majority of cases of cancers.
- Majority of cancer related deaths are due to lifestyle, diet, and infections.
- Preventative measures include reduced smoking, balanced diet, increased testing, and vaccinations.

14.1 CHEMOTHERAPY TYPES

Naturally derived extracts and isolated chemicals have been used for treating various diseases for centuries [1]. Chemical agents used for curing or treating cancers are called chemotherapeutics. Traditional chemotherapeutics act by killing rapidly dividing cells generally through targeting DNA or processes critical for cell division [2]. Nontraditional therapies target vulnerabilities specific to cancer cells, including alterations in gene and proteins [3]. Chemotherapeutics are classified according to their modes of action and include alkylating agents, antimetabolites, microtubule targeting agents, topoisomerases, and antibiotics.

Alkylating agents act by adding alkyl groups to reactive constituents of biomolecules including proteins and nucleic acids. Alkylation of guanine in DNA results in abnormal base pairing, DNA cleavage or cross-linking of the strands. DNA damage eventually leads to cell death or apoptosis resulting from an inability to repair the damage. Alkylating agents include the first chemotherapeutic agent, nitrogen mustard gas, and more recent agents such as temozolomide, chlorambucil and mephalan [2]. Alkylating agents have been very useful for treating many hematologic and solid cancers. Another group of DNA targeted chemotherapies are the antimetabolites that act by inhibiting nucleic acid synthesis. Antimetabolites promote cancer cell death by either incorporating into nucleic acids or blocking the enzymes needed for DNA or RNA synthesis. Methotrexate acts as an antimetabolite by inhibiting dihydrofolate reductase, an enzyme important for producing tetrahydrofolic acid, an important intermediate for the synthesis of nucleotides precursors. The fluoropyrimidine antimetabolite, 5-fluorouracil incorporates into RNA molecules preventing RNA synthesis.

Topoisomerases inhibitors target cancer cells by preventing the unwinding of DNA, which is critical for replication, transcription, and cell division. Two major topoisomerases are present in the human cells, TOPI and TOPII, and drugs

Pharmacognosy. DOI: http://dx.doi.org/10.1016/B978-0-12-802104-0.00014-7

that target either protein have been used to treat cancers. TOPI, or type I topoisomerase, makes single-stranded cuts in DNA, while TOPII makes double-strand breaks. One of the earliest identified TOPI inhibitor is camptothecin which was extracted from the Chinese plant, *Camptotheca acuminate*. Camptothecin and other derivatives, such as topotecan and irinotecan, inhibit topoisomerase activity by binding to TOPI-DNA reaction intermediate. The mechanisms of action of TOPII inhibitors, such as doxorubicin, etoposide, and daunorubicin, include DNA intercalation and enzyme modifications.

Microtubule-targeting chemotherapies disrupt mitotic progression and cytoskeletal dynamics and include the vinca alkaloids and taxanes. The vinca alkaloids prevent microtubule formation or induce depolymerization, while the taxanes stabilize microtubules or increase polymerization. The vinca alkaloids were originally isolated from the periwinkle, *Catharanthus roseus*, and were used to treat diabetes, high blood pressure, and cancer. The first described vinca alcaloids, vinblastine, was very effective in treating Hodgkin's disease, leukemia, and other cancers in mouse models and clinical trials were first stated in 1960. Other vinca alkaloids, including vincristine, vinorelbine, and vinedesine, are used in the clinic for treating various cancers. Taxanes have been used to treat a number of cancers, such as ovarian, lung, breast, and prostate. Paclitaxel, the first characterized taxane, was isolated from the bark of the pacific yew tree, *Taxus brevifolia*. The semisynthetic derivatives of paclitaxel, docetaxel, and/or cabazitaxel are FDA-approved for the treatment of advanced prostate and breast cancers, head and neck, and colon cancers.

Antibiotics are derived mostly from *Streptomyces* bacteria, and like the other classes of chemotherapy, act on DNA to kill cancer cells. A major group of antibiotic chemotherapy is the anthracyclines, which include doxorubicin (Adriamycin), daunorubicin, idarubicin, and epirubicin. The anthracyclines have four main mechanisms by which they induce toxicity to cancer cells: inhibition of DNA or RNA synthesis by intercalating between bases; inhibition of topoisomerase II enzyme; reactive oxygen species (ROS) generation; and interfering with histones-DNA interaction. Doxorubicin has been widely used in the clinic for treatment of breast, cervical, stomach, and endometrial cancers, as well as acute leukemia and non-Hodgkin's lymphoma [2]. Daunorubicin was the first anthracycline to be used to treat cancer, where it was shown to be effective in adult acute lymphocytic leukemia [4]. A major disadvantage in using anthracycline for cancer therapy is the high risk of cardiotoxicity. Other bacterially derived chemotherapeutics agents include actinomycin D, bleomycin, mitomycin C, and mitoxantrone. Actinomycin D was the earliest antibiotic used to treat cancer, where it increased survival rate of childhood Wilm's tumor to 90% [5,6].

Cancers can develop in almost any tissue of the body. Cancer cells have specific traits that allow them to grow and spread to other organs. The stage and grade of a tumor is dependent on morphological features of the tissue and whether the cancer has spread to other parts of the body.

14.2 WHAT IS CANCER?

Cancer is a group of diseases characterized by abnormal cell growth and proliferation and the ability to invade and spread beyond the tissues in which they originate. The process of carcinogenesis involves stepwise accumulation of genetic alterations that progressively transform a normal cell to malignant derivatives. According to Hanahan and Weinberg, cancer cells have specific traits that confer malignant behavior. These hallmark traits include reduced dependence on exogenous growth signals, insensitivity to growth suppressive signals, resistance to apoptotic cell death, limitless replicative potential, sustained angiogenesis, and invasive and metastatic capability. Cancer cells can achieve growth autonomy through production of their own growth factors, modulation of growth factor receptors, or activation of intracellular growth signaling pathways. Insensitivity to growth suppressive signals and evasion of apoptosis occurs through alterations of the cell growth cycle or deregulation of the programmed cell death circuitry. Cancer cells are capable of many cell doublings without undergoing senescence. This limitless replicative potential is achieved normally through maintaining telomere length, which is usually lost after a number of cell divisions in normal cells. The generation of new blood vessels, or angiogenesis, is essential for providing the necessary nutrients and oxygen for expansive cancer growth. This angiogenic capability arises in early to mid-stage of cancer progression and is coordinated through a shift in the balance between pro-angiogenic and antiangiogenic molecules, including vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1). The most dangerous of these traits are the ability to invade and metastasize to distant tissues. Majority of cancer deaths are as a result of metastasis of cancer cells to vital organs such as the liver, bone, brain, or lung. Invasive cells typically lose adhesive contact with their neighbors, modify contact with the extracellular matrix (ECM), and acquire the ability to degrade the underlying ECM. Gaining access to the vasculature and surviving in circulation are other features of metastatic cancer cells.

Eventually, the cancer cells arrive at the distant organ and are able to exit the vasculature and form new colonies, completing the metastatic cascade.

14.3 TYPES OF CANCER

Virtually all cells belonging to various tissue types in the body can develop into a cancer. Depending on cell of origin, cancers can be categorized into different types including carcinomas, sarcomas, cancers of the central nervous system (CNS) and hematopoietic system. Carcinomas arise from epithelial cells, which form the linings of glands and the surfaces of organs. Carcinomas can be further subdivided according to the morphological features and the types of epithelial cells from which they arise. For example, cancers resembling glands or producing glandular products are called adenocarcinomas, whereas squamous cell carcinomas have features of protective epithelial tissue that line cavities or channels such as the skin or cervix. Adenosquamous carcinoma is a mix of adenocarcinoma and squamous cell carcinoma. In anaplastic carcinomas the cells lack any distinct features resembling the cells or tissue from which they arise. Large cell carcinomas are composed of large rounded cells with abundant cytoplasm and small cell carcinoma are about three times the size of a lymphocyte with very little cytoplasm. Lung carcinoma can be divided into small-cell or nonsmall-cell carcinoma, where small-cell carcinoma accounts for 15% of lung cancer diagnosed in the United States. Small-cell carcinoma invariably occurs in smokers and is very aggressive with high doubling rate and a predisposition to metastasize. Sarcomas are cancers that arise from cells that make up connective tissue such as fibroblasts, bone, muscle, and fat cells. There are 50 different types of sarcoma which include osteosarcoma which originates in the bone and many soft tissue sarcomas including gastrointestinal stromal tumor, fibrosarcoma, rhabdomyosarcoma, and angiosarcoma. Sarcomas are rare and represent about 1% of the new cancer cases in the United States. Hematopoietic cancers include various types of leukemia, lymphomas, and myelomas which are derived from white blood cells, including B and T cells and plasma cells. Hematological cancers are often referred to as liquid tumors because they are present in the body fluids and are often detected by blood tests. Leukemia is a group of cancers that arise from immature or progenitor white blood cells which can either be acute or chronic depending on the growth rate. Four major kinds of leukemia types have been described and include acute lymphoblastic leukemia, chronic lymphocytic leukemia, acute myelogenous leukemia, and chronic myelogenous leukemia. Lymphomas are cancers that occur in mature lymphocytes including B and T cells. Two major categories of lymphoma include Hodgkin lymphoma and non-Hodgkin lymphoma (NHL), with NHL accounting for about 90% of cases. Cancers of the nervous system are classified as neuroectodermal because of the ectodermal origin of this tissue. Examples of cancers arising from cells in the nervous system include glioma and medulloblastoma. Gliomas arise from glial cells and can be categorized as astrocytoma, oligodendroglioma, or ependymoma based on their morphological appearance. Gliomas are the most common brain tumors and can be low grade and well differentiated or high grade and undifferentiated with worse prognosis. Glioblastoma (GBM) is a high grade astrocytoma with median survival of 12−18 months.

14.3.1 Pathological Features of Cancer

In order to assign a diagnosis, pathologists examine biopsied tissue for morphological features that are hallmarks of cancers. These features of the tumor will also be used to determine the grade and stage of the cancer. Tumor grade is indicative of how quickly the cancer will grow and spread and is based on how abnormal the cells and tissue look microscopically. Differentiated tumors have cells and tissue organization that closely resemble cells and tissue from nondiseased organs. Undifferentiated tumors have cells and tissue that lack normal architecture and grow and spread faster than differentiated tumors. Tumor tissue is often assigned a score which is based on tissue extent of differentiation and other factors depending on the tissue from which the tumor arose. For example, in breast cancer, the Nottingham grading system is based on features which include tubule formation, nuclear structure, and rate of mitosis, as well as differentiation status. Low grade tumors are generally well differentiated and often are associated with better prognosis, while high grade tumors are less differentiated and require more aggressive treatment. The staging of a cancer is based on the size of the tumor and cell type and whether it has spread to lymph node or other organs. One of the most widely used staging systems is the TNM system, where the T refers to the size and extent of the primary tumor, N refers to lymph node spread, and M refers to whether the cancer has spread to other organs. The tumor can be further described using numbers (T0−T4), with the larger numbers indicating the size of the main tumor. The number of lymph nodes that have cancer can also be assigned numbers (N0−N3). Most often healthcare providers report the stage of cancer using the simple stage 0−IV: where stage 0 means abnormal cells are confined, or in situ;

stage I–III implies cancer is larger and spread to nearby tissues; and stage IV means the cancer is spread to other parts of the body.

Stage	Definition
Stage O	Carcinoma in situ
Stage I–III	Higher numbers indicate more extensive disease
Stage IV	Cancer has spread to distance tissues or organs

National cancer institute, 2015. Accessed in 2015 and available at, http://www.cancer.gov/about-cancer/diagnosis-staging/staging/staging-fact-sheet#q3

Genetic mutations give rise to cancerous cells. Three classes of cancer causing genes include oncogenes, tumor suppressor genes (TSGs), and stability genes.

14.3.2 Genetic Alterations in Cancer

Cancer cells arise from a series of genetic alterations that confer upon them the traits discussed above. Some of the most frequently altered genes have been characterized and their roles in carcinogenesis have been the focus of intense investigation over the past four decades. Three classes of cancer genes have being characterized: the proto-oncogene, TSG, and stability gene. An oncogene results in transformation of a normal cell into a cancer cell and typically arises from overexpression or activating mutations of a proto-oncogene. Examples of oncogene include *ras* genes (H-ras, N-ras, K-ras), *myc* genes (c-myc N-myc), epidermal growth factor receptor family, Src kinases, and raf kinase. Proto-oncogenes are typically involved in regulating cell growth and proliferation. Oncogenic K-ras, of the small GTPases family is prevalent in many human tumors and is seen in almost all cases of pancreatic cancer. TSGs protect cells from acquiring cancer traits and loss of such a gene often leads to cancer development. TSGs typically repress the cell cycle or induce programmed cell death. Examples of TSGs include retinoblastoma protein, p53, and PTEN. Stability genes are involved in maintaining genomic integrity and as such orchestrate DNA repair processes, recombination events during cell division, and chromosome segregation. Examples of stability genes include the BRCA1 and ATM, which are frequently mutated in breast cancers and leukemia, respectively.

A single mutational event in an oncogene or TSG is not sufficient to promote carcinogenesis. Carcinogenesis is a multistep process which involves ongoing clonal evolution with multiple mutational events conferring malignant phenotypes to cancer cells. This multistep process was articulated by Peter Nowell who proposed a model where a normal cell becomes neoplastic through acquiring a mutation that confers growth advantage. This growing neoplastic population is subjected to environmental pressures that favor its elimination. However, a member of this neoplastic population acquires more mutations that allow additional growth advantages. Subsequent mutational events further improve the fitness of the original clone and lead to malignant progression in the tumor. This multistep progression can also be observed through the histopathological examination of the diseased tissues. In the colon, e.g., early tumor shows excessive or hyperplastic growth characterized by thicker epithelia. Later during disease progression is the formation of adenoma also known as polyp, which is characterized by hyperplasia and abnormal tissue morphology or dysplasia. At the polyp stage the tumor is still considered benign and after a number of years of further abnormal growth, the adenoma progress to carcinoma then to the metastatic disease. The genetic events leading to colon carcinoma have been well described. The earliest event in colon carcinogenesis is the loss of the APC TSG at chromosome 5q. Later during tumor progression the activation of K-ras oncogene and loss of p53 TSG along with other events such as DNA hypermethylation have been identified. Similar characterizations of genetic events have been described in prostate, lung and pancreatic cancers.

14.4 LEADING CANCERS WORLDWIDE

Over the past three decades cancer incidences worldwide have gradually increased, more so in the industrialized Western world. However, cancer mortality rates have been declining owing to improvements in disease detection and treatment. In 2012, the International Agency for Research on Cancer (IARC) estimated 14 million new cases and 8 million deaths from cancers worldwide. North America and Australia/New Zealand regions have the highest incidences of cancers in both women and men, while Western Africa and South-Central Asia have the lowest incidences

in men and women, respectively. The most prevalent types of cancers are lung, breast, bowel, and prostate, which account for over 40% of new cases.

The prevalence of cancer types varies across regions of the world and can be attributed to differences in diet, lifestyle, environmental exposure, and infections. In the developing world, infection is a major contributing factor to two of the four leading cancers in men and women. Infectious agents, such as hepatitis B virus (HBV), human papillomavirus (HPV) and *Helicobacter pylori*, are the causative agents for liver, cervical, and stomach cancers, respectively. Liver cancer is the second leading cause of cancer deaths in developing countries. In 2012, 745,500 deaths were estimated, with China accounting for 50% of the total. Majority of the liver cancers worldwide are hepatocellular carcinoma and they are primarily caused by HBV. Other risk factors include consumption of food with the aflatoxin (a fungal derived toxin), obesity, type 2 diabetes, heavy alcohol consumption, and smoking. Public health measures can be used to prevent liver cancer, such as implementation of routine HBV vaccination program in infants. Reports suggest that universal HBV childhood vaccination in Taiwan, which began in 1984, has resulted in an 80% decline in liver cancer incidences [7,8].

Cervical cancer is the fourth leading cause of cancer deaths worldwide, where 90% of deaths occur in the developing world. This disproportionate difference in deaths is linked to the limited access to the Papanicaloau (Pap) screening test in the developing world. Changing sexual behaviors, such as multiple partners, have also contributed to increasing incidences of cervical cancer among younger generations in more liberal developed countries. However, recent developments in vaccines against the HPV virus have led to early interventions to prevent cervical cancer. Esophageal cancers are largely influenced by lifestyle practices such as high alcohol intake, smoking, and obesity. The highest incidences are in Asia, and East and Central Africa, while the lowest rates are in Western Africa and in parts of Europe and South America. The prevalence of esophageal squamous cancer correlates with tobacco and alcohol use, which is on the rise in Asian countries such as Taiwan. A decline in esophageal squamous cancer is seen in North America and Europe where there is decreased tobacco and alcohol use. The main risk factors for esophageal adenocarcinoma include obesity and chronic gastroesophageal reflux disease, and secondary factors are smoking and a diet low in fruits and vegetables.

Globally, lung cancer is the leading cause of cancer related deaths in men. Lung cancer is the leading cause for death in women in developed countries and follows breast cancer as the most common cause of cancer deaths in women developing countries. In 2012, lung cancer accounted for 13%, or an estimated 1.8 million patients, of newly diagnosed cancers [9]. The highest incidences of lung cancer were in Europe, Eastern Asia, and North America, while the lowest incidences were in sub-Saharan Africa. Worldwide incidences of lung cancer are mostly attributed to cigarette smoking, accounting for 80% of cases in men and 50% in women. Restricting tobacco use has significantly reduced the cases of lung cancer in many developed countries such as the United States and Finland. However, in the developing world where tobacco use has recently become an epidemic, the cases of lung cancer continue to rise. Exposure to indoor and outdoor air pollution and environmental and occupational carcinogens, such as asbestos, also contribute to lung cancer cases. Chinese women have relatively high rates of lung cancer which are thought to be related to the indoor air pollution from coal stoves and cooking fumes. Early detection tests for lung cancer, such as chest X-rays, have not been effective in reducing the death rates. Unfortunately, only about 15% of lung cancers are detected at the early stages. Treatment options for patients are dependent on the type and stage of the cancer. Survival in early stage non-small cell lung cancer patients have been improved by chemotherapy, radiation, and surgery, whereas in advanced cases, chemotherapy and targeted drugs are combined. Notwithstanding, lung cancer is still very lethal with the 5-year survival rate for all stages about 16% in the United States.

Breast cancer is the most diagnosed and leading cause of cancer related death in women worldwide. Developed countries account for 50% of all cases owing to the higher availability of early detection tests. Known risk factors for breast cancer include age, family history of breast cancer, high breast tissue density, and mutations in breast cancer susceptibility genes, BRCA1 and BRCA2. Reproductive practices such as delayed pregnancy, long menstrual history, use of oral contraceptives, and hormone replacement therapy contribute to breast cancer risk. Although cases of breast cancer are rising, global breast cancer deaths have remained stable. This may be due to increased screening by mammography and breast examinations that allow for earlier detection and treatment. However, there are large disparities in the death rates across the globe with 5-year survival as high as 89% in the United States and as low as 12% in Gambia. The earliest form of breast cancer is referred to as ductal carcinoma in situ (DCIS), where the cancer is confined to milk ducts of the breast and has not invaded to other parts of the breast. Upon detection, patients with DCIS may be effectively treated by lumpectomy and radiation. Advanced stages of breast cancer involve the invasion of the cells to other parts of the breast and local lymph nodes. These cases are usually treated with mastectomy, to remove the entire breast and the local lymph nodes, chemotherapy, and radiation. Classification of breast cancer

subtypes based on the protein expression has helped to determine the course of treatment. Invasive cancers are grouped based on the expression of the hormone receptors, estrogen (ER) and progesterone (PR), and HER2/neu. Hormone receptor-positive cancers are slow growing and can be treated with hormone therapy drugs such as Tamoxifen or aromatase inhibitors. Hormone receptor-negative cancers do not express ER or PR, are usually fast-growing and often occur in patients that have not undergone menopause. Her2-positive cancers express high quantities of the protein and are usually treated with drugs that target Her2 such as lapatinib or the antibody trastuzumab (Herceptin). In contrast, Her2 negative tumors do not express Her2 and are nonresponsive to treatment with drugs that target Her2. Triple positive cancers express hormone receptors as well as Her2 and can be treated with drugs that target hormone modulating drugs as well as agents that target Her2. Triple negative cancers do not express the hormone receptors or Her2 and they grow and spread quickly. Aggressive chemotherapy and radiation are the only treatment options for these patients.

Prostate cancer is the second most diagnosed case of cancer in men worldwide and in developed countries it is the most frequently diagnosed. Rising rates of prostate cancer incidences reflect the increased use of the prostate-specific antigen (PSA) test to detect the disease. In 2012 prostate cancer was the fifth leading cause of cancer related deaths worldwide. Men of African descent in the Caribbean are more susceptible to prostate cancer related death than in any other region. This high risk may reflect a specific genetic susceptibility or differences in diet. Known risk factors for prostate cancer include age, race, and family history. Some studies suggest associations between diet high in processed meats and low in vegetable may be risk factors for prostate cancer. Prostate cancer is often asymptomatic; however there are profound changes at the tissue level. PSA testing and pathologic examination of the tissue provide prognosis and help to guide therapy. The Gleason score is assigned to a tissue specimen based on the abnormal arrangement of the cells in the tissue. Low Gleason scores ($\leq$6) are associated with a favorable prognosis, while higher scores are considered aggressive and treatment is recommended. The standard treatments for localized high-risk prostate cancer include androgen ablation therapy, prostatectomy and radiation [10,11]. For advanced metastatic prostate cancer, aggressive chemotherapy, hormonal therapy and radiation are utilized. The most widely used hormonal therapeutic agents include enzalutamide and abiraterone. Docetaxel and cabazitaxel are used as first-line and second-line chemotherapies, respectively [10].

Many drugs used to treat cancers are derived from natural sources. Semisynthetic analogs of natural compounds often improve efficacy, bioavailability, and safety.

14.5 DRUGS THAT TREAT AND PREVENT CANCER

The approaches to treat cancer are highly dependent on the extent of progression of the disease. Localized disease without any evidence of invasion or spread to nearby organs is often removed through surgical resection followed by radiation and chemotherapy. Advanced, metastatic cancers are inoperable and aggressive chemotherapy is often employed. Many drugs are employed in the treatment of cancers, and the specific agents or combinations thereof are tailored according to the pathology of the tumor and the tissue of origin. This section will be focused on drugs, natural or synthetic, used to treat various cancers.

14.5.1 Natural

Many of the chemotherapeutic agents commonly used for treating cancers are naturally derived. The sources for these anticancer agents include plants and bacteria. One of the most widely used plant-derived anticancer agent is paclitaxel, which was identified as the active component of the Pacific yew from US National Cancer Institute screening efforts in the 1960s. The compound was toxic against a wide panel of cell lines and the primary target was determined to be microtubules. The first reported use of paclitaxel in patients was for ovarian cancer and the drug was subsequently approved for treating breast and ovarian cancers. Vinca alkaloids, vinblastine and vincristine, are derived from the periwinkle plant, *Catharanthus roseus*, and like the taxols target microtubules. However, the vinca alkaloids prevent microtubule polymerization to promote cancer cell death. Bacterially-derived anthracycline chemotherapies widely used in cancer treatment include doxorubicin and daunorubicin. Both compounds are produced by *Streptomyces*, although daunorubicin is more abundant owing to its production by a wide variety of wild-type strains. Daunorubicin is the precursor of doxorubicin in the biosynthetic pathway and genetic engineering strategies have led to increased yields of the more efficacious doxorubicin, which is marketed as Adriamycin. Topoisomerase II is the major target of both

compounds, and binding to this protein induced DNA strand breaks and eventually apoptosis. Bleomycin was isolated from *Streptomyces verticullus* and kills cancer cells by inducing double-strand breaks in DNA. It was discovered in 1962 and has been used to treat lymphoma, testicular, ovarian, and cervical cancers.

14.5.2 Synthetic

Platinum-based chemotherapies, such as cisplatin, carboplatin, and oxaliplatin, are synthetic drugs used in the treatment of testicular, bladder, breast, lung, ovarian, colorectal, and pancreatic cancers, among others. The platinum-based drugs kill cells by forming adducts with the DNA in rapidly dividing cancer cells. The DNA damage caused by the platinum drugs activates various pathways that leading to programmed cell death or apoptosis. The precise mechanism leading to cell death from platinum-based therapy is still unclear; however these drugs have been known to activate signaling pathways that control DNA damage repair and stress, cell cycle, and growth. Cisplatin was the first member of the platinum drugs used to treat cancer, but the toxic side effects posed a challenge requiring the synthesis of analogs to circumvent these issues. About two decades after cisplatin was approved for use in patients, its derivative, carboplatin was approved for treatment of ovarian cancer. The nephrotoxicity associated with cisplatin was no longer an issue with the use of carboplatin. A major limitation of chemotherapy observed during the course of treatment is resistance which can be intrinsic or acquired. The third FDA-approved platinum-based drug for treating cancer was oxaliplatin, which exhibited superior toxicity profiles and efficacy in resistance cancers such as colon cancer. Further synthetic developments in platinum based drugs have generated more soluble analogs that can be administered orally [12].

Another category of synthetic drugs widely used in the treatment of advanced cancer patients is the nucleoside analogs such as 5-flurouracil (5-FU) and gemcitabine antimetabolites. 5-flurouracil was rationally designed and synthesized by Charles Heidelberger and Robert Duschinsky in 1957 based on the observation that tumors utilized uracil to a greater extent than normal tissues. The first report of clinical use of 5-FU was in 1963, with response in cancer of the bowel, breast, stomach, ovary, pancreas, bladder, and cervix. Gemcitabine was synthesized at Eli Lilly by Larry Hertzel as a potential antiviral compound. However, it was shown to be very effective against leukemia, solid tumors *in vivo* in mouse models [13,14]. Numerous clinical trials have been conducted with gemcitabine, including advanced breast, bladder, nonsmall cell lung, and pancreas cancers [15–18]. Gemcitabine is the standard of care for pancreatic cancer and late stage bladder cancer.

Many chemotherapeutic drugs are analogs of naturally occurring compounds. Examples of synthetic chemotherapeutic analogs include etopiside and teniposide, which are topoisomerase inhibitors derived from podophyllotoxin, a toxin found in American Mayapple. Etoposide is used in many cancers, while teniposide is mainly used in acute lymphocytic leukemia. Derivatives of the DNA topoisomerase I inhibitor, camptothecin, irinotecan and topotecan, are also widely used chemotherapies often used in combination regimens. Irinotecan is used in combination with 5-FU and leucovorin (FOLFIRI) for treating colon cancer. Topotecan is an orally available, water soluble salt used for ovarian, cervical, and small cell lung cancers. Other chemotherapeutic analogs include paclitaxel derivatives, docetaxel, and/or cabazitaxel. The semisynthetic chemotherapies are generally designed to have improved efficacy and safety profiles.

14.6 RECENT RESEARCH NATURAL ISOLATES AND EXTRACTS

Therapeutic agents derived from natural sources, such as plants, marine organisms, and microbes, have been used to treat many ailments for centuries. Phytochemicals are believed to not have such severe side effects as synthetic agents. Therefore, investigation of isolates or extract for anticancer properties is a very active area of cancer research. Many studies have been conducted on the potential of plant-derived extracts to either prevent or treat cancer. This section will highlight recent studies on the anticancer properties of naturally derived compounds or extracts and their mechanisms of action.

14.6.1 Epigallocatechin-3-gallate

Plant extracts contain a variety of compounds of different classes with reported activities against cancer. The anticancer activity of green tea, *Camellia sinensis*, has been attributed to the catechin polyphenols. The most abundant catechin in green tea is epigallocatechin-3-gallate (EGCG), which has been extensively investigated for its activity against many cancers including brain, prostate, cervical, and bladder *in vitro* and *in vivo*. EGCG targets many signal transduction pathways in cancer cells, but the precise mechanism for its anticancer activities is not known. Some of the pathways

targeted by EGCG *in vitro* and proposed for its chemopreventative or anticancer effects are mitogen-activated protein kinases (MAPKs), growth factors and cell cycle proteins, NF-κB, and apoptosis signaling [19]. EGCG inhibits ERK1/2, JNK and p38 MAPKs in cell lines from colon cancer [20]. Conversely, it was shown that the anticancer effect of EGCG in breast and prostate cancer cell *in vitro* is due to activation of MAPKs [21,22]. Other reported anticancer effects of EGCG on prostate cancer cells include the sensitization of LNCaP cells to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) induced apoptosis. Combination of EGCG with TRAIL led to increased levels of proapoptotic proteins from the intrinsic and extrinsic pathways and decreased expression of prosurvival proteins such as the Bcl_2 and Bcl_{XL} [23]. EGCG has also been shown to effectively delay tumor progression *in vivo* in a transgenic mouse model of prostate cancer and a xenograft model of breast cancer [24–26]. Several clinical trials have been conducted to examine the chemopreventative or therapeutic efficacy of green tea extracts and EGCG in prostate, liver, cervical, and lung cancers with inconclusive results [27–31].

14.6.2 Apigenin

Apigenin is a flavonoid present in many vegetables and herbal spices including parsley, celery, basil, chamomile, cilantro, and oregano [32,33]. Plant flavonoids have been shown to decrease the risk of development of cancers and have been widely researched for chemoprevention. An epidemiological study in men has indicated the consumption of five flavonoids including apigenein, myricetin, quercetin, kaempferol, and luteolin decreases the incidence of all types of cancer, as well as the mortality from gastrointestinal and respiratory cancers [34,35]. A Finnish study also reported that, in both women and men, consuming a diet rich in flavonoids decreases the risk of cancers, more so in lung cancer, over a 24-year-long follow-up period [36]. Apigenin has many biological properties, including antioxidant, antimutagenic, anticarcinogenic, antiinflammatory, and antiproliferative activities. Many molecular targets for apigenin have been described in human cancers, making it difficult to attribute a specific mechanism of action for its anticancer properties [33]. A comprehensive screen has recently identified 160 human cellular targets of apigenin which are found in three main functional categories: GTPase activation, membrane transport, and mRNA metabolism and alternative splicing [37]. Apigenin has been investigated for its anticancer properties in numerous cancer cell lines. In HT-29 colon cancer cells apigenin induces G2/M cell cycle arrest by binding to ribosomal protein RPS9 resulting in downregulation of the cyclin dependent kinase CDK1 [38]. Similarly, apigenin treatment induces apoptosis and blocks cell cycle at the Go/G1 and G2/M checkpoints in oral squamous cell carcinoma *in vitro* by decreasing expression of key cell cycle proteins including CDK1 [39]. In prostate cancer cells apigenin inhibits growth through various mechanisms including loss of androgen receptor in androgen-responsive LNCaP cell lines [40]. Cell cycle arrest due to loss of cyclin D1, D2 and E and their activation partners also contribute to apigenin-induced growth inhibition in prostate cancer cells [40–42]. Further, apigenin induced apoptosis *in vitro* in LNCaP and androgen-resistant DU145 and PC-3 cell lines and *in vivo* through targeting various regulators of the programmed cell death pathway including Bax, Bcl2, and the caspases [43–46]. Other targets of apigenin in prostate cancer cells are the NF-κB, PI3K/Akt, MAPK, p21, and p53, which all converge on the apoptotic pathway [33,41,42,45–48]. Apigenin has also been shown to sensitize doxorubicin-resistant liver cancer cells to doxorubicin and resistant colon cancer cell lines to the BH3 mimetic ABT-263 through targeting Akt signaling [49,50].

14.6.3 Genistein

Epidemiological studies indicate that soy consumption has been associated with reduced risk for breast cancer in Asian women [51,52]. Reports also indicate that the protective effect of a diet high in soy is lost in second generation Asian women who adopt a Western diet [52,53]. The most common isoflavone found in soy is genistein, the putative anticancer agent. Accordingly, many studies have been conducted on the mechanism of action of this phytochemical. Genistein is in a class of compounds called pythoestrogen due to its structural similarity to estrogen and its ability to mimic or antagonize estrogen action by binding to estrogen receptors. Although, some of the anticancer properties of genistein may be elicited by targeting the estrogen receptor, other estrogenic independent actions of the compound have been described [54]. Genistein was first shown to be an inhibitor of targeting tyrosine kinases [55]. *In vitro*, genistein promotes cell death in different cancer types including gastric, colon, breast, pancreas, and prostate [56–61]. In leukemic cells genistein induced apoptotic cell death after 24 h treatment, whereas shorter treatments induced cell cycle arrest [62]. Conversely, normal lymphocytes were unaffected by genistein treatment under these same conditions, suggesting a safe toxicity profile for this agent. Early reports on the effect of genistein on gastric cancer cells demonstrated that growth inhibition resulted from cell cycle arrest at the G2/M stage [56]. Some cell cycle genes reported to be

targets of genistein include p21, p27, p16, cyclin B1, Cyclin D1, and CDk1 [63]. Genistein-induced cell cycle arrest is likely linked to DNA damage resulting from inhibition of topoisomerase II activity [64,65]. The mechanism of apoptotic cell death by genistein in MDA-MB231 breast cancer cells was shown to be partially dependent on the activities of NF-κB and Akt, key survival genes in cancer cells [63,66]. Other reports indicate that genistein shift the balance of the Bcl family proteins, Bcl-2 and Bax, to promote apoptosis [67,68]. Akt signaling was shown to be the primary mechanism by which genistein prevents the formation of tumors in the transgenic adenocarcinoma mouse prostate model (TRAMP) [69].

The combinations of genistein with cisplatin, doxorubicin, or docetaxel chemotherapies was shown to synergistically enhance cytotoxicity and cell death in prostate, breast, lung, and pancreatic cancers [66,70]. Suppression of NF-κB was shown to be the primary mechanism mediating the sensitization of the cancer cells to the chemotherapeutic agents. In a xenograft mouse model of prostate cancer, the anticancer and antimetastatic effects of docetaxel chemotherapy were potentiated by genistein [71]. In this model, the ostepontin and receptor activator of NF-κB (RANK) signaling axis was shown to mediate the inhibition of metastastic spread of prostate cancer cells to the bone by combination of docetaxel with genistein. Diffuse large cell lymphoma is characterized by elevated NF-κB signaling, and combining standard of care chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisone) with genistein synergistically reduced tumor growth in xenograft model through this pathway [72]. One advantage of combining genistein with chemotherapy is the potential to use lower doses of the chemotherapy, thereby decreasing the toxic side effects.

While genistein was shown to be a potent chemopreventive agents in the TRAMP mouse model, in mice that already harbored low grade tumors, genistein increased the progression and metastatic spread of the disease [73,74]. The effect of genistein in this progression model was shown to be age- and dose-dependent, indicating that the timing of genistein exposure might have different effects of cancer progression [74]. Similarly, in a carcinogen induced rat model and transgenic (MMTV-neu) mouse models of breast cancer, genistein was shown to be chemopreventive during the prepubertial stage, but was ineffective in delaying tumor progression [75,76]. Further, some studies suggest that genistein counteracts the cytotoxic effects of chemotherapeutic agents, tamoxifen and letrozole, in hormone responsive breast cancer cell lines [77–81]. Therefore more careful studies are needed to examine the proper therapeutic window for genistein in cancers, particularly the hormone responsive types. On the clinicaltrials.gov webpage, trials for genistein in various cancers are being conducted or have been concluded. A number of early phase clinical trials have been conducted in prostate cancer with either no effect or disease stabilization [82]. In a study genistein treatment in women with early-stage breast cancer resulted in a gene expression signature indicative of increased proliferation and overexpression of the fibroblast growth factor receptor 2 (FGFR2) [83]. Another phase 2B study suggests that genistein might be hazardous in premenopausal women who are at high risk of developing breast cancer [84].

14.6.4 Kaempferol

Kaempferol is a natural flavonol, a class of flavonoid, found in many fruits vegetables and herbs, including grapes, tomatoes, broccoli, tea, and ginkgo biloba leaves. This biologically active compound exhibits many pharmacological activities including antioxidant, antiinflammatory, antimicrobial, antidiabetic, and anticancer activities. The anticancer activity of kaempferol has been reported in cancer cells from different organs including breast, ovarian, gastric, lung, pancreatic cancer, and blood cancers [85,86]. Kaempferol interferes with many signaling pathways required for the survival of cancer cells. In breast cancer cells, kaempferol induces apoptosis through an extracellular signal regulated kinase (ERK) dependent mechanism [87]. Kaempferol can also induce apoptosis in breast and ovarian cancer cells through activation of p53 and suppression of cell cycle genes [88,89]. In addition to decreasing ERK and Akt activities, kaempferol also increases ROS production in human glioma cells leading to induction of apoptosis [90,91]. Glioablastoma cells were also rendered more sensitive to doxorubicin upon cotreatment with kaempferol. Similarly, kaempferol increases sensitivity of glioma cell to TRAIL-induced apoptosis through reduction of Akt activity and survivin protein [92]. Kaempferol was shown to be as potent as 5-FU in promoting apoptosis in pancreatic cancer cells, and when combined with 5-FU, has an additive effect on cell death [86]. Keampferol as a phytoestrogen has been studied as potential targets for hormone-dependent cancers such as breast and prostate. However, kaempferol was effective in both hormone dependent and independent breast and prostate cancers [93,94]. Kaempferol anticancer activity is not just restricted to inducing cell death, as some studies demonstrate the antiangiogenic and antimetastatic properties of this natural product [89], and it has been reported the antiangiogenic activity of kaempferol in ovarian cancer cells was due to the downregulation of VEGF [95,96]. The antimetastatic properties of kaempferol were linked to inhibition of ERK

and Akt signaling and decreased activity of matrix metalloproteinases including MMP-2, MMP-3, and MMP-9 [96–98].

14.6.5 Resveratrol

Resveratrol was originally identified for inhibiting cyclooxygenases (COXs), the key enzymes involved in prostaglandin synthesis, conferring potent antiinflammatory and antioxidant properties. This natural polyphenol is mainly found in grapes, berries, and red wine. Recently, resveratrol was linked to the strong cardioprotective effect of red wine which has been observed in the French population. The effects of resveratrol have also been studied in other diseases, including cancer. As a potential chemopreventative agent, resveratrol is seen as a natural, safe alternative over pharmaceutical agents. Resveratrol was shown to be effective against tumor initiation, promotion, and progression in many types of cancer [99–101]. One of the first reports of the anticancer potential of resveratrol indicated that it reduced the activity of COX-1, without affecting COX-2, and reduced inflammation in a model of carrageenan-induced paw edema. Further, in human promyelocytic leukemia cells (HL-60), resveratrol reduced 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced free radical formation and promoted terminal differentiation. Finally, resveratrol was shown to prevent 7,12-dimethylbenz(a) anthracene (DMBA)-induced formation of preneoplastic lesions in the mouse mammary gland as well as delayed tumor progression in a two-stage mouse skin cancer model where DMBA and TPA were used as cancer initiator and promoter, respectively. Despite many preclinical data demonstrating the effectiveness of resveratrol as a chemopreventive agent, translating the findings into the clinic has proven quite challenging. One of the challenges has been achieving pharmacologic dose similar to what is seen in individuals consuming a diet rich in the compound. A recent report suggests that a low dose of resveratrol, equivalent to a large glass of red wine, was more effective than the doses used in a previous phase I clinical trials [102–105]. Another challenge for employing resveratrol as a chemopreventive in the clinic is identifying the appropriate biomarker. Resveratrol may target many other pathways or proteins other than COX-1, including peroxisome proliferator-activated receptor (PPAR), endothelial nitric oxide synthase (eNOS), and silent mating type information regulation 2 homolog 1 (SIRT1), NFκ-B, Wnt, and PI3K/Akt pathways [100,106,107]. What pathways are relevant for the anticancer effects of resveratrol and whether these are conserved across cancer types is not known. The efficacy of low dose resveratrol in mouse and human colon cancer was associated with increased activated adenosine monophosphate kinase suggesting a potential biomarker for resveratrol chemopreventive activity [102]. Given the multiple purported health benefits of resveratrol, particularly cardioprotective, anticancer, antiinflammatory, and antiaging, there will be continued research to further understand its mechanism(s) of action.

14.6.6 Curcumin

Turmeric from the rhizome *Curcuma longa*, is a widely consumed dietary curry spice. Curcumin, a polyphenol compound, is the active component of and constitutes 2–8% of turmeric preparations [108]. Curcumin is relatively safe and has been used extensively in Ayurvedic medicine for its multiple benefits including antioxidant, analgesic, antimalarial, and antiinflammatory properties [109]. The first reported use of curcumin to treat human disease was published in 1937 in the Lancet for patients with biliary disease, where daily dosing for 3 weeks resulted in improved gall bladder function [110]. Preclinical studies demonstrated the chemopreventive effects of curcumin in experimental models of *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal cancer and DMBA-induced mammary cancer in rats [111,112]. Curcumin also prevented colon adenoma formation in mice genetically predisposed to colon cancer, due to mutation in the APC gene [113]. The effects of curcumin have been studied in the clinic for its anticancer properties against multiple myeloma, breast, colon, and pancreatic cancers [110,114]. In a phase II clinical trial, curcumin showed clinical biological activity in pancreatic patients, with one patient having stable disease for more than 18 months and another had a brief tumor regression accompanied by an increase in serum cytokine levels [115]. Oral curcumin (4 g/day) reduced the number of aberrant crypt foci, precursor of colon carcinoma, by 40% in smokers participating in a phase IIa clinical trial [116]. A significant limitation of curcumin use in patients is the low bioavailability, requiring doses as high as 8–12 g [116].

Curcumin is pleiotropic with multiple molecular targets, including transcription factors, growth factors, cytokines, receptors, and enzymes [110]. NF-κB signaling is a major target of curcumin in many cancer cell types *in vitro* including colon, breast, pancreatic, prostate, and lymphoma [117–120]. In colon cancer cells, curcumin inhibited PMA, TNF-α, and fecapentate-induced COX-2 expression through inhibition of the NF-κB inducing kinase (NIK)/IkB kinase signaling complex and suppression of NF-κB transactivation [117]. Curcumin targets key proteins in cell survival,

proliferation, and apoptotic pathways in different cancers, including Akt, Bax, Bcl-2, and Bcl-xL [118,119,121]. Curcumin also sensitizes cancer cells to chemotherapy agents and apoptosis-inducing agents in an NF-κB-dependent mechanism. In prostate and breast cancers, curcumin potentiates the effect of paclitaxel leading to reduced tumor growth and metastasis in xenograft models *in vivo* [120,122]. Curcumin also sensitizes pancreatic, colon, and bladder tumors and multiple myeloma to standard therapeutic agents in mice [123–127]. These data suggest that combining curcumin with lower doses of chemotherapy may reduce some of the side effects of chemotherapies in the clinic.

14.6.7 Parthenilode

Feverfew (*Tenacetum parthenium*), the traditional medicinal plant, has been used for treating migraines, inflammation, stomachache, fever, and rheumatoid arthritis. One of the main components of feverfew involved in the biological properties is parthenolide (PTL). Parthenolide is a sesquiterpene lactone found mostly in the flowers and leaves of the feverfew at 0.1–02% of dry weight. The antitumor activity of parthenolide was first described in 1973 as the active agent in the extract from *Magnolia grandiflora* [128]. The compound has shown antitumor activities against many cancer types including AML, ALL, brain, breast, colon, pancreas, prostate, and skin. Importantly, parthenolide exhibits cancer specific activity and is nontoxic to normal cells making it an attractive candidate for drug development [129–131]. However, the high lipohilicity of the compound limits the bioavailability, requiring the synthesis of analogs with improved solubility [132]. One such analog is dimethylaminoparthenolide (DMAPT), which has been shown be an effective antitumor agent in models of leukemia, prostate, lung, breast, bladder, and pancreatic cancers [132–136]. PTL targets many pathways to promote toxicity or cancer cell death, including NF-κB [137–139], signal transducer and activator of transcription (STAT), c-Jun N-terminal kinase (JNK), and ROS. PTL inhibits NF-κB signaling through direct binding to the IκB kinase (IKKβ) and p56 forming a covalent bond with the sulfhydryl side chains at the cysteine-179 and cysteine-38, respectively [139,140]. In either case, the binding of PTL to IKKβ and p65 leads to decrease NF-κB binding to DNA and subsequently apoptosis. STAT-3 activity is upregulated in many cancers, including leukemia, breast, colorectal, prostate, skin, and hepatocellular carcinoma, where it supports tumor cell proliferation. Parthenolide suppresses STAT-3 activity in hepatocellular carcinoma cells and sensitizes the cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced cell death [141,142]. The exact mechanism of suppression of STAT-3 activity by parthenolide is unknown, and it has been speculated that it forms covalent bonds with the sulfhydryl group in JAK-2 kinase. Parthenolide-induced sensitization to TRAIL-mediated apoptosis was also attributed to JNK activation and NF-κB inhibition [143]. The JNK/NF-κB axis regulating TRAIL-mediated apoptosis was conserved across many cell lines compared to the STAT-3 which was restricted to hepatocellular carcinoma [143]. Cancer cells often exhibit aberrant redox balance, often with higher oxidative state. Chemical entities that further promote ROS generation, result in the production of harmful oxidants that may damage cellular components and eventually cell death. Parthenolide has been shown to promote oxidative stress in prostate cancer cells through activation of NADPH oxidase and sensitizes these cells to radiation-induced death [130]. ROS generation was also shown to be important for parthenolide-induced death in hepatocellular carcinoma cells, which was attenuated by supplementing the antioxidant *N*-acetyl-L-cysteine (NAC) [144].

Parthenolide's anticancer effect is not just restricted to promoting cell death, but it also exhibits antiangiogenic and antimetastatic activities [145–148]. Some reports indicate that PTL inhibits angiogenesis through inhibiting matrix metalloproteinase-9 IL-6 and VEGF expression or activity [146,148,149].

14.6.8 Plant Extracts

14.6.8.1 Pomegranate Fruit Extract

Pomegranate, *Punica granatum* L is widely consumed in drink and juices, used as a dietary supplement and in folk medicine. The fruits and seeds have been considered sacred in many of the world major religions and mythologies [150]. Pomegranate is rich in beneficial phytochemicals including the catechin phenolics, flavonols, anthocyanins, and ellagic acid [151–153]. Pomegranate fruit extracts (PFE) exhibit various biological activities with potential therapeutic uses against inflammation, cancer, diabetes, osteoporosis, coronary heart disease, Alzheimer's, AIDS, and male infertility [153–156]. A number of studies have demonstrated chemopreventative as well as therapeutic activities against prostate cancer *in vitro* and *in vivo*. PFE inhibits cell proliferation and induces apoptosis in PC3 human prostate cancer cells by altering the levels of pro-apoptotic, antiapoptotic proteins, and also cell cycle proteins [157]. Pomegranate juice and fruit skin extracts were shown to induce apoptosis in androgen-independent prostate cancer cell

lines by inhibition the NF-κB inflammatory signaling pathway [158]. Similar effects of pomegranate extract on NF-κB signaling were observed in HT-29 human colon cancer cells in addition to attenuation of TNF-α-induced COX-2 expression [152]. Fermented PFE and a solvent-extracted polyphenol-rich extract inhibited the proliferation of estrogen dependent and independent breast cancer cell lines, MC7 and MM-MDA-231 [159]. Other reports indicate that PFE has antiinvasive properties in both prostate and breast cancer cell lines, implicating possible therapeutic utility against tumor metastasis [159–161]. Various *in vivo* studies have demonstrated the efficacy of PFE in inhibiting tumor growth. Pomegranate juice, PFE, or ellagitannin-enrich pomegranate extracts decrease tumor formation and growth in xenograft transplantation models of prostate cancer in immunocompromised mice and also in a transgenic mouse model [157,162,163]. Importantly, oral PFE impairs tumor metastasis and development of poorly differentiated tumors in the transgenic TRAMP model of prostate cancer through targeting PI3K/Akt signaling [162]. Together, these studies indicate that pomegranate extract may serve is a chemopreventive and chemotherapeutic agent in prostate cancer. A few studies have examined the therapeutic potential of pomegranate in humans with very promising results [164,165]. Using PSA doubling as a surrogate for therapeutic efficacy, pomegranate juice consumption significantly increased the PSA doubling, without any adverse events, in men following surgery and radiation. In one study, participants without lymph node or metastatic spread were given 8 ounces of pomegranate juice daily for a period of 13 months resulting in a PSA doubling time of 15 months at baseline to 54 months after treatment [164]. A more recent randomized, double-blind phase II dose exploration study using two doses of pomegranate extract increased the PSA doubling time from 11.9 to 18.8 months in the low dose group to 12.2–17.5 in the high does group after 18 months of treatment [165].

14.6.8.2 Marijuana

Cannabis sativa, or marijuana, has been used widely for recreational, medicinal, and religious purposes and also industrially for making several products derived from hemp, the soft fiber from the stalk. Recreational use has always been subjected to criminalization, but recently popular opinion has shifted toward legalizing its use. The Netherlands and a few states in the United States have legalized the recreational use of the plant in limited amounts. The biological activity in marijuana is mostly attributable to the group of terpenophenols called the cannabinoids. Most of pharmacological activity is assigned to delta-9-tetrahydrocannabinol (Δ^9-THC) owing to its high potency and abundance in the plant. Although the original research focus on the cannabinoids was for the psychoactive properties, other areas of interest including treatment for pain associated with multiple sclerosis or spinal cord injury, Crohn's disease, antiemetic for HIV medication, and management of Gilles de la Tourettes syndrome have arisen [166]. The primary mode of action is on the endocannabinoid system which consists of two G-protein-coupled receptors, CB1 and CB2. The CB1 is present in the CNS where it mediated the neurologic effects of the cannabinoids. CB2 is expressed mostly in immune cells and less expressed in the CNS. Numerous studies on the effects of cannabinoids on various types of cancers have been reported [167,168]. Early reports by Munson et al. demonstrated that Δ^9-THC and Δ^8-THC inhibited the growth of Lewis lung adenocarcinoma cell *in vitro* and in mice [169]. Subsequently, the anticancer effect of cannabinoids has been demonstrated in breast, lung, prostate, skin, and colon cancers as well as in glioma [170]. More importantly, cannabinoids also exhibit selectivity to glioma and breast cancer cells, sparing normal cells, and avoiding some of the side effects of traditional chemotherapy [171,172]. Synthetic or cannabis-derived cannabinoids exhibit anticancer effects through multiple mechanisms including inducing cell cycle arrest, apoptosis, or autophagy [173]. The first report of cannabinoid-induced apoptosis was in glioma models where administration of Δ^9-THC and WIN-55,212-2 led to tumor regression and prolonged survival in mice and rats [174]. Treatment of the glioma cell *in vitro* led to apoptosis through prolonged ceramide production and ERK activation [174]. Prostate cancer cells treated with synthetic cannabinoid WIN-55,212-2 were arrested in G_0/G_1 phase of the cell cycle, downregulated cyclins and cyclin-dependent kinases, ultimately resulting in apoptotic cell death in an ERK1/2 MAP kinase-dependent manner [175]. Subsequently, another report indicated that WIN-55,212-2 induced cell death in gastric cancer by a similar ERK1/2 MAP kinase-dependent mechanism [176]. Other mechanisms of apoptotic induction by cannabinoids have been described, including the accumulation of ceramide and ROS, ultimately leading to caspase activation and loss of mitochondrial membrane integrity. Cannabinoids also induce cell death in some cancer cells through autophagy, which involve the lysosomal degradation of soluble proteins and organelles during stress conditions [173]. Some reports indicate that cannabinoid-induced autophagy occurs concomitant with apoptosis and is upstream of apoptosis [177]. Cannabidiol, the nonpsychoactive cannabinoid, was found to be the most antiproliferative cannabinoid when tested against a panel of cancer cell lines from human breast, colorectal, prostate, and gastric adenocarcinoma, and rat glioma, leukemia, and transformed thyroid cells. Cannabidiol also inhibits the growth and metastasis of xenograft MDA-MD-231 breast cancer cell. The antiproliferative

effect was shown to be in part dependent on the CB_2 receptor and elevation of intracellular Ca^{2+} and ROS [178]. Other studies demonstrate that synthetic agonist to the CB1/2 JWH-133 and WIN-55,212-2 inhibit breast cell proliferation and migration *in vitro* and reduced tumor growth, proliferation, angiogenesis, and metastasis in xenograft MDA-MB-231 tumors. Similarly, the CB1/2 synthetic agonists delay, and reduced tumor growth in the polyoma middle T (PyMT) transgenic breast cancer mouse model [179]. The antitumor mechanism of action of the CB1/2 agonists in breast cancer was shown to be due to the downregulation of cyclooxygenase-2 and induction of apoptosis [179]. Other reports indicate that the synthetic CB1/2 agonists JWH-015 and WIN-55,212-2 also inhibit nonsmall cell lung cancer growth and metastasis. Highlighting the importance of the CB1/2 receptors in cannabinoid anticancer effects, Δ^9-THC has been shown to promote tumor growth and metastasis in mouse 4T1 breast cancer cells that express low to undetectable levels of these receptors. The tumor promoting mechanism of Δ^9-THC in these low CB1/2 expressing cells was due to immunosuppression through promoting the Th2 immune response [180].

14.7 PRACTICE QUESTIONS

1. How do traditional chemotherapeutics differ from nontraditional chemotherapeutics?
2. List the classification of chemotherapeutics and discuss each.
3. How do cancer cells achieve growth autonomy?
4. What are the different types of cancers and discuss their pathology?
5. What are the leading cancers worldwide?
6. Discuss two drugs that treat cancer, one natural and the other synthetic.
7. Much recent and current research is conducted on plant extracts for anticancer properties, discuss three of these.

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Chapter 15

Bioactive Plant Molecules, Sources and Mechanism of Action in the Treatment of Cardiovascular Disease

A.C. Liwa[1,2], E.N. Barton[3], W.C. Cole[1] and C.R. Nwokocha[3]

[1]University of Calgary, Calgary, AB, Canada, [2]Catholic University of Health and Allied Sciences, Mwanza, Tanzania, [3]University of the West Indies, Kingston, Jamaica

Chapter Outline

Teaching Goals

The role and compelling evidence of efficacy of bioactive natural products in ameliorating pathological conditions of hyperlipidemia, atherosclerosis, and hypertension.

An understanding of selected well-studied molecules and sources with compelling evidence of CVD efficacy in vivo and/or clinical settings.

An appreciation of: (1) the physiological mechanisms of cholesterol homeostasis, and the synthetic and natural products with efficacy in cholesterol reduction, and (2) the physiological regulation of blood pressure, and the synthetic and natural products with efficacy in blood pressure reduction.

15.1 INTRODUCTION—THE PROBLEM AND NEED FOR NOVEL CARDIOVASCULAR DISEASE THERAPIES

Cardiovascular diseases (CVDs) such as ischemic heart disease, cerebrovascular disease (ischemic and hemorrhagic stroke), hypertension, and heart failure are a leading cause of global morbidity, mortality, and health care spending. Significant gains have been made in the past 20 years in reducing the incidence of CVDs in high income countries where the affected are predominantly greater than 60 years old [1,2]. However, the burden in low and middle income countries remains high, accounting for almost 80% of global mortality owing to cardiovascular causes, and it occurs predominantly in working-age individuals [1,2]. The economic burden of CVDs is currently valued at 7% of gross domestic product in low and middle class countries, and it is expected to rise with an anticipated increase in premature mortality due to noncommunicable diseases by 2020 [3]. The present and projected economic and social consequences of CVDs cannot be accommodated by health care organizations in low and middle income countries, and represent an unsustainable burden on the economy of high income nations.

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This impending crisis of global social and economic burden has prompted member states of the United Nations to adopt a goal of a 25% reduction in deaths between the ages of 30 and 70 years owing to noncommunicable diseases such as CVDs by 2025 [4]. How and whether this ambitious goal can be attained remains uncertain. The recent reduction in CVD mortality in high income countries has been achieved through a combination of: (1) population-wide risk factor reduction (primary prevention, e.g., reductions in hyperlipidemia, salt intake, and blood pressure); (2) improved identification of individuals at risk; and (3) the application of costly, but efficacious therapies for secondary prevention of CVDs (including statins, angiotensin converting enzyme inhibitors, thrombolytics, angioplasty, and bypass surgery) [5–9]. Unfortunately, public risk factor reduction is not sufficient in isolation [10], and it is unlikely that the required health care costs of improved identification and secondary prevention can be sustained in high or low/medium income countries. These economic limitations, as well as the limited number of novel CVD drugs introduced by the pharmaceutical industry in the past 20 years [11], have fostered increased worldwide interest in natural products as sources of low cost alternative therapies for the prevention and/or treatment of CVDs.

In this chapter, we review the natural products containing bioactivities for which there is compelling evidence of efficacy in ameliorating hyperlipidemia, atherosclerosis, and hypertension. Space limitations do not permit a comprehensive review of all sources and bioactive molecules that have been identified. Rather, here we focus on selected well-studied molecules and sources with compelling evidence of anti-CVD efficacy in animal studies and/or clinical settings.

15.2 HYPERLIPIDEMIA AND ATHEROSCLEROSIS

15.2.1 Pathophysiology of Hyperlipidemia and Atherosclerosis

Lipids are a major class of hydrophobic molecules in the human body, and play key roles in cell structure (e.g., glycerophospholipids), cell-to-cell communication (e.g., eicosanoids including prostaglandins, leukotrienes, thromboxanes), intracellular signaling (e.g., diacylglycerol, phosphatidylinositol phosphates), and energy storage (triglycerides (TGs)) (for additional details on lipids, lipid homeostasis, hyperlipidemia, and risk assessment see refs [12–14]). The main lipid molecules include fatty acids and metabolites, such as TGs, di- and mono-glycerides, phospholipids, and cholesterol. As plasma lipids are insoluble in water, they are associated with apolipoproteins during transport as lipoprotein particles in the circulation. Lipoproteins are classified into seven groups having varied density, size, and content of TGs, cholesterol, phospholipids, and apolipoproteins ordered from large to small: chylomicrons, chylomicron remnants, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL-C), intermediate density lipoprotein (IDL), high-density lipoprotein (HDL-C) [15]. LDL-C particles contain ApoB, a robust and reliable indicator of CVD risk [16], whereas HDL-C particles contain ApoA as the principle structural protein (also ApoC and E [15]).

The key features of our current understanding of cholesterol homeostasis are shown in Fig. 15.1 (see [17] for a detailed review). Cholesterol is acquired in the diet via Niemann-Pick C1-like 1 (NPC1L1) transporters in apical membrane enterocytes of the small intestine [18], or generated endogenously from acetyl-coenzyme A via a pathway in which the rate-limiting conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) into mevalonate is catalyzed by the enzyme HMG-CoA reductase (HMG-CoA-R) [17]. Dietary lipids are incorporated into chylomicrons in the intestine, TGs are metabolized by muscle and adipose cells through the actions of lipoprotein lipase in endothelial cells, and circulating chylomicron remnants carrying cholesterol absorbed in the small intestines are cleared by the liver [17]. Cholesterol homeostasis is dependent on a coordinated activity of three pathways: (1) intracellular cholesterol esterification mediated by the enzyme acyl-CoA cholesterol acyl transferase (ACAT2) which permits cholesterol accumulation in lipid droplets; (2) cholesterol synthesis; and (3) the clearance and elimination of LDL-C as bile acids which involves LDL-receptor-mediated uptake into hepatocytes where the enzyme, cholesterol 7-α-hydroxylase (i.e., CYP7A1, cytochrome P450 family 7, subfamily A, polypeptide 1) mediates the rate-limiting step in the oxidation of cholesterol into primary bile acids [12,19,20]. The ATP-binding cassette proteins ABCG5 and ABCG8 operate as a functional complex to limit dietary sterol accumulation by mediating sterol secretion from intestinal epithelial cells into the lumen, and by promoting secretion of hepatic sterols into the bile [21]. In contrast, ABCA1 (cholesterol efflux regulatory protein) and ABCG1 are responsible for cholesterol efflux out of cells leading to the generation of HDL-C particles. The liver X nuclear receptor α (NXR) and farnesoid X nuclear receptor (FXR; or NR1H4) are ligand-activated transcription factors that respond to intracellular oxysterols and bile acid levels, respectively, and act as a heterodimeric complex to control the expression of genes encoding key enzymes and transport proteins involved in cholesterol homeostasis [12,22]. For example, dietary cholesterol stimulates LXR upregulation of *ABCG5* and *ABCG8* expression, and elevated bile acid levels stimulate FXR which promotes the expression of SHP (short heterodimer partner) that acts to suppress the expression of CYP7A1 leading to reduced bile formation [12,23]. In contrast, low cellular cholesterol

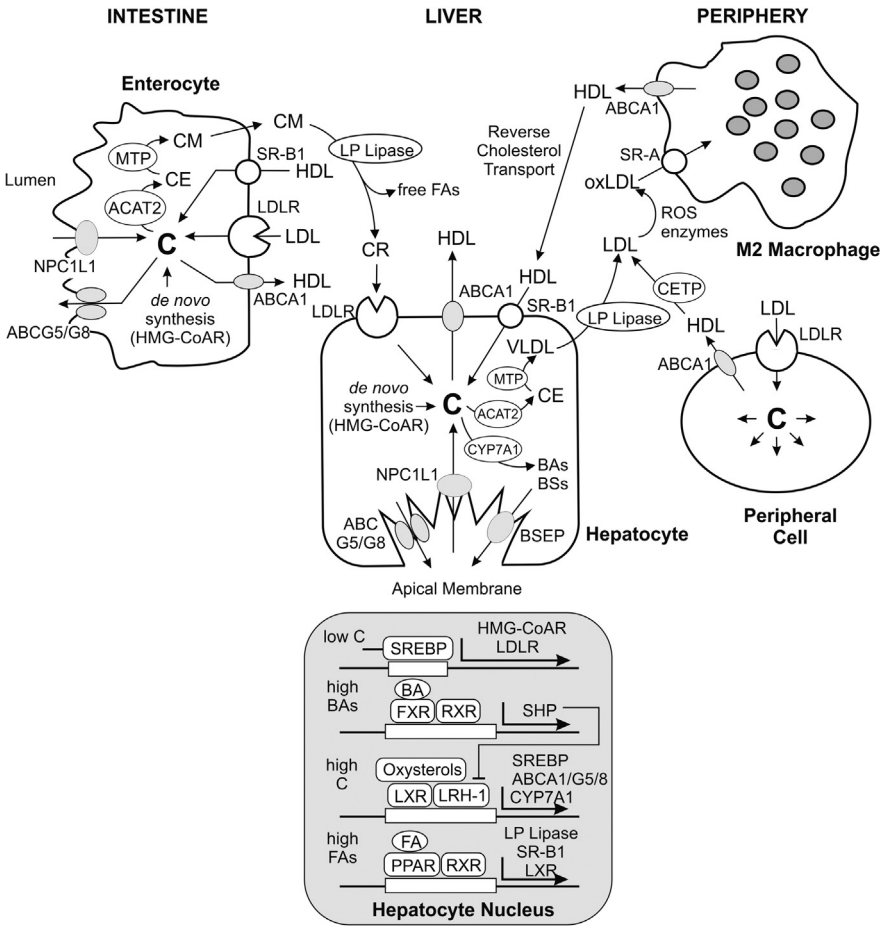

FIGURE 15.1 Key mediators of cholesterol transport, converting enzymes and intermediates in the intestine, liver, and periphery. In enterocytes and hepatocytes, cholesterol (C) is obtained from dietary sources via uptake from the intestinal lumen and from the bile by Niemann-Pick C1-like 1 transporters (NPC1L1) in the apical membrane, from de novo synthesis via 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR), and from circulating HDL and LDL particles via scavenger receptor B1 (SR-B1) and LDL receptors (LDLR), respectively. Intracellular cholesterol is esterified by the enzyme acyl-CoA cholesterol acyl transferase 2 (ACAT2) in enterocytes and then incorporated with triglycerides and Apo-B48 into chylomicrons (CM) by microsomal triglyceride transfer protein (MTP) for transport to the liver. In hepatocytes, ACAT2 esterifies cholesterol for incorporation with triglycerides and Apo-B100 into VLDL particles by MTP for export and delivery to the periphery via the circulation. Triglyerides in chylomicrons and VLDL particles are hydrolyzed by endothelial lipoprotien lipases (LP Lipase) to generate free fatty acids and glycerol that are absorbed by peripheral cells, leaving behind chylomicron remnants (CR) and LDL (and intermediate density) particles, respectively. CRs and LDL are taken up by hepatocytes and peripheral cells via LDLRs. Efflux of unesterified cholesterol back into the intestinal lumen from enterocytes and from hepatocytes into bile ducts occurs via heterodimer transporter ABCG5 and G8. Bile acids (BAs) are synthesized in hepatocytes from cholesterol via cytochrome P450-mediated oxidation (CYP7A1) and conjugated with glycine or taurine to form bile salts (BSs) for transport into the bile ducts by bile salt export protein (BSEP) in the apical membrane. Cholesterol is exported from hepatocytes and peripheral cells (including macrophage and foam cells of atherosclerotic plaques) via ABCA1 (and G1) transporters that transfer the cholesterol to Apo-A1 to form HDL particles. HDL particles produced in the periphery may contribute to reverse cholesterol transport to the liver for excretion, or the cholesteryl esters from HDL particles may be exchanged for triglycerides in LDL (or VDL) particles by cholesterylester transfer protein (CETP). Reactive oxygen species (ROS) and enzymes act on LDL particles to generate oxidized LDL that is accumulated in macrophages (and foam cells) following uptake by class A scavenger receptors (SR-A) or removed from the plasma by endothelial cells via oxidized low-density lipoprotein receptor 1 (LOX1; not shown).

Inset: Cholesterol, bile acids, and fatty acids modulate transcription of genes responsible for cholesterol homeostasis in the hepatocyte nucleus. When cholesterol (C) levels are low, SREBPs undergo cleavage and the N-terminal domain acts as a transcription factor for target genes containing sterol response elements that encode multiple enzymes for the synthesis and uptake of cholesterol; e.g., HMG-CoAR and LDL receptor (LDLR). Bile acids (BAs) are ligands for farnesoid X receptors (FXRs) that dimerize with retinoid X receptors (RXR) to evoke expression of the small heterodimer partner (SHP) protein that functions to inhibit transcription of the CYP7A1 gene responsible for the conversion of cholesterol to bile acids via interaction with the liver homolog-1 transcription factor (LRH-1). Liver X receptors (LXRs) in hepatocytes (i.e., LXRα) are activated by elevated oxysterol levels to dimerize with LRH-1 or RXRs (not shown) to stimulate transcription of multiple genes promoting cholesterol metabolism and transport including ABCA1, G1, G5 and G8, CETP, CYP7A1 (LRH-1), LP lipase, LXRα, and SREBPs. Peroxisome proliferator-activated receptor alpha (PPAR) is a nuclear receptor transcription factor that is sensitive to fatty acids (e.g., arachidonic acid) generated in conditions of energy deprivation that dimerizes with RXRs to promote transcription of genes involved in fatty acid transport and utilization, such as LP lipase, SR-B1, and LXR.

levels stimulate sterol regulatory element-binding protein (SREBPs) cleavage, leading to increased expression of HMG-CoA-R and additional downstream enzymes in the endogenous biosynthesis pathway [17]. HDL-C plays a key role in maintaining normal lipid levels through its contribution to reverse cholesterol transport [24]. HDL-C particles transport cholesterol from muscle, fat cells, and arterial macrophages in the periphery to steroidogenic organs for use in hormone production, or to the liver where they bind to scavenger receptors (class B type 1) for elimination as bile acids [24]. Exciting recent findings indicate a role for microRNAs (e.g., miR-33) in regulating HDL biosynthesis and cholesterol efflux by inhibiting the expression of *ABCA1* and *ABCG1*, as well as suppressing fatty acid oxidation [17]. Specifically, experimental suppression of miR-33 was shown to promote reverse cholesterol transport and atherosclerotic plaque regression (see [17] for key references). For this reason, manipulation of microRNAs via bioactive molecules from natural products to promote reverse cholesterol transport is an important area for development of antiatherosclerosis therapies.

Hyperlipidemia is the most common condition involving abnormal levels and/or composition of circulating plasma lipids (i.e., dyslipidemia). Hyperlipidemia can result from genetic causes (primary hyperlipidemia), such as a mutation in a receptor or transport protein, or it can be acquired (secondary hyperlipidemia) owing to the presence of another pathology such as diabetes. It is a chronic condition that is most often asymptomatic for decades, but is associated with dramatically increased risk of developing atherosclerosis and compromised blood flow leading to ischemic heart disease, stroke, and peripheral artery disease [25].

Minimal CVD risk is considered to be achieved with the following plasma levels:

- LDL-C <70 mg/dL
- HDL-C >60 mg/dL
- TGs <150 mg/dL
- Total cholesterol <200 mg/dL.

Moderate CVD risk is associated with the following plasma levels:

- LDL-C 130–159 mg/dL
- HDL-C 50–59 mg/dL
- TGs 150–199 mg/dL
- Total cholesterol 200–239 mg/dL

High CVD risk is associated with the following plasma levels:

- LDL-C 160–189 mg/dL
- HDL-C <40 mg/L men and <50 mg/dL women
- TGs 200–499 mg/dL
- Total cholesterol ≥240 mg/dL

(values recommended by the National Cholesterol Education Program Adult Treatment Panel III [26]).

Atherosclerosis is recognized as a chronic inflammatory disease resulting from elevated levels of ApoB-LDL-C, with oxidized LDL trapped in the subendothelial space playing a key role in the pathogenesis of the disease [27–29]. Atherosclerosis results from the accumulation of LDL-C within the arterial wall: ApoB-LDL-C particles leave the bloodstream and accumulate within the subendothelial space where they are trapped by interacting with matrix proteoglycans and undergo spontaneous or cell-mediated oxidation and hydrolysis [27–29]. The presence of oxidized LDL within the subendothelial space of the arterial wall is thought to stimulate endothelial cell activation and subsequent adhesion molecule expression and cytokine release [27–29]. This results in the migration of inflammatory mononuclear leukocytes, monocytes and T lymphocytes into the wall where they are activated by cytokines, such as macrophage colony-stimulating factor and tumor necrosis factor, and through direct contact with extracellular matrix components [27–30]. Macrophages derived from penetrating monocytes initially function to clear the excess lipid via type A scavenger receptors, but their capacity is overwhelmed in the long term resulting in increased numbers of fat-containing macrophages (foam cells) and fatty streak/plaque expansion within the vessel wall. In parallel, metalloproteinase activity and extracellular matrix protein production by fibroblasts and smooth muscle cells are stimulated by resident inflammatory cells, and when combined with an enhanced deposition of calcium within the expanding plaques, the result is sclerosis, i.e., hardening of the arterial wall [27–30].

The primary clinical complications of atherosclerotic plaques include narrowing of the arterial lumen and plaque rupture leading to coronary artery disease, myocardial infarction, thromboembolic stroke, and peripheral artery disease [30]. As atherosclerosis is typically a chronic, progressive disease, it follows that lowering plasma ApoB-LDL-C levels

may substantially delay or even prevent the onset of atherosclerosis, particularly in the coronary circulation [31]. Moreover, it is now considered possible to elicit the regression of atherosclerotic plaques through a combination of dramatic plasma Apo-B and lipid reduction, suppression of the inflammation and increased oxidative stress within the arterial wall, and increased HDL-C levels that facilitate reverse cholesterol transport out of the plaques for elimination [32].

At present, lipid-lowering through dramatic reductions in LDL-C is the major treatment option for hyperlipidemia, and it is associated with an established decline in the risk of cardiovascular events in patients with or without coronary artery disease [29–33].

Established therapies for atherosclerosis are based on ameliorating hyperlipidemia and hypertension, and suppressing thrombotic complications. No contemporary therapy addresses the inflammatory condition that drives the progression of atherosclerosis; novel combinatorial therapies involving lipid lowering agents, as well as suppression of tumor necrosis factor α and interleukin-1 receptor signaling are promising possibilities [34]. Traditional therapies based on natural products may in some instances already accomplish this goal.

15.2.2 Synthetic Drugs for Hyperlipidemia/Atherosclerosis

Several approaches employing synthetic compounds are available for management of hyperlipidemia, including the best-selling pharmaceutical drug in history, the HMG-CoA-R inhibitor atorvastatin.

15.2.2.1 HMG-CoA Reductase Inhibitors

Reversible, competitive inhibitors of HMG-CoA-R, known as *statins* (e.g., lovastatin, simvastatin), are the most widely used cholesterol-lowering drugs. Statins suppress cholesterol synthesis, as well as indirectly upregulating LDL receptors in the liver leading to increased LDL-C clearance [35]. Statins are structurally similar to HMG-CoA, but bind to the enzyme in the nanomolar range preventing subsequent binding of HMG-CoA in the micromolar range [36,37].

Statins are effective in reducing incidence of CVD in patients with and without indications of overt CVD leading to lower mortality [38,39] (but see [40] for critical view of statins). The clinical benefits of statin therapy are postulated to extend beyond their hypolipidemic effect; i.e., statins have putative pleiotropic actions; they are thought to be antiinflammatory, protective of endothelial function and nitric oxide (NO) bioavailability, inhibit platelet aggregation, suppress matrix metalloproteinase expression and activity, and suppress cell dedifferentiation and proliferation within the arterial wall [41]. Notably, statins have been demonstrated to halt the progression of atherosclerosis, and although the data are limited, aggressive statin use with high doses of the most efficacious drugs may even cause plaque regression [42,43].

Despite the generally positive view of statins, they are not without criticism [40]. This negativity arises from issues including statin intolerance [44], their lack of effect on HDL-C or lipoprotein a levels [45], overstated efficacy [40,46], high cost and well-documented adverse side effects including increased risk of diabetes [47,48], cancer [49], cognitive impairment [50,51] and muscle weakness, possibly due to mitochondrial dysfunction [52,53]. Many adverse effects arise because the mevalonate pathway is involved in the synthesis of coenzyme Q10, heme-A, and isoprenylated proteins that play key roles in cellular signaling, physiology, and homeostasis, and cholesterol is an intermediate in the synthesis of steroid hormones, vitamin D, and bile acids and other hormones and autocoids [52].

15.2.2.2 Fibrates

Fibric acid derivatives (e.g., clofibrate, gemfibrozil, fenofibrate, bezafibrate, and ciprofibrate) are employed clinically, but their mechanism of action is complex and debated. The primary mechanism is thought to be via interaction with peroxisome proliferator-activated receptor alpha (PPAR-α). PPAR-α activation results in up- or downregulation of target genes involved in lipid transport and metabolism, but the clinical significance of these effects remains to be delineated [45,46,54]. Fibrates are more effective in lowering elevated plasma TG-rich lipoproteins. LDL-C levels generally decrease especially in individuals with elevated baseline plasma concentrations, and HDL-C levels are usually increased when baseline plasma concentrations are low [54].

15.2.2.3 Bile Acid Sequestrants

Bile acid sequestrants have a reputable history as effective and safe hypolipidemic agents. They have been the drug of choice for patients with moderately elevated LDL-C, including children and premenopausal women [55]. Sequestrants such as colestipol and colestrymine are positively charged indigestible resins that bind to negatively charged bile acids in the gastrointestinal tract forming insoluble complexes that cannot be reabsorbed [56]. The resultant decrease in endogenous bile acids in turn stimulates liver LDL receptor upregulation and increased LDL-C clearance to support a rise in bile acid synthesis which leads to an indirect reduction in plasma LDL-C levels. Bile acid sequestrants were shown to lower LDL-C by 15−26%, and induce a slight increase of HDL-C in controlled trials [57]. Despite the proven efficacy and lack of systemic effects, bile acid sequestrants have a high rate of discontinuance due mainly to gastrointestinal side effects [58].

15.2.2.4 Cholesterol Absorption Inhibitors

Ezetimibe was the first in the class of compounds that inhibit intestinal absorption of cholesterol from dietary and biliary sources by impeding the transport of cholesterol across the intestinal wall [59]. The mode of action involves binding with high affinity to the enterocyte cholesterol transporter NPC1L1 [46]. Inhibition of cholesterol absorption in the intestines leads to decreased hepatic cholesterol levels and increased clearance of LDL-C from the plasma. It is used primarily as an add-on to statin therapy to yield additional decreases in LDL-C [33].

15.2.2.5 Niacin

Niacin (nicotinic acid, Vitamin B_3) is a water-soluble vitamin that serves as a precursor for two essential coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) [60,61]. NAD- and NADP-linked enzymes are involved in virtually every aspect of metabolic processes. Niacin reduces TG and LDL-C and elevates HDL-C levels by mechanisms that are not clearly understood, but include: (1) decreased mobilization of fatty acids from adipose tissue by inhibiting the hormone-sensitive lipase-mediated lipolysis of TGs, and (2) inhibition of microsomal diacylglycerol acyltransferase (DGAT), a key enzyme in TG synthesis, which suppresses hepatic TG synthesis and limits ApoB lipidation resulting in delayed translocation of ApoB across the endoplasmic reticulum membrane and a reduction in ApoB-LDL-C particle release [60,61]. Although niacin has the most diverse effects on lipid profile, its use is complicated by the side effect of flushing and itching sensation [62].

15.2.3 Natural Product Therapies for Hyperlipidemia/Atherosclerosis

Despite the success of statins and other lipid lowering drugs, interest remains high in natural sources of bioactive compounds for the management of hyperlipidemia because of their low cost and the potentially serious side effects of the synthetic agents [63−66]. Indeed, several natural bioactive compounds with substantiated efficacy are well-established traditional medicines.

15.2.3.1 Garlic

Garlic (*Allium sativum*) has an important historical role in the treatment of CVD, it is one of the best studied sources of bioactive molecules with hypolipidemic and antiatherosclerotic properties, and it is one of the most commonly used herbs in the management of hyperlipidemia, as well as other CVDs [67]. The main bioactive component in raw garlic is allicin, but several other sulfur containing molecules are also present (e.g., *S*-allylcysteine, *S*-allylmercaptocysteine, allixin). Varied extracts of garlic have been shown to possess cholesterol and plasma lipid lowering effects in both laboratory animals and man [68,69], and to reduce atherosclerotic lesions in laboratory animals fed a high-cholesterol diet (e.g., [70]). In the latter case, aged garlic extract reduced the surface area of thoracic aorta exhibiting fatty streaks, decreased vessel wall cholesterol content, and suppressed the elaboration of lipid-filled lesions in carotid arteries of hypercholesterolemic rabbits at sites of balloon catheter injury by inhibiting smooth muscle phenotype and proliferation, and lipid uptake into the vessel wall [71]. Meta-analysis of published trials document a significant, albeit moderate reduction (i.e., compared to statins) in plasma lipids, especially total cholesterol (8% decline) and LDL-C (9% decline) (as well as a slight rise in HDL-C levels, but no effect on TGs) with garlic therapy of greater than 2 months with minimum adverse effects (e.g., slightly increased bleeding risk and gastrointestinal discomfort; contraindicated in patients taking antiplatelet medications) [69,72−74]. Some of the variability in clinical trial results can be ascribed to differences in garlic preparations, with aged garlic extract, produced by long-term

storage of raw garlic slices for greater than 1.5 years in ethanol, superior to garlic powder or oil owing to a greater content of *S*-allylcysteine [69]. The mechanism(s) of action of garlic is not known with certainty, but suppression of cholesterol synthesis through an inhibition of squalene monooxygenase and HMG-CoA reductase, and an inhibition of LDL oxidation are postulated [72–74].

15.2.3.2 Phytosterols

Phytosterols and their saturated forms, phytostanols, are bioactive plant-specific phytochemicals that are structurally similar to cholesterol. Relatively high levels of phytosterols (e.g., sitosterols; campesterol, γ-sitosterol) are present in lipid-rich plants foods such as olive oil, nuts, legumes, and seeds [75,76]. The intestinal absorption efficiency of phytosterols and phytostanols is very low (<5% and <0.3%, respectively [75]) compared to that of cholesterol. In plasma, phytosterols and phytostanols are carried in lipoproteins similarly to cholesterol, so because of the abundance of LDL (70–80%) they circulate mainly in LDL. Phytosterols and phytostanols are thought to interfere with intestinal cholesterol absorption; i.e., they are competitive inhibitors of cholesterol uptake [76,77]. The molecular basis of the inhibition is unknown, but suggested mechanisms include displacing cholesterol from mixed micelles, increased expression of genes encoding sterol transporter proteins (*NPC1L1*, *ABCG5*, and *ABCG8*) that promote cholesterol efflux from enterocytes into the intestinal lumen, decreased cholesterol reesterification rate in enterocytes, and increased cholesterol removal from the body via transintestinal cholesterol efflux [75–77].

Although plant sterols have been employed for many years to reduce cholesterol levels, their use has come under increased scrutiny and debate with an improved understanding of the mechanisms of uptake, actions, and consequences of phytosterol ingestion [78–80]. Concern principally originates from evidence that accumulated plant sterols have toxic effects, and that patients with sitosterolemia, and animals with mutant or absent *ABCG5* and *ABCG8* transporter genes, which have increased absorption, decreased secretion, and higher plasma neutral sterol levels exhibit signs of premature atherosclerosis; i.e., plant sterols may be proatherogenic when in excess [80].

15.2.3.3 Saponins

Saponins are naturally occurring surface-active glycosides produced by plants, lower marine animals, and some bacteria. Saponins occur constitutively in a great many plant species, in both wild plants and cultivated crops. In cultivated crops, the triterpenoid saponins are generally predominant, while steroid saponins are common in wild plants used as herbs [81]. Plant saponins have been shown to inhibit cholesterol absorption from the intestinal lumen in experimental animals and consequently to reduce the concentration of plasma cholesterol. This may be the result of interactions with cholesterol in the digestive tract or a direct effect of plant saponins on cholesterol metabolism. For example, saponin fractions from garlic or ginseng were shown to decrease total and LDL cholesterol plasma concentrations without changing HDL cholesterol levels in hypercholesterolemic animal models [82,83].

15.2.3.4 Berberine

A high percentage of LDL-C is cleared from the circulation by LDL receptor-mediated uptake in the liver, so plasma cholesterol levels are directly influenced by the level of LDL receptor expression in hepatocytes. For this reason, hepatic LDL receptors represent an important therapeutic target to control lipid levels and prevent atherosclerosis [84]. Berberine is an alkaloid first isolated from the Chinese herb huanglian (*Coptis chinesis*), and shown to increase hepatic LDL receptor mRNA and protein expression [85]. Furthermore, in a placebo-controlled study, berberine therapy for 3 months was found to reduce total plasma cholesterol (TC) by 29%, TG by 35% and LDL-C by 25% [85]. Berberine is also found in Ranunculaceae plants, e.g., in dried root samples of goldseal (*Hydrastis canadensis* L.), a plant native to the eastern region of North America. Goldenseal contains three alkaloids berberine, canadine, and hydrastine. Berberine and canadine increase LDL receptor message expression and reduce cholesterol levels in Golden Syrian hamsters [84].

15.2.3.5 Guggulsterone

Guggul (gum guggul) is a resin produced by the mukul mirth tree (*Commiphora mukul*). Guggul extracts are widely used as cholesterol-lowering agents in Asian countries. Guggul was used as an adjunct to dietary therapy in patients with hyperlipidemia, and it was demonstrated to decrease the levels of total cholesterol, LDL, TGs compared with the

placebo group, but no changes were seen in the levels of HDL [86]. Guggulipid extracted from guggul contains the sterols, guggulsterones E and Z, as the major bioactive compounds [87]. Guggulsterone-Z is an FXR ligand that acts as an antagonist; FXR antagonism results in increased CYP7A1 transcription and increased cholesterol catabolism, as well as promoting bile acid reabsorption in the intestine [88]. Guggulsterones were also demonstrated to be effective antioxidants making them beneficial against LDL oxidation in atherogenesis [89].

15.2.3.6 Green Tea

The tea plant *Camellia sinensis* contains several biologically active polyphenols (for a detailed description see section 15.3.3.1) including catechins such as epigallocatechin-3-gallate (EGCG) and (−)-epigallocatechin (EGC) [90]. Green tea consumption was shown to alleviate cardiovascular dysfunction by reducing total and LDL cholesterol, blood pressure reduction, and CVD mortality in clinical studies and in numerous animal studies (reviewed in [90−95]). The mechanism by which the cholesterol-lowering action is achieved is not known with certainty, but ECGC and EGC increased CYP7A1 promoter activity, and ECGC increased CYP7A1 message expression [96], as well as reducing the activity of the apical sodium-dependent bile acid transporter of enterocytes in the distal intestine which disrupts bile acid recycling, promotes bile acid synthesis and thereby reduces cholesterol levels [97].

15.2.3.7 Traditional Chinese Medicine

Although the specific condition of hyperlipidemia is not a precise entity in traditional Chinese medicine, there is a robust list of natural products that have been used for more than 1000 years to treat the syndromes of "dampness," "turbid phlegm," and "blood stasis" and more recently have been shown to possess hypolipidemic potency [98−100]. Xie et al. [99] and Sham et al. [100] have reviewed various Chinese state-approved traditional formulations and identified the most common herbal components with a hypolipidemic profile in animal or clinical studies. In most cases, the active molecules, mechanisms of action, and/or pharmacokinetic characteristics are not known with certainty. Representative examples include: (1) the dried fruits of hawthorn tree (*Crataegus pinnatifida*) with flavonoids and triterpenic acids (e.g., oleanic acid) that are thought to inhibit HMG-CoA reductase activity [101], repress intestinal acyl-CoA:cholesterol acyltransferase activity [102], and enhance PPARα expression [103]. (2) Dried fleeceflower root tubers (*Fallopia multiflora*) containing anthraquinones, stilbene glucosides, and polysaccharides that may inhibit intestinal lipid absorption, increase the activities of lipoprotein lipase and hepatic lipases, inhibit fatty acid synthase, and enhance LDL receptor expression [99]. (3) Dried stem tubers of common water plantain (*Alisma plantago-aquatica; Alisma orientale*) and powder extracts of hawthorn containing triterpenoids and polysaccharides that decrease liver cholesterol synthesis, lower plasma LDL and cholesterol, inhibit intestinal acylCoA:cholesterol acyltransferase activity, and possess antioxidant capacity [99,100,102]. (4) Dried roots and rhizome of red sage or danshen (*Salvia miltiorrhiza*) that contain multiple tanshinones, including tanshinone IIA which may have PPARγ antagonist activity [104] and FXR/liver X receptor α coagonist activity that reduce plasma levels of TG, LDL and total cholesterol levels while elevating HDL levels in rats on a high fat diet [105]. (5) Extracts of Lotus leaves (*Folium nelumbinis nucifera*) that contain alkaloids, phenolic acids (e.g., gallic acid) and polyphenols (e.g., rutin, the glycoside of quercetin and disaccharide rutinose) which decrease fatty acid synthase, acyl-CoA carboxylase and HMG-CoA reductase expression, and activate AMP-kinase signaling to inhibit the activity of these lipogenic enzymes in mice on a high fat diet [106]. (6) Extracts of notoginseng (*Panax notoginseng*) roots that contain 27 different saponins (including ginsenosides, notoginsenosides, and gypenosides), as well as polysaccharides, flavonoids, and phytosterols [107,108]. Notoginseng therapy was shown to increase FXR and LXRα target gene expression and reduce serum concentrations of LDL-C, TGs, and total cholesterol in rats on a high fat diet [109].

15.2.3.8 Natural Sources of HMG-CoA Reductase Inhibitors

The first statins identified were mevastatin (mevinolin) isolated from *Penicillium citrinum* [110], and lovastatin (also referred to as monacolin K) isolated from *Aspergillus terreus* [111]. Lovasatin is also present in oyster mushrooms (*Pleurotus ostreatus*) [112], red yeast rice [113], and ripe (but not raw) leaves of *Camellia sinensis* used in the Chinese tea Pu-erh [114]. Red yeast rice is obtained by cultivation with the mold *Monascus purpureus*, the latter being the source of several compounds that inhibit HMG-CoA reductase including monacolin K (lovastatin) [113]. Patients with CVD consuming red yeast rice showed a decreased incidence of nonfatal myocardial infarction or death from cardiac causes compared to placebo [115]. The combination of red yeast and olive oil polyphenol extract reduced LDL cholesterol in patients and lowering of total cholesterol, LDL, TG, and ApoB, with LDL reduced by

24% and oxidized LDL by ~20% [116]. Red yeast rice, when combined with fish oil and lifestyle changes, was as effective as a moderate dose of simvastatin in lowering LDL-C levels [117], and is considered to be an appropriate lipid-lowering therapy in subjects with a history of statin-associated myalgias [117–119]. Indeed, the cardioprotective effects of red yeast were in some instances greater than lovastatin, implying that other monacolins, phytosterols, or yet to be identified active ingredients are also important. The presence of lovastatin (monacolin K) in red yeast rice supplements led the Food and Drug Administration of the United States to consider these supplements to be a drug and to restrict their sales. The levels of lovastatin and other monacolins present in red yeast extracts from 12 proprietary sources varied considerably and some contained the nephrotoxicant citrinin [120]. The findings regarding the effectiveness of red yeast rice are encouraging, but improved standardization and oversight are essential before general clinical use can be considered [121].

Anti-HMG-CoA reductase activity has also been isolated from extracts of vine or Malabar spinach (*Basella alba*), green amaranth (*Amaranthus viridis*), and wild betel (*Piper sarmentosum*); compared to simvastatin, *Basella alba* extract was slightly less effective at inhibiting the enzyme in vitro (i.e., ~74% vs ~85% inhibition for simvastatin), but the specific molecule(s) involved was not identified [122,123].

15.2.3.9 Other Approaches

Other methods of lowering lipids include an antagonism through an upregulation of hepatic LDL receptors. This results in an increased uptake of plasma cholesterol for excretion, as well as, enhancing its degradation to bile acid, as is apparent for *Crataegus* hawthorn treatments [124]. Antiatherosclerotic effects were also noted using an ethanolic extract of common knotgrass (*Polygonum aviculare*) and concluded to be mediated via an inhibition of the MAPK pathway [125]. Aqueous extract of *Ficus glumosa* leaves was reported to have hypolipidemic and antiatherosclerotic properties by reducing total cholesterol, LDL, VLDL, as well as hepatic and aortic triglyceride levels [126].

15.3 HYPERTENSION

Current estimates indicate that greater than 1 billion individuals have hypertension worldwide, and in ~95% of these patients the disease has an unknown etiology (i.e., "essential" hypertension) [127]. Hypertension is the leading risk factor for premature mortality due to CVD, and blood pressure reduction provides the largest benefit for premature CVD-specific mortality [10,127].

- Hypertension is defined as a mean systolic blood pressure (SBP) ≥140 mm Hg or a mean diastolic blood pressure (DBP) ≥90 mm Hg
- Prehypertension—SBP 120–139 or DBP 80–89 mm Hg
- Stage 1 hypertension—SBP 140–159 mm Hg or DBP 90–99 mm Hg
- Stage 2 hypertension—SBP ≥160 mm Hg or DBP ≥100 mm Hg

(The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report [128]).

Reduced rates of hypertension and CVD mortality have been achieved with improved: (1) national public education initiatives that promote reductions in sodium consumption, address deficiencies in diet and physical activity, and indicate the consequences of obesity to health; and (2) optimization of clinical detection and treatment [129]. However, because of the lack of a defined etiology of hypertension, treatment is empiric and a significant number of patients do not attain blood pressure goals, or exhibit treatment-resistant hypertension even when taking two or more standard antihypertensive drugs [130].

15.3.1 Pathophysiology

Essential hypertension is thought to result from a complex age-dependent interaction between genetic, epigenetic, and environmental factors leading to an irreversible remodeling of resistance arteries and arterioles and elevated blood pressure [131,132].

In normal conditions, blood pressure is controlled through a complex interplay of multiple direct and indirect regulatory mechanisms, including:

- baroreceptors that sense intravascular pressure.
- natriuretic signaling peptides released by the brain and heart in response to elevated pressure.
- the renin–angiotensin–aldosterone system (RAAS) that regulates plasma volume, enhances noradrenaline release from sympathetic nerve terminals, and modulates vascular tone.
- the kinin–kallikrein axis that modulates salt reabsorption in the kidney and vascular tone
- the sympathetic nervous system that modulates heart rate, cardiac and vascular contractility via α_1-adrenoceptors, and renin release from specialized juxtaglomerular epitheloid cells via β_1-adrenoceptors.
- endothelium-dependent vasodilators (e.g., NO) and vasoconstrictors (endothelin and thromboxane A_2) that modulate vascular tone.
- mechanisms intrinsic to vascular smooth muscle cells of resistance arteries and arterioles in the periphery and kidney (renal afferent arteriole) that evoke myogenic tone development in response to intravascular pressure.
- pressure natriuresis in which renal perfusion pressure elevation stimulates sodium excretion.

(see Refs. [131–136]).

It is generally held that a sustained rise in blood pressure is dependent on a change in the relationship between renal perfusion pressure and sodium excretion (i.e., a shift to the right in the pressure–natriuresis relation), and it appears to be invariably altered in animal models and patients with hypertension [134–136]. The molecular mechanisms of pressure natriuresis are not known with certainty, but an altered natriuretic response to pressure increase can be caused by activation of RAAS or sympathetic drive, decreased glomerular filtration or functional nephrons, impaired tubular sodium reabsorption or renal NO and prostaglandin release, and infiltration of the kidney by macrophages and T cells in an adaptive immune response that leads to increased oxidative stress, inflammatory cytokine release, and increased local angiotensin II synthesis in the kidney [134–137].

Increased oxidative stress is an established hallmark of hypertension and, as indicated above, in the progression of atherosclerosis. As many traditional therapies employing natural products are thought to involve a suppression of oxidative stress, it is worthwhile to consider the causes of oxidative stress in the vasculature in detail. Oxidative stress results from a reduction in antioxidant capacity due to low concentrations of endogenous antioxidants, reduced activity antioxidant enzyme activity, and/or increased production of reactive oxygen species (ROS) that overwhelms endogenous protective mechanisms [138,139]. ROS include molecules such as superoxide anion, hydroxyl radical, hydrogen peroxide, hypochlorite radical, and the peroxyl radicals. Any increase in the concentrations of these reactive species can be harmful if not balanced by appropriate antioxidant activity. Imbalances leading to excessive ROS levels resulting in a disruption of redox hemostasis, cell signaling, lipid peroxidation, protein and DNA damage, enzyme oxidation, as well as increased endothelial permeability, leukocyte adhesion and monocyte migration, and a resulting rise in vascular inflammation, increased vascular reactivity, platelet aggregation, and thrombus formation [140]. Other reported effects include the ability of ROS to stimulate cell proliferation, differentiation, and migration, as well as a direct modulation of vascular tone owing primarily to endothelial damage and consequential reduction in the release of vasodilator factors, especially NO [141–143]. Excessive ROS production and increased oxidative stress are thought to play a key role in many chronic conditions such as cancer, neurodegenerative, and CVDs, including hypertension [144–147]. ROS, and the superoxide radical in particular, can result in the activation of redox sensitive transcription factors such as NF-κB and the expression of a range of adhesion molecules at the luminal surface of the endothelium [148]. The latter promotes invasion of monocytes and promotes the atherosclerotic process. ROS also oxidize LDL in the vascular wall to form oxLDL, as well as causing lipid peroxidation, which facilitate atherosclerotic lesion formation and plaque buildup [148,149]. Superoxide impairs the vasodilatory actions of NO through its interaction with this key endothelial factor. NO release by the endothelium plays a key role in controlling vascular smooth muscle proliferation, suppressing vascular smooth muscle tone, and mediating vascular homeostasis [148,150,151]. Superoxide reduces the bioavailability of NO by interacting with and converting NO into peroxynitrate [152]. Peroxynitrite in turn uncouples endothelial NO synthase causing it to become a superoxide-generating enzyme [151,152]. The loss of NO and presence of peroxynitrate promotes increased vascular tone, a loss of endothelium-dependent vasodilation, and increased vascular resistance, hypertension, and heart failure [146,147,153].

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes (NOX) are the main source of ROS in vascular tissues [154–157], but lipoxygenases, xanthine oxidase, cytochrome P450 oxidases, uncoupled nitric oxide

synthase (NOS), monoamino oxidases (MAO), and the mitochondrial electron transport chain can also contribute to the ROS load [158,159]. NOX enzymes represent a family of multisubunit enzymes that generate superoxide radicals ($^{\bullet}O_2^-$) through a reduction of oxygen using NADPH or NADH as the source or donor of electrons [156,160]. NOX were originally thought to be expressed only in phagocytic cells (neutrophils, monocytes, and macrophages) in which they generate ROS that are key for nonspecific host defense [161–163]. However, this view has been updated to include NOX isoforms found in nonphagocytic cells, such as adventitial cells, vascular smooth muscle cells, and endothelial cells, that generate intracellular ROS via vascular NOX isoforms 1, 2, 4, and 5 [148,162,164,165]. Upregulation of the activity and/or expression of NOX 1 and NOX 2 leads to inflammation and oxidative stress in the cardiovascular system [159,165,166]. NOX5 has been implicated in PDGF-induced endothelial cell proliferation and the formation of capillary-like structures; depletion of NOX5 suppresses tube formation, while increasing NOX5 increases enothelial nitric oxide synthase (eNOS) activity, but reduces the bioavailability of NO through interaction with superoxide and the formation of peroxynitrate [152]. Therefore, overexpression of NOX5 in the endothelium can also lead to an impairment of vasodilation [152,165]. Blood flow, shear stress, vasoactive agonists (e.g., angiotensin II), cytokines, interleukins, and several growth factors have all been shown to stimulate vascular NOX activity and/or upregulate NOX expression [143,153,165].

NOX enzymes have been implicated as a major cause of vascular diseases, with multiple pathophysiological actions within the different layers of the arterial wall that can be enhanced by inflammatory mediators, adhesion molecules, thrombosis, and mechanical stress [140,155,157,164]. NOX activity is thought to contribute to the progression of atherosclerosis as well as hypertension [151], with NOX1 in particular implicated in the development of hypertension; NOX1 levels are upregulated in multiple rat models of hypertension and downregulation of NOX1 activity with siRNA treatment against one of its subunits (p22phox) inhibits angiotensin II-evoked hypertension with a concomitant reduction in NOX1 expression [165]. NOX proteins are differentially expressed in different vascular beds, with NOX4 reported to be upregulated in cerebral vessels in chronic hypertension [167,168]. Regional differences in superoxide generation have been reported, with greater levels in intracranial compared with the systemic vessels due to increased levels of NOX expression [169–173]. NOX inhibition may be an important form of therapy in the management of CVDs arising from oxidative damage, endothelial dysfunction, and hypertension. NOX inhibitors have been successfully employed as therapeutic agents in the treatment of neurodegenerative diseases resulting from oxidative damage [173–175]. Indeed, therapies targeting the source of ROS generation would appear to be a more effective strategy compared to scavenging ROS already present in the vascular wall.

15.3.2 Synthetic Drugs for Hypertension Therapy

Current hypertensive therapy is provided by diuretic drugs (including thiazides, loop diuretics and sodium channel antagonists, aldosterone antagonists), angiotensin-converting enzyme blockers, angiotensin II AT1 receptor antagonists, renin inhibitors, and Ca^{2+} channel antagonists [128–130,132]. Despite the effectiveness of these compounds, blood pressure may not be adequately controlled even when these drugs are used in combination [128,130,132].

One avenue for therapy that has been of long-standing interest is the use of antioxidants. Under physiological conditions, ROS levels are kept low via endogenous antioxidant defense systems or through the inhibition of enzymes that generate ROS [138,143]. Cellular and membrane localized antioxidant scavenging mechanisms are present in endothelial cells and vascular smooth muscle cells. Nonenzymatic antioxidants include uric acid, vitamin C, vitamin E, and glutathione (GSH) [161]. Vitamin C is water soluble, found in the cytoplasm, and not only scavenges ROS, but also protects vitamin B and GSH from free radicals in the cell membrane. Vitamin E is lipid soluble and is the most important antioxidant found in cell membranes. GSH, a low molecular weight thiol antioxidant serves as a substrate for the glutathione peroxidase enzyme [161]. The latter catalyzes the conversion of lipid hydroperoxides and H_2O_2 and itself, is converted to GSH disulfide or GSSG upon oxidation. One therapeutic approach to suppress excessive ROS levels and regulate cellular redox state has been the use of antioxidant vitamin supplements, including vitamin C and vitamin E. These antioxidants have provided impressive results for experimental control of oxidative stress in animal models of chronic diseases, but their performance in the clinic has been very disappointing [138,176–182]; neither vitamin A (ascorbic acid) or E (α-tocopherol) supplementation was effective in CVD therapy in recent clinical trials which questions the usefulness and clinical benefits of antioxidant vitamin supplementation [138,181].

Endogenous Antioxidant Mechanisms.

- Superoxide dismuthase (SOD), catalase, and glutathione peroxidase are the most important enzymatic antioxidant systems in the vasculature.
- SOD and catalase work in tandem to eliminate oxygen radicals; SOD converts oxygen radicals to H_2O_2, with the latter then utilized by catalase to form H_2O [183,184].
- The essential enzymatic antioxidants of the endothelium are heme oxygenase, thioredoxin system, SOD, catalase and glutathione peroxidase; they all serve to reduce ROS levels and maintain redox homeostasis, but their mechanisms of action differ.
- Heme oxygenase has indirect antioxidant effects through the degradation of pro-oxidant free heme derived from hemoproteins, and the generation of biliverdin and bilirubin which have antioxidant properties [185]. Constitutive heme oxygenase isoform HO-2 is ubiquitously expressed in endothelial cells, whereas expression of the inducible isoform, HO-1, is stimulated by heme, hypoxia, cytokines, oxidized LDL, angiotensin II, NO, peroxynitrite, and H_2O_2 [185].
- The thioredoxin system is a ubiquitous oxidoreductase that consists of thioredoxin reductase together with thioredoxin and NADPH that have antioxidant and redox-sensitive regulatory roles in endothelial cells [186].

Inhibition of NOX has been considered as a viable option for development of novel therapies, in part because this strategy would target the generation of excessive ROS. Indeed, several known synthetic drugs may act in part by suppressing NOX activity or expression, and these could be exploited as antihypertensive therapies. For example, atorvastatin, a cholesterol-lowering drug with antioxidant properties may inhibit NOX, as has been observed for apocynin [174] and betulinic acid [175]. Interestingly, a metabolite of the angiotensin II type 1 receptor antagonist losartan (EXP3179) was recently shown to suppress NOX-mediated superoxide production by inhibiting protein kinase C activity and the activation of NOX [187]. The NOX inhibitor fluorofenidone was found to attenuate tubulo-interstitial injury and oxidative stress in fibrotic rat kidneys via the PI3K/Akt signaling pathway [188]. Indapamide, a thiazide-like diuretic was reported to decrease blood pressure, inflammation, and oxidative stress by downregulating the expression of the NOX subunit p47phox [189]. A drug combination of telmisartan, valsartan, and cilnidipine was shown to attenuate NOX1 gene expression in vascular tissues [190,191]. Nicorandil may in part lead to an amelioration of endothelial dysfunction through an attenuation of p47phox expression, resulting in an inhibition of NOX and eNOS uncoupling [192]. These findings indicate the validity of the approach, but successful therapies based on NOX inhibition will require compounds demonstrating isoform specificity [193].

15.3.3 Natural Products for the Treatment of Hypertension

15.3.3.1 Phenolic Acids and Polyphenols

Plants produce several thousand structurally diverse phenolic acids and polyphenol derivatives that can be separated into four principle groups: (1) the phenolic acids (mono-, di-, and trihydroxybenzoic acids); (2) the flavonoids and related molecules; (3) terpenoids (i.e., isoprenoids; e.g., cannabinoids from *Cannabis sativa*, ginkolide and bilobalide from Gingko biloba, and curcumins from turmeric (*Curcuma longa*); and (4) nitrogen-containing alkaloids and less abundant sulfur-containing molecules (e.g., berberine, nicotine, strychnine, yohimbine). The flavonoids have been further subdivided into six subclasses, including: (1) anthocyanidins (e.g., cyanidin from berry fruits); (2) flavanones (e.g., hesperetin and naringenin from citrus fruits); (3) flavones (e.g., apigenin from apples and chamomile (*Anthemis nobilis*); (4) flavonols (kaempferol, quercetin from apples, grapes and green tea); (5) isoflavones (e.g., daidzein, genistein from legumes such as soy beans); and (6) flavanols (e.g., catechin, epicatechin from grapes (wine), tea plant (*Camellia sinensis*), and cocoa seeds (*Theobroma cocoa*) (reviewed in [194–197]; see also extensive listings and sources in the U.S. and European databases at http://fnic.nal.usda.gov/food-composition/phytonutrients and http://www.phenol-explorer.eu, respectively). Although flavones and flavonols can occur as their core phenolic compounds, flavonoids are in general conjugated with sugars that enhance stability, but reduce their dietary bioavailability [195]. Flavonoids also undergo polymerization and oligomerization to form complex proanthocyanidins and tannins, structures that range from dimers to a maximum of 17 flavonol core units following oxidation by enzymes or fermentation [197]. Proanthocyanidins are subdivided into 16 species including the procyanidins, oligomers of the flavan-3-ols catechin and epicatechin, and the prodelphinidins that are oligomers of the gallocatechins [198].

An impressive body of epidemiological, medical anthropological, experimental, and clinical evidence has been generated in support of the view that polyphenols and phenolic compounds derived from natural sources, principally plants,

improve vascular vasodilator function, and are protective against hypertension and CVDs [197−205]. A pertinent representative example are the Kuna Indians of the San Blas Islands of Panama. These indigenous peoples have very low blood pressure and an absence of CVDs that were attributed to their traditional diet dominated by fruits and cocoa with a high polyphenol content [201,205]. Indeed, a variety of studies provide compelling evidence that dietary polyphenols can reduce hyperlipidemia and atherosclerosis, improve endothelium-dependent vasodilation and lower blood pressure, and reduce the level of cardiovascular inflammatory and oxidant stress (reviewed in [197]). Although substantial advances have been made, we still have a very limited understanding of specific molecules involved and the mechanism(s) of action by which these compounds mitigate hypertension and other CVDs. This lack of mechanistic understanding is due in part to the overwhelming diversity of chemical structures, their complex and varied characteristics of absorption, metabolism, and distribution in living systems, and our limited knowledge of the effective concentrations and bioavailability of each specific polyphenol at their sites of action. For example, many studies have employed parent polyphenol compounds (i.e., the aglycones or sugar conjugates) at concentrations from 100 μmol/L to several mmol/L in single cell or ex vivo tissue experiments, without considering that the parent molecules, or their metabolites arising from catabolism after ingestion, are most often detected in the nmol/L range in vivo [197,198,206−208]. Furthermore, some flavonoids and related compounds pass to the large intestine where they are structurally modified by the colonic microbiome and the resulting metabolic by-products may then be absorbed and contribute to the biological effect [198]. Thus, it is likely that many relevant compounds have not been assessed and/or that the mechanisms of action identified for some compounds in in vitro experiments may be of limited relevance to understanding their actions in vivo [197,198,206]. These are problems that must be addressed in the future to provide accurate scientific justification for the therapeutic use of individual or combinations of polyphenols.

Polyphenols have long been considered to act via a direct antioxidant action by scavenging ROS within the extracellular matrix and in cells. However, this view has been strongly questioned because the plasma concentrations of the compounds are in most instances insufficient to replicate the scavenging activity demonstrated in vitro [197,198,206,209], and the kinetics of their reactions with the predominant ROS appear to be too slow for biological relevance [210]. Although a detailed molecular explanation for the biological effects of polyphenols is currently lacking, emerging evidence suggests that they may act through multiple distinct mechanisms (reviewed in [197]). These identified mechanisms include: (1) interactions with cell membranes that modify membrane structure, membrane signaling events within lipid rafts, or prevent lipid peroxidation [197,211]; and the (2) modulation of a select group of enzymes relevant to cardiovascular function. For example, the flavanol (−)-epicatechin via its *O*-methylated metabolites, 3′- and 4′-*O*-methyl epicatechin, suppresses NOX activity (likely NOX4) in cultured endothelial cells treated with oxidized LDL treatment by suppressing a shift from eNOS to inducible nitric oxide synthase (iNOS) expression, reducing cytotoxicity, and increasing NO release [212−214]. Several flavonoids enhance NO bioavailability by stimulating eNOS activity [204,215−218]. These findings are consistent with in vivo evidence of increased endothelium-dependent, flow-mediated dilation following (−)-epicatechin or cocoa flavonoid consumption in healthy young and older adults [219,220]. Flavonoids have also been shown to inhibit lipoxygenases [221,222], angiotensin-converting enzyme [223−227], and metalloproteinase activity, as well as reducing tyrosine nitrosylation by peroxynitrate [228]. Taken together, these actions have the potential to decrease angiotensin II-stimulated NOX activity and ROS generation, reduce oxidative stress, and suppress vascular inflammation that contribute to the pathology of atherosclerosis and hypertension. (3) Polyphenol compounds can also alter transcription factor signaling to change the expression of key molecules such as NF-κB that plays a key role in vascular inflammation and atherosclerosis [229,230] (reviewed in [197,231]).

Hydroxycinnamic acids (HCAs) are prevalent phenolic acids within plants that are in general derived from cinnamic acid. The most common are *O*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, caffeic acid, and ferulic acid. They may be present as free carboxylic acids, esters (following condensation with compounds such as quinic acid, flavonoids or carbohydrates; e.g., 5-*O*-caffeolylquinic acid from condensation of caffeic acid with quinic acid), or amides (following condensation with amino acids or amines). HCAs are in many grains, fruits, vegetables [232], nuts [233], and medicinal plants [234]. For example, coffee, green and black tea, cocoa, berries, citrus fruits, apples and pears, grapes and wine, kale, spinach, chicory, artichoke, leafy herbs, potatoes, and grains such as barley, wheat, rye, and maize, all contain several HCAs. Caffeic acid is in general the most widespread, but many other cinnamates are also present at varied levels depending on the plant species [232]. HCAs have been considered to be protective against atherosclerosis and hypertension based on their in vitro antioxidant properties (e.g., [235]). For example, caffeic acid (between 0.5 and 5.0 μM) and other derivatives prevent oxidation of LDL in vitro [236,237], but again, it is not clear if the plasma levels of HCAs are sufficient to permit antioxidant activity in vivo. Ferulic acid at 10−1000 μM was shown to reduce blood pressure and restore endothelium-dependent relaxation in N(ω)-nitro-L-arginine methyl ester-treated Wistar (50 mg/kg/day to suppress eNOS generation of NO) and spontaneously hypertensive rats via restoration of basal and stimulated

NO bioavailability [238–240]. The authors speculate that the antioxidant capacity of ferulic acid was pertinent to the mechanism of action, but this is not known with certainty. Moreover, sodium ferulate did not affect mean arterial pressure in hypertensive patients, although it did potentiate the blood pressure lowering effect of angiotensin converting enzyme inhibitor captopril [241]. Rosmarinic acid (ester of caffeic acid and 4-coumaroyl-4′-hydroxyphenyllactate) was also shown to enhance endothelium-dependent vasorelaxation, and in a manner consistent with that of an aqueous extract of *Melissa officinalis* L. known as lemon balm, a traditional herb of the Mediterranean region [242].

15.3.3.2 Apocynin

Apocynin (4′-hydroxy-3′-methooxyacetophenone) present in the roots of the Nepalese kutki plant, *Picrorhiza kurroa*, is an ortho-methoxy-substituted catechol that inhibits NOX [243]. Apocynin was initially thought to be a selective inhibitor of NOX, in particular NOX2, and to have direct antioxidant activity [193]. However, its antioxidant activity has been questioned [173,244]; it may act by blocking NOX assembly in the membrane [245].

15.3.3.3 Phycocyanobilin

Spirulina is an edible blue green algae that belongs to the cynobacteria group [246,247]. It has reported antioxidant and antiinflammatory properties, and is a potent inhibitor of NOX [248]. Phycocyanobilin in Spirulina can function as an NOX inhibitor and contains the covalently bound chromophore phycocyanobilin (PCB). It is a potent free radical scavenger with the ability to reduce not only oxygen centered radicals but other reactive species such as hydroxyl, peroxyl, hypochlorite, and peroxinitrite radicals [246]. PCB (a chromophore) is a biliverdin derivative that is converted by the ubiquitously expressed enzyme biliverdin reductase when in mammalian cells to form phycocyanorubin, a compound similar in structure to bilirubin [248].

15.4 CONCLUSIONS

The economic and human costs of the current CVD epidemic are unacceptable, especially given that the majority of cases are related to lifestyle and dietary choices. Achieving the United Nations' target of a 25% global reduction in deaths due to CVDs by 2025 is a daunting goal. It is our view that success will only be achieved through greater knowledge of the genetic, dietary, and environmental causes of CVDs, dramatic improvements in global public health education, and the deveopment of novel, low cost therapies based on bioactive compounds from natural sources.

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15.5 KNOWLEDGE ASSESSMENT QUESTIONS

1. How are lipids transported in that are the differences between the varied transport particles? Which are good and which are bad transport particles in terms of CVD risk?
2. What are the key events in atherosclerosis?
3. Indicate three mechanisms affected by bioactive compounds in natural products that result in an improvement in cholesterol homeostasis and reduced CVD risk.
4. What are the key mechanisms responsible for blood pressure control?
5. Why are ROS thought to increase CVD risk?
6. What class of bioactive compounds are thought to provide the best protection against CVD and list their major sources?
7. What is the greatest challenge in CVD risk reduction?

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Chapter 16

Plant Metabolites and More Treating Various Ailments: Natural Products Treating Diseases

S. Badal
University of the West Indies, Kingston, Jamaica

Chapter Outline

Teaching Goals

To have a comprehensive understanding of homeostasis
To understand the origin of disease conditions
How does inflammation contribute to healing and diseases at the same time?
What are the various disease conditions presented?
What and how do plant extracts and metabolites improve these conditions?
What and how do traditional drugs improve these conditions?
The challenges involved in current approaches toward drug development

16.1 INTRODUCTION

As human beings, in order to survive we adapt to our environment. Our bodies are equipped with systems and processes that are able to identify and modify harmful extreme conditions. However, such abilities can become compromised as a result of several factors: be it age, our environment, or genetic predispositions. Because of such susceptibilities, we then rely on external help which has over the years been primarily sourced from natural products, predominantly from plant sources. The rich history of plant extracts and metabolites used toward treating various ailments is well documented and was described in Chapter 1, Background to Pharmacognosy. As such, many current drugs are of plant origin or are synthesized from natural templates. This usage has increased over the years [1] indicating the efficacy and perhaps

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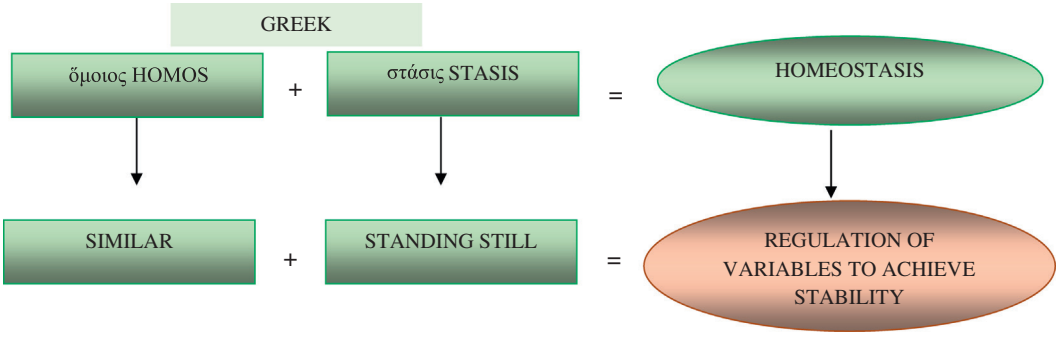
safety associated with such molecules. These can and have been shown to mitigate against diseases and so offer improved quality and longevity of life.

Homeostasis is the process the body uses to maintain a controlled environment for optimal biological processes as well as to safeguard against harmful stimuli. In other words, homeostasis enables stability and constancy of the environment of cells, tissues, organs, and organisms from micro to complex. The concept of homeostasis was first introduced by French physiologist Claude Bernard in 1865 and was then subsequently termed by Bradford Cannon in 1926.



The process of homeostasis is achieved through three primary participants.
These three primary participants include:

The receptor: Picks up changes in the environment and sends message to control center
The control center: Has ideal range for environment and determines appropriate response
The effector: Executes response to regain environment stability

Examples of homeostasis include:

The organism's ability to regulate and survive in extreme temperatures, achieve pH balance, maintain blood glucose levels (BGLs), and so much more. As mentioned earlier, there are several factors that can affect the overall process of homeostasis. To explain this, let us take a look at the processes involved in achieving normalized BGLs demonstrated in Fig. 16.1.

When BGLs go above the normal range, the homeostatic receptors that identify this are the pancreatic β-cells. When BGLs are too low, the homeostatic receptors to identify and initiate a response are the pancreatic α-cells. The control center is the brain which knows the ideal levels of blood glucose and determines the course of action. The release of insulin, the effector, is that course of action when BGLs are too high, this triggers a series of signals that ultimately lead to glucose metabolism and subsequently normalized BGLs. On the other hand, the release of glucagon, the

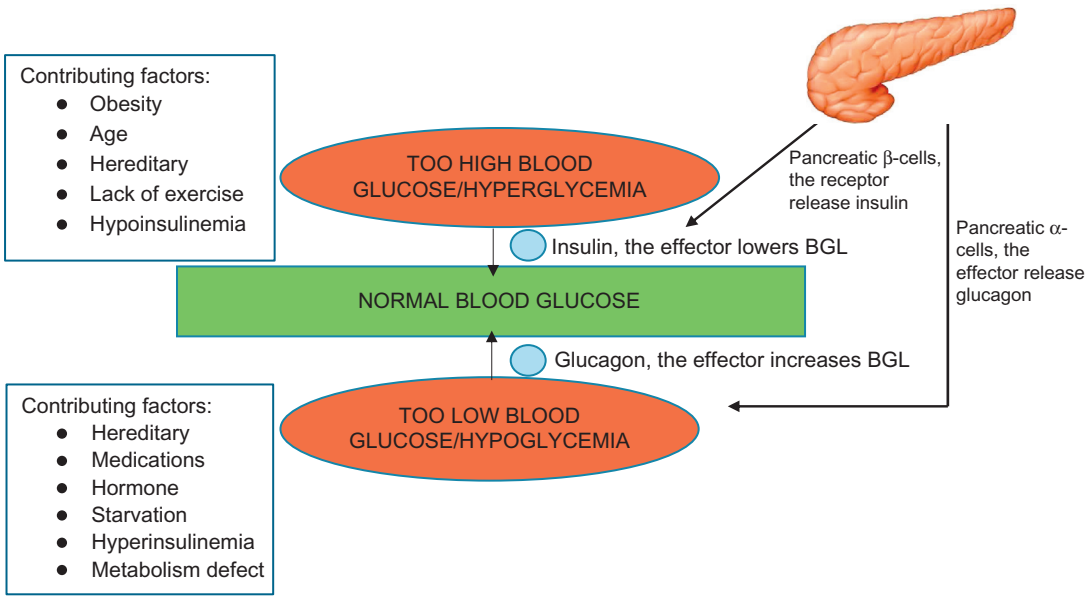


FIGURE 16.1 Homeostatic conditions utilized in achieving normal blood glucose concentrations.

effector, is the course of action when BGLs are too low, this triggers the breakdown of glycogen to glucose and so subsequently elevates the BGLs. In the event of compromised receptors, control centers, and/or effectors like the pancreatic β-cells, normalizing BGLs becomes a challenge and this can result in diabetes.

Compromise of the homeostatic machinery results in disease conditions.

Such compromise can be inherited as in the case of type 1 diabetes where more often than not, the body's production of insulin is below normal or nonexistent resulting in too high BGLs also known as hyperglycemia. On the other hand, type II diabetes is more associated with a combination of factors, hereditary and environmental, in which case the body either produces below average levels of insulin or insulin production is ideal but the body is unable to utilize the insulin, also known as insulin resistance. The high abdominal fat storage often evident in obese persons is common with insulin resistance. Such occurrences are even more evident in persons with less than ideal physical activity. High simple carbohydrate diets have been shown to over time parallel overworked pancreatic β-cells resulting in reduced insulin production. The risk of developing type II diabetes is also increased among the elderly whose pancreatic insulin production is often less than normal and so the levels of insulin needed to normalize BGLs in a timely manner is compromised among other factors.

As mentioned earlier, compromise in the homeostatic environment can and does lead to various disease conditions as seen in the example above. What are other disease conditions and how do plant metabolites and more mitigate against these? Let us explore these in categories but before we do, one of the body's primary mechanisms of response/effector to a number of these conditions is through the process of inflammation. It is therefore important to have a brief understanding of this before we proceed.

16.1.1 Inflammation

Inflammation is part of a series of biological responses of the network of blood tissue to stimuli (pathogens, damaged cells, or irritants) perceived to be a threat to the organism's homeostatic environment. The process of inflammation occurs with the ultimate goal of achieving healing.

16.1.1.1 Acute Inflammation

In the event of acute inflammation, the symptoms become evident fairly quickly, usually within a few minutes or hours upon stimuli encounter but the symptoms then disappear once the stimuli are removed. The acronym PRISH (Pain, Redness, Immobility (loss of function), Swelling, and Heat) is often times used to describe the series of symptoms experienced with acute inflammation. Acute inflammation was considered a disease up until the late 18th century when John Hunter (1728–1793, London surgeon and anatomist) came to the realization that it was only due to a response to injury, a process beneficial to the host: "But if inflammation develops, regardless of the cause, still it is an effort whose purpose is to restore the parts to their natural functions."

The body is equipped with a network of cells whose sole purpose is the protection against harmful stimuli. Together these cells are able to identify, process, and remove harmful stimuli in addition to initiating a cascade of signals necessary for future protection. They include: macrophages, dendritic cells, histiocytes, Kupffer cells, and mastocytes and from here on out we will refer to them as the combat team (CT). On the surface of these cells are pattern recognition molecules (PRMs) also referred to as pattern recognition receptors (PRRs) and these are able to identify unique molecules to pathogens, termed pathogen-associated molecular patterns (PAMPs). At the onset of a stimulus, the CT is activated which results in one or more of their PRMs recognizing one or more PAMPs. Once recognition is complete, a series of pro-inflammatory mediators are released. These can include one or more of the following: lysosome granules, arachidonic acid, bradykinin, histamine, IFN-γ, GM-CSF, OSM, specific IL members, Leukotriene B4, nitric oxide, prostaglandins, TGF-β, TNF-α, among others. Bradykinin and prostaglandins cause vessel dilation and accumulation of inflammatory mediators at the site of infection which result in redness and heat due to the increased blood flow. These mediator molecules also play a role in increasing the permeability of blood vessels which facilitates the migration of leukocytes, mainly neutrophils and macrophages, outside of the blood vessels (extravasation) to the site of infection. This often results in swelling of the area due to accumulation of fluid. The pain is due to chemicals released that stimulate nerve endings and loss of function is due to many factors. Once the perceived threat is addressed, then there is a release of antiinflammatory agents that combat the pro-inflammatory ones restoring the homeostatic environment of the respective tissue. These series of reactions can also be viewed in Fig. 16.2.

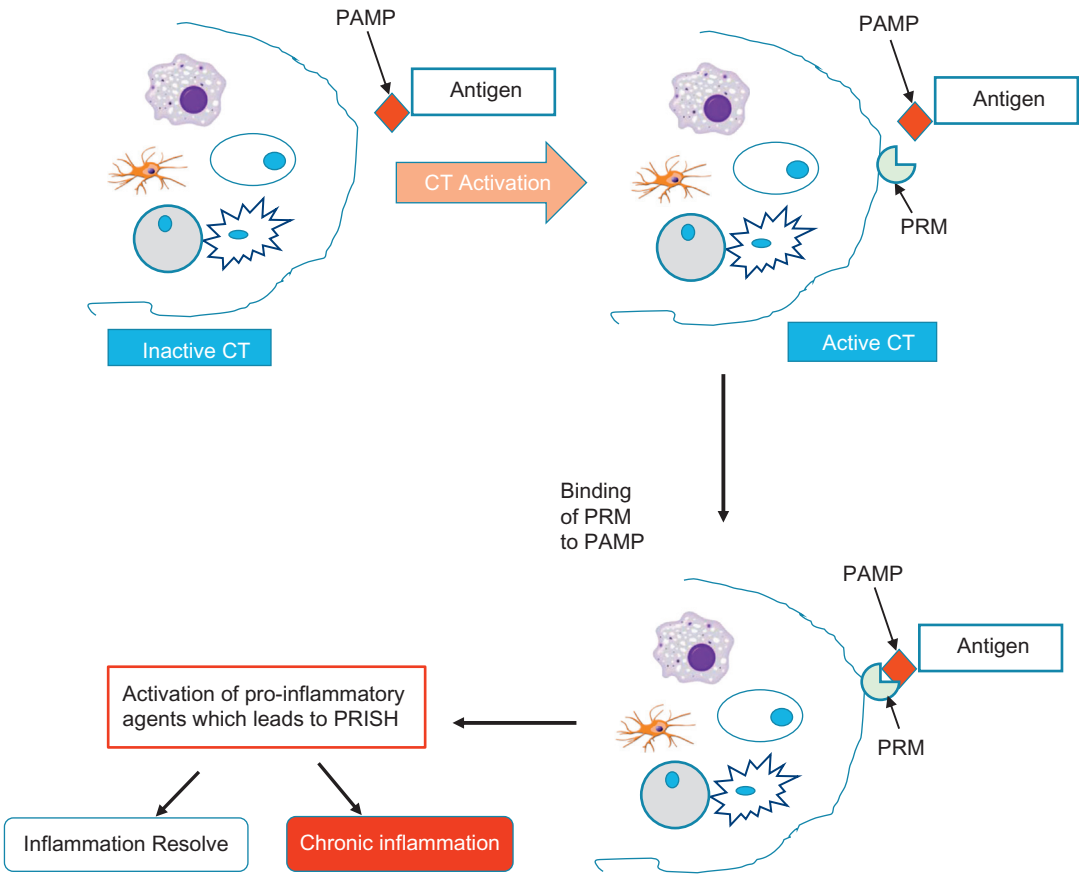


FIGURE 16.2 The basic steps involved in acute and chronic inflammation.
The combat team is represented here as a team of macrophage, dendritic, histiocyte, Kupffer, and mastocyte cells. In the presence of an antigen, the CT becomes activated when PAMP (on CT) binds to the PRM (on antigen). This binding initiates a series of signals which can lead to inflammation resolve, however in the case of equilibrium imbalances, chronic inflammation can ensue.

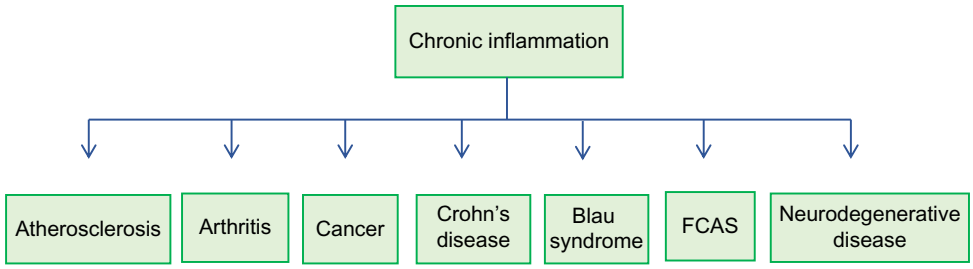


FIGURE 16.3 The various diseases associated with chronic inflammation.

16.1.1.2 Chronic Inflammation

So if inflammation is a normal response of the body to achieve ideal tissue function and a homeostatic environment then why do we need antiinflammatory drugs? Simple, although the process of inflammation has its ideal intent, as with other biological processes, interruptions within the pathways involved with normal cellular processes, whether by external factors or inherited genes, can disturb the equilibrium of ideal cellular functions resulting in unideal outcomes. Prolonged inflammatory response, be it via persistent stimuli or a dysregulation of the inflammatory resolve mechanism leads to chronic inflammation. This is deemed to be the root cause of many diseases (Fig. 16.3) such as atherosclerosis, arthritis, cancer, Crohn's disease, Blau syndrome (NOD2), and familial cold autoinflammatory syndrome (FCAS), in addition to chronic neurodegenerative diseases, such as Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD).

There are numerous pro-inflammatory agents and while research is still limited, there is an existing common trend to some chronic inflammatory morbidities implicating a select group of biomarkers that can be modulated in the hope of preventing or treating these conditions.

16.2 DISEASES

16.2.1 Background

Diseases are atypical, pathological conditions that affect part or all of an organism characterized by particular symptoms [2]. Disease conditions can be as a result of extrinsic or intrinsic factors. Ultimately, life's quality and longevity are negatively affected as symptoms include: pain, suffering, dysfunction, particular handicaps, emotional distress, social challenges, and even death. Such impact is not only confined to the disease bearer but caregivers as well [3,4]. We therefore strive for optimum health, function, and happiness. When any of these are threatened, we seek amelioration. In the case of diseases, such amelioration often resides in drug entities though there is a concern about the disparity in available drugs and research to global needs [5].

While there are numerous drugs on the market, many if not most of these are not effective, as stated by Alan Rose, vice president of genetics at GlaskoSmithKline, "The vast majority of drugs, more than ninety percent only work in thirty or fifty percent of the people, I wouldn't say that most drugs don't work. I would say that most drugs work in 30 to 50 per cent of people. Drugs out there on the market work, but they don't work in everybody" [6]. Therefore, using simple genetic tests to identify the most appropriate drug for patients is one of the proposed ways in moving forward as described by Alan Rose.

Also, disease conditions are multifactorial so optimal treatment cannot be one-dimensional but has to take into account the various points of distress, as described in subsequent chapters, e.g., treatment toward human immunodeficiency virus (HIV). There is now a paradigm shift from the lock and key approach, where one drug for one target for one disease is no longer accepted but instead, exploring multiple drug entities that can mitigate numerous points maybe best to optimize ideal outcomes. Network pharmacology [7], briefly introduced in Chapter 1, Background to Pharmacognosy, is one such approach where synergism is key. Also, while natural products have demonstrated elevated safety compared to their synthetic counterparts there are still cause for concern for some, especially in light of recent findings.

Research on current drugs and new drugs has to shift from lock and key mode to a synergistic one in order to elevate ideal outcomes. Synergy is working together.

- Nine leading causes of death [8]
- Ischemic heart disease
- Stroke
- Chronic obstructive lung disease
- Lower respiratory infections
- HIV
- Diarrheal Disease
- Diabetes
- Road Injury
- Hypertension

16.2.2 Caused by External Causes/Infectious

An infectious disease is as a result of invasion of a host by agents, also referred to as pathogens, whose actions are harmful to the host resulting in phenotypic expressions characteristic of a disease. Since the pathogens can be transmitted to other hosts either directly through insects or animals, contaminated food, or environmental exposure, the condition is termed infectious, hence infectious diseases. While the threat level is relatively high for infectious diseases around various parts of the world, it is important to note that most microorganisms do not cause diseases. Actually, many offer some protection against the harmful ones through mere competition of resources and stifling their replication [9].

16.2.2.1 Causes of Infection

Some pathogens inadvertently cause a disease in any susceptible host making them true pathogens, whereas the opportunistic strains rarely cause infections in individuals with healthy immune systems. An example of this is the fungus *Pneumocystis carinii* which causes a rare type of pneumonia, only seen among immunosuppressed young men. Infection is, however, not only dependent on the strain of the pathogen or the susceptibility of the host but on other factors like the environment. External temperatures can affect exponentially the incubation time of the vector-borne infective agent within its vector organism pathogen. Other conditions like precipitation, sea level elevation, wind, and duration of sunlight also play a role [10].

Causes of Infection:
- Susceptible host
- Type of microorganism/pathogen
- Environmental Factors: temperature, precipitation, sea level elevation, wind, sunlight

Infectious organisms

Six Main Infectious Agents:
- Bacteria
- Viruses
- Fungi
- Protozoa
- Helminthes
- Prions [9].

16.2.2.2 How to Treat Infections

Treating *bacterial infections* compared to other pathogens is relatively easy. This is because bacteria possess features that are unique to their prokaryotic nature and so can be better separated from their eukaryotic host environment. These treatments are categorized depending on their mode of action, in that some inhibit cell wall synthesis, others inhibit protein or nucleic acid synthesis, or enzyme-catalyzed reactions. Penicillin and cephalosporins all interfere with the synthesis of the peptidoglycan layer in prokaryotic cell walls; chloramphenicol, the tetracyclines, and erythromycin, bind to prokaryotic ribosomes and inhibit protein synthesis.

On the other hand, treating *viral infections* is more challenging and also poses a greater threat to the host as viruses use the host's metabolic enzymes in their replica. Consequently, in the past, antivirals mainly treated symptomatic effects until the host's immune system recovered control and was able to eradicate the pathogen. As the research community continues to battle such infections like HIV, the development of more targeted-specific treatments toward viral infections is more eminent. An understanding of the virus' life cycle contributes to the development of treatments that can target the virus at specific points, such as Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs), Epivir-HBV.

Like antiviral treatments, treatments of *fungal*, *protozoa*, and *helminthic diseases* also pose a challenge because molecules that inhibit the growth of these eukaryotic organisms tend to be toxic to the host. The growth of fungi and protozoa is rapid hence drugs are developed to target vital entities within their replicative or biosynthetic pathways. As such, common antifungals impede sterol syntheses (the azole derivatives) or interrupt the cell membrane (polyenes like amphotericin B) while many antihelminthic drugs only target the nonreplicative adult worm. These drugs often times mitigate critical biological processes, such as energy production and muscle function (e.g., the benzimidazoles and avermectins), or at points that play a role in egg production or larval development.

We will briefly discuss further in this section two infectious diseases that pose a threat to mankind, the long-standing HIV and the most recent return of Ebola which caused a panic around the world earlier in 2014. We will also take a look at the Chikungunya virus which recently resulted in an epidemic in the Caribbean; while this virus does not pose as great a threat as the previously two mentioned ones, a greater understanding of the virus is needed since there is currently no treatment and consequently, quality of life is compromised, more so for some than others.

The HIV belongs to the lentivirus which is a subgroup of retrovirus and it is responsible for the acquired immunodeficiency syndrome (AIDS) [11]. The CDC [12] reported that there were roughly two million new cases of HIV in 2013

and approximately 35 million people who now live with the virus globally. In 2010 around one and a half million persons died with AIDS and since the epidemic, an estimate of 39 million persons died with AIDS. In the absence of treatment, the HIV virus is able to do this by enabling the removal of particular components of the human defense machinery while facilitating its latency which enables it to replicate itself as it exponentially weakens the immune system to a point of no return.

- The HIV remains dormant through a number of processes
- Once inside the cell, the viral genome uses its reverse transcriptase enzymes to converts itself into a double-stranded DNA
- This then gets incorporated into the host DNA through a virally encoded integrase.
- The virus now becomes latent as the immune system of the infected host is unable to detect the virus threat
- The virus continues to replicate itself and gets incorporated into other host cells

Symptoms:

Early

- Headache
- Diarrhea
- Nausea and vomiting
- Fatigue
- Aching muscles
- Sore throat
- Red rash mainly on torso
- Fever

Late

- Immune system almost totally compromised making host susceptible to other infections/disease conditions.

There are currently no cures for HIV since the virus is practically incorporated within the host's genome making this a rather challenging task. However, to date there are roughly 30 approved drugs that treat the condition under five different classes, where the first approved drug was azidothymidine (AZT). Other names for Anti-HIV are: the cocktail, antiretroviral (ARV), and highly active antiretroviral therapy (HAART or ART).

The five classes of drugs are:

- NRTIs also called nukes: block the virus' ability to correctly use its reverse transcriptase enzyme for the synthesis of DNA material. Examples: Epivir-HBV, Videx, Viread, Ziagen, Epivir, Retrovir, Zerit, Emtriva, Hivid, and Videx EC.
- Non-NRTIs (NNRTIs) also called nonnukes act in a similar manner to nukes; the only difference is they act directly on the reverse transcriptase enzyme, also blocking the production of new viral DNA material which also include the previously listed drugs under NRTIs.
- Protease Inhibitors (PIs), these inhibit the protease enzymes which the virus uses to increase viral copies through shorter viral strands. These include: Kaletra, Lexiva, Prezista, Norvir, Aptivus, Reyataz, Viracept, Agenerase, Crixivan, Fortovase, and Invirase.
- Entry/Fusion inhibitors work by blocking the entrance of the virus into new cells. The virus is able to enter healthy cells through receptor sites which are present on both the host CD4 cells and itself. The entry fusion inhibitors interfere with this process. These include Fuzeon and Selzentry and experimental drugs; cenicriviroc and ibalizumab.
- Integrase inhibitors block the virus from adding its DNA into the DNA of the host CD4 cells and current treatments are: Isentress, Stribild, Tivicay, Triumeq, and Vitekta. Treatments are usually a combination of three drugs divided into two classes aimed at: reducing viral load through attack of the virus at various points; protection of the immune system; and reducing HIV drug resistance.

Recent research shows the following natural extracts and isolates as promising anti-HIV leads.

- Alertoxins from the endophytic fungus
- Alternaria tenuissima [13]
- Perylenequinones [14]
- Marine extracts from red algae, *Galaxura filamentosa*, and Cnidarian jelly fish, *Cassiopia ~~A~~andromeda* [15]
- Plant peptides, cyclotides [16]
- Palmitic acid (PA) the naturally occurring fatty acid isolated from *Sargassum fusiforme* and analog, 2-bromopalmitate (2-BP) [17].

The Ebola virus disease (EVD) also known as the Ebola hemorrhagic fever first appeared simultaneously in 1976 in Nzara, Sudan, and Yambuku, Democratic Republic of Congo. Current total confirmed cases since the virus first appeared in 1976−2012 are approximately 2400 of which 1600 deaths occurred, almost 70% [18]. The EVD is of major concern now given the recent March 2014 outbreak in Sierra Leone and Liberia [18]. There is still much more to be understood about the virus and much needed research into plant and other natural metabolites and their potential efficacy toward this condition.

What we do however know is that

Processes involved in EVD:

- Virion attaches to the host specific cell surface receptor
- Virus envelopes into host cellular membranes.
- The viruses' genome replication results in full-length positive-strand antigenomes
- These are transcribed into genome copies of negative-strand virus progeny [19].
- Mature progeny particles infect other cells.

Symptoms:

Early:

- Fever
- Fatigue
- Muscle pain
- Headache
- Sore throat

Late:

- Vomiting
- Diarrhea
- Rash
- Impaired kidney and liver function
- Both internal and external bleeding (e.g., oozing from the gums, blood in the stools) can ensue.

There is currently no Food and Drug Administration (FDA) approved treatments though the following medications are undergoing research: Favipiravir [20], BCX4430 [21], Brincidofovir [22], Lamivudine [23], and JK-05. While most were used to treat recent infections and success has been received in many instances [24,25,26], there is still not sufficient evidence in support of any medication warranting immediate FDA approval. Survival rates of persons with the virus are improved with rehydration using oral and/or intravenous fluids along with treating nonspecific symptoms.

Chikunguya (CHIKV) resulted in a fairly recent outbreak affecting persons mainly from Africa, Asia, and the Caribbean [27]. The name Chikungunya originates from the Kimakonde language, meaning "to become contorted." It was first described during an outbreak in southern Tanzania in 1952 but has been emerging since 2006 first in the Indian subcontinent, then Thailand [28], and most recently the Caribbean [27].

This RNA virus belongs to the alphavirus genus of the family Togaviridae and unlike the previously discussed viruses, it is transmitted by mosquitoes, *Aedes albopictus* and *A. aegypti* [29]. The incubation period is usually 2−12 days and its symptoms include fever (above 39°C), strong joint pain which can lasts for months and even years, muscle pain, headache, fatigue, nausea, vomiting, and a rash. Inflammation of the eyes may occur and is also known as iridocyclitis, or uveitis, as well as retina lesions [30].

Symptoms:

- Fever (above 39°C),
- Strong joint pain which can lasts for months and even years
- Muscle pain
- Headache
- Fatigue
- Nausea
- Vomiting
- Rash
- Inflammation of the eyes may occur and is also known as iridocyclitis, or uveitis as well as retina lesions [30].

The typical duration for the fever is 2 days which then ends abruptly, however, other symptoms, e.g., headache, insomnia, and an extreme degree of prostration can last longer [31]. There are currently no vaccines and drugs to treat this illness so preventative measures are encouraged, in that "don't get bitten by a mosquito." Also, Panadol is recommended to minimize the discomfort along with rest. Among the homeopathy community, papaya leaf is encouraged to be taken as a paste and there have been reported cases of success, though this begs the question of success being due to the leaf or one's own immune system. Since this is a self-limiting condition, research is needed to verify the efficacy of papaya leaf paste against the virus and also a vaccine is definitely needed to combat the virus [32].

16.2.3 Other Infectious Diseases

Other infectious diseases that are more prevalent and a cause for concern oftentimes in lower to middle income countries comprise: leishmaniasis, malaria, schistosomiasis, tuberculosis, Chagas' disease, leprosy, lymphatic filariasis, onchocerciasis, hepatitis C, diarrheal diseases, ascariasis, rabies, yaws, and necatoriasis [5]. Together these diseases pose a cause for global concern. Of noted interest are tuberculosis (TB) and malaria.

- TB is caused by the mycobacterium, *Mycobacterium tuberculosis*, which targets the lung.
- *Incidences and mortality*: one-third of the global population and an approximate death rate of 1.4 million in 2013 [33].
- *Early symptoms include*: cough, sneeze.
- *Chronic symptoms include*: chronic cough, blood tinged sputum, fever, night sweats, and weight loss.
- *Transmission*: persons with TB can transmit bacteria through bodily fluids when coughing or sneezing.
- *Treatments*: Bacillus Calmette-Guérin (BCG) vaccine

- Malaria is caused by the parasitic protozoan belonging to the genus *Plasmodium* and targets the liver where it reproduces.
- *Incidences and mortality*: 219 million reported cases in 2010 from which 660,000 died [34].
- *Symptoms*: headache, fever, shivering, joint pain, vomiting, hemolytic anemia, jaundice, hemoglobin in the urine, retinal damage, and convulsions
- *Transmission*: *Anopheles* mosquito bite that previously bit infected persons
- *Treatments*: one or more of the following as tailored by doctor: artemisinin, mefloquine, lumefantrine, or sulfadoxine/pyrimethamine, quinine, and doxycycline

16.3 AGE-RELATED

There are some diseases that are more prevalent among the aging population (senescence). Therefore difficulties that arise within the aging populous predispose such individuals to these conditions more so than others. Approximately two thirds of the roughly 150,000 people who die each day across the world die of age-related causes. This occurrence is even greater in industrialized nations, reaching 90% [35].

Some of these diseases include:

- Hypertension
- Atherosclerosis
- Type 2 diabetes
- Cancer
- Glaucoma
- Cataracts
- Arthritis
- Alzheimer's

Many age-associated diseases are intertwined with each other and so clear-cut separation is usually not the case. To expound, hypertension is many times connected to atherosclerosis, diabetes, and glaucoma. Environmental conditions too can enhance or reduce a person's susceptibility to certain disease conditions. For every heartbeat, blood is pumped throughout the body via arteries; when the pressure of the blood flow is constantly above the ideal range, then one is said to have *hypertension*, also called high blood pressure. As persons age, the walls of the lining of their arteries

change. Therefore, the large blood vessels become less elastic which subsequently causes an increase in the velocity of the pulse waves. This increase in pulse wave velocity then increases late systolic blood pressure and myocardial oxygen demand; such occurrences can also cause restricted organ perfusion (blood flow to organs).

High blood pressure can cause damage to the lining of the blood vessels. The aged populous is more susceptible to atherosclerosis as their vasculature lining tends to undergo prolonged plaque deposits resulting in hardening of the blood vessels. Atherosclerosis can also be a genesis of hypertension. *Atherosclerosis* is therefore a part of the complex myriad of conditions belonging to cardiovascular diseases (CVDs) and occurs when the lining of blood vessels becomes hardened due solely to plaque deposits. Oftentimes atherosclerosis leads to myocardial infarction, stroke, and ischemic gangrene. The process of atherosclerosis is usually initiated when there is damage to the lining of the endothelium primarily due to high blood pressure, smoking, or high cholesterol. Damage to the endothelium typically results in low density lipoproteins (LDLs) crossing the damaged area, entering the walls of the arteries.

As described in the section above, the aged populous is susceptible to diabetes especially the type II form. It is also known that persons with diabetes are likely to develop hypertension as diabetes does affect the lining of the arteries and can cause atherosclerosis which can then lead to hypertension. Diabetes, hypertension, and atherosclerosis are also linked to other conditions like renal and ocular failure. Vasculature damage compromises potassium excretion in the kidneys and so elderly persons are prone to hyperkalemia. Other associations of hypertension, atherosclerosis, and diabetes are:

- Renal artery stenosis,
- Obstructive sleep apnea
- Primary aldosteronism (hormonal induced hypertension)
- Thyroid disorders.

Cancer is a disease that has been associated with the elderly although its occurrences in recent times are more evident in the younger populous. This condition is discussed further in Chapter 14, Chemotherapeutics.

Another age-related disease is *glaucoma* which causes damage to the optic nerve due to prolonged elevated pressure levels in the eye and gets worse over time. The condition tends to be hereditary but may not be evident until later on in life. Glaucoma has been referred to as the "silent thief of sight," as vision loss occurs gradually over a period of time and symptoms are usually only evident when the disease is in its advanced stage [36]. Glaucoma is the second leading global cause of blindness behind cataract [37]. One in 200 people aged 50 and younger are affected with the disease while 1 in 10 over the age of 80 are affected, highlighting the association of the condition to the aged population [38].

The other aged-related disease condition is *cataract*, which too is an ocular-associated disease. Cataract causes a blurring of the ocular lens leading to a reduction in vision and blindness. Visual loss develops when the opaqueness of the ocular lens obstructs light passage prior to being focused on the retina at the back of the eye [39]. Cataract is caused by oxidative stress and protein glycation. Proteins involved in the protection of the ocular lens are compromised by oxidative damage; these oxidized proteins cause opacity. In treating cataracts, protecting against oxidative stress and restoring those proteins associated with antioxidant activities are key as elevating the levels of the following are important:

- Glutathione
- Superoxide dismutase (SOD)
- Glutathione peroxidase
- Glutathione *S*-transferase
- Catalase

While decreasing the following are also effective:

- Lipid peroxidation
- Aldose reductase
- Protein glycation

Arthritis is an autoimmune condition and occurs when the body's own self defense mechanism is misappropriated and instead of addressing harmful stimuli, the body attacks itself. An estimated 53 million Americans were diagnosed with some form of arthritis [40]. Most arthritic conditions are causes of inflammation resulting in damage to joint areas through the loss of cartilage elasticity, the attacking or infection of the synovial membrane. Though there are over 100 different types, the most common type among the elderly is Osteoarthritis. Arthritic limitations include:

- Reduced walking distances
- Climbing of stairs

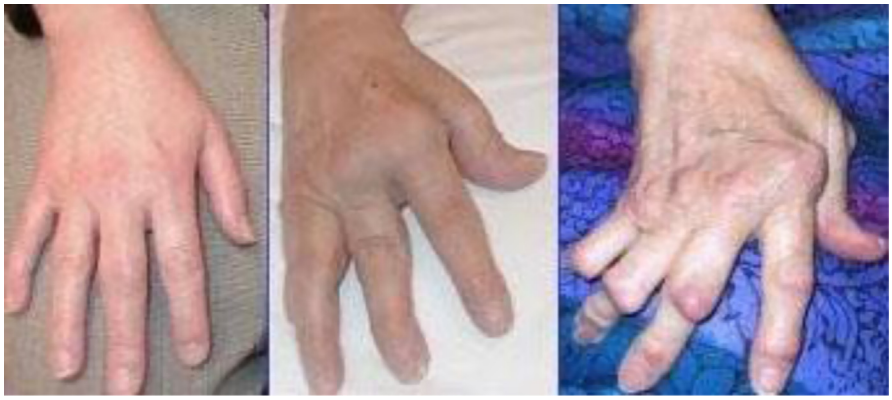


FIGURE 16.4 The progression of deformities in the hand associated with rheumatoid arthritis.

- Bending of the knees
- Inability to keep up with social activities and gatherings [41]
- Various deformities and disabilities [42] as seen in Fig. 16.4

Also known as *Alzheimer's* or Alzheimer disease, AD is named after Dr. Alois Alzheimer, a German doctor. It is responsible for approximately 65% of all dementia cases [43]. Data shows that AD is responsible for a population-attributable risk between 5% and 15% on 5-year mortality for ages 65 and older, and represents the fifth leading cause of death in the similar age range [44,45]. The survival from the time of AD diagnosis varies between 3 and 8 years [46]. In 1906, Dr. Alzheimer observed alterations in the brain tissue of a woman who had died of a rare mental illness. He later discovered a number of abnormal clumps (now called amyloid plaques) and tangled bundles of fibers (now called neurofibrillary tangles). The main features of AD include:

- Plaques in brain
- Tangles in brain
- The loss of connections between nerve cells in the brain.

AD is a chronic neurodegenerative disease that often starts slowly and gets progressively worse over time. Symptoms of Alzheimer's commonly start with short-term memory loss and as the condition progresses, other symptoms include:

- Challenges with speech
- Getting lost easily
- Changes in disposition like loss of enthusiasm
- An inability to manage self-care
- Normal daily activities
- Withdrawal from one's environment
- Lost bodily functions
- Death [43,47]

16.3.1 Plants Extracts Treating Age-related Diseases

Plant extracts have a rich history of usage in the treatment of many age-related diseases. For centuries garlic has been used to lower *blood pressure levels* and over 2000 clinical studies have validated this folklore claim. It is advised by David Hoffmann, of Sebastopol, California that taking a clove of garlic a day will help significantly in preventing or reversing the effects of high blood pressure [48].

Given that the genesis of *atherosclerosis* is heavily connected to inflammation, treatments of this condition are primarily antiinflammatories. Turmeric has been shown to have antiinflammatory activity [49] and further demonstrated antiatherosclerosis activity by protecting liver microsomes from lipid peroxidation in atherosclerotic rabbits [50] and inhibited LDL oxidation in atherosclerotic rabbits [51]. Turmeric has to date approximately 235 compounds, principally phenolic compounds and terpenoids, which have been known to have antiinflammatory properties, and so it has been used toward treating atherosclerosis. Some of the identified compounds from this spice include:

- Diarylheptanoids and diarylpentanoids
- 8 phenylpropene and other phenolic compounds
- 68 monoterpenes
- 109 sesquiterpenes
- 5 diterpenes
- 3 triterpenoids
- 4 sterols
- 2 alkaloids
- 14 other compounds

Many plants have been used to treat *diabetes* and the antidiabetic activities of some have been confirmed. From a review article written by Modak [52], a list of these plants from India can be viewed below:

- *Allium sativum*
- *Eugenia jambolana*
- *Momordica charantia*
- *Ocimum sanctum*
- *Phyllanthus amarus*
- *Pterocarpus marsupium*
- *Tinospora cordifolia*
- *Trigonella foenum-graecum*
- *Withania somnifera*

Momordica charantia, also known as ceresy, is frequently used as an antidiabetic and antihyperglycemic agent in India as well as other Asian countries. Extracts of various parts of the plant including the fruit pulp, seed, leaves, and whole plant have demonstrated hypoglycemic properties in animal models [53]. A list of formulated herbal medicaments and their ingredients can be viewed in the review article by Modak [52], some of these include: Diabecon, Diasulin, Pancreatic tonic, bitter gourd powder, and Dia-care.

A number of herbal remedies have been used and reported to be effective toward the treatment of *glaucoma*. Some of these include:

- Bilberry
- Curcumin
- Calabar bean
- Jaborandi oil
- Rose hip
- Cayenne
- Marijuana

As stated prior, many of these have demonstrated a number of biological activities making them effective in not just treating one ailment but many. For example, bilberry, *Vaccinium myrtillus* L., which is known for its improvement of vision, yet it has also been shown to lower BGLs and have antiinflammatory, antioxidant, and lipid-lowering properties making it useful toward treating conditions associated with inflammation like atherosclerosis, diabetes, CVD, cancer, dementia, and other age-related conditions [54]. Bilberry is rich in *anthocyanins* and it is believed that its properties are heavily linked to these secondary metabolites.

The following plants show promise in treating cataracts:

- *Pueraria lobata*
- *Allium cepa*
- *Trigonella foenum-graecum*
- *Zingiber officinalis*
- *Foeniculum vulgare*
- *Curcuma longa*

Natural flavonoids which are abundant in the abovementioned plants, have been shown to prove efficacious in treating cataracts by multiple mechanisms of actions. Onion's anticataract activity is believed to be achieved through its ability to increase levels of SOD and glutathione peroxidase, and inhibition of aldose reductase [55]. In vitro research showed that

μM levels of the flavonol quercetin and its metabolite [56] elevated lens transparency and inhibited oxidation-induced sodium and calcium influx in rat lens tissue culture [57]. Similar observations were made for the flavonoid venoruton, a mixture of mono-, di-, tri-, and tetrahydroxyethylrutosides that also reduced leakage of lactate dehydrogenase in rat lens organ culture [58]. More information on these can be viewed in the review article by Stefek [59].

Many natural products demonstrate antioxidant capacity like the flavonoid, Quercetin. However, recent findings indicate that its oxidized form is more toxic than free radicals.

Rheumatoid arthritis (RA) is a condition primarily associated with inflammation and so turmeric is also known to endow some alleviation of discomfort that arise from this arthritic condition. Additionally, ginger and sarsaparilla are also known to be effective toward RA and some evidence confirms this [60]. In a study conducted at the University of Miami, a concentrated extract of ginger was shown to lower pain and stiffness in knee joints of patients with RA by 40% when compared to those given a placebo [61]. They concluded that ginger could replace nonsteroidal antiinflammatory drugs (NSAIDS). *S. sarsaparilla* was shown to inhibit carrageenan-induced paw inflammation in rats, as well as cotton pellet-induced exudation [62]. *S. sarsaparilla* contains many saponins, phytosterols, starch, resin, cetyl alcohol, volatile oils, and many acids.

Traditionally, *Ginko biloba* has been used to treat AD, and ginkgolides have been confirmed to possess antioxidant, neuroprotective, and cholinergic properties that together proved efficacious toward AD. Clinical trials have shown that extracts of *G. biloba* showed parallel bioactivities to prescribed drugs, tacrine, and donepezil, while demonstrating minimal side effects [63]. Other plants with documented memory improving activities include, *Salvia officinalis* (sage) and *Melissa officinalis* (balm). Recent research has linked AD to insulin deficiency and resistance [64] and so the use of coconut oil in treating AD is now the hot topic, especially since coconut oil has been shown to improve the body's use of insulin [65].

Tacrin has been taken out of the market in many countries. The most important drug now is galanthamine

16.4 BIOCHEMICAL DERAILMENTS, METABOLIC DISEASES

Metabolism is the process the body uses through various reactions aided by enzymes to produce energy from the food we eat which consists of carbohydrates, proteins, and fats; these are broken down to their monomeric units: sugars, amino acids, and fatty acids, respectively. The monomeric units are either used immediately as energy or are stored in one or more of the following organs: liver, body fat, and muscle. When there is an imbalance in any of these processes, you then have a metabolic disorder (MD) which can be genetic or acquired through dietary and lifestyle factors. The MD of primary concern to this chapter is diabetes, which is also a repercussion of inflammatory processes, especially the type 2 form, and was briefly introduced previously.

An approximate 250 million individuals worldwide succumb to diabetes and this number is expected to double by 2030 [66]. More than 80% of diabetes deaths occur in low- and middle-income countries [67]. Type 2 diabetes is a major cause of morbidity and mortality and was found to be the seventh leading cause of death in the United States in 2006 [68]. An estimated 24 million people in the USA live with diabetes and roughly 90–95% of them have the type 2 form [69]. The total estimated health care costs associated with diabetes in 2007 was $174 billion [68]. Diabetes is also the leading cause of kidney failure, nontraumatic lower-extremity amputations, and blindness among adults in the United States [69]. It is believed that lifestyle factors such as diet and exercise can significantly impact the prevalence or lack thereof of this disease. But in the meantime, much research is being done to acquire solutions.

There are two forms of diabetes, type 1 and type 2, where type 1 is due primarily to hereditary factors, while type 2 is more associated with lifestyle conditions. Both conditions are characterized by hyperglycemic effects as a repercussion of insulin deficiency but the type 2 form is also characterized by insulin resistance [70,71]. This form is more predominant than type 1 and global estimates of diabetes in the absence of intervention was expected to grow significantly within the next few decades [72] and so it has.

Originally known as insulin-dependent diabetes mellitus (IDDM), type 1 diabetes (T1DM) is an autoimmune disorder, one that is caused by a progressive destruction of insulin producing pancreatic β-cells which results in hyperglycemia [73,74]. To cure patients of T1DM, it is believed that ablation of the β-cell-specific autoimmune reaction and the use of β-cell replacement therapy is necessary [74].

Currently, one of the primary sought after cures for diabetes is transplantation of the pancreatic islet which is minimally invasive and has great potential to restore normoglycemia and achieve complete independence from exogenous insulin in T1DM patients [74,75,76]. Recent in vitro studies showed that plant extracts from: Garlic (*A. sativum* L., Alliaceae), Persian shallot (*Allium ascalonicum* L., Alliaceae), and Sage (*Salvia officinalis* L., Lamiaceae) were found to control diabetes from antioxidant and hypolipidemic effects [77]. In vivo studies showed that extracts from the *Cassia javanica* plant displayed promising hypoglycemic effects which compared with known hypoglycemic drug, Glibenclamide [208]. Extracts from Fenugreek and Termis seeds powder, along with their mixture, were found to offer beneficial properties for preventing diabetic complications in animal models [78].

Other plant extracts with promising antidiabetic properties include *Ficus carica* Linn [79]. *Sarracenia purpurea* and some of its active ingredients have been identified as an alternative and complementary treatment for diabetic complications associated with glucose toxicity [80]. In a review article written by Afifi and Kasabri [66], several plants were identified as having undergone some research for antidiabetic properties and over 1200 species have been identified. Some of these include: *Camellia sinensis, Pimpinella anisum, Zingiber officinale, Matricaria recutita, Salvia fruticosa, Trigonella foenum-graecum, Nigella sativa, Lupinus albus, Teucrium polium, Allium sativum, Cinnamomum zeylanicum*, and *Olea europea.*

Current plant metabolites are being researched for antidiabetic properties. Conophylline was isolated from the leaves of the plant *Erytamia microphylla* and was found to induce differentiation of β cells from precursor cells and most recently demonstrated suppression of islet fibrosis in vivo. Using the structure of epoxyquinomici, the authors also designed a compound, DHMEQ, as a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB, a protein that controls DNA transcription) inhibitor, it was found to improve the success of islet transplantation in vivo [81]. Epicatechin alkaloid derivatives for pharmacological interventions to reduce or prevent diabetic complications are extremely important. The consumption of whole grain foods has been associated with decreased risk of type 2 diabetes through improved gut barrier function. Improved gut barrier function seems to be connected to a reduction of endotoxic bacterial lipopolysaccharides (LPS) into circulation. Lower amounts of LPS in blood are believed to alleviate peripheral inflammation [82]. Nuclear receptors are also considered important to the treatment and perhaps cure of diabetes given their involvement in regulating critical processes such as metabolic homeostasis [83]. As flavonoids have been identified as effectors of nuclear receptors, they have been deemed promising leads for pharmaceutical and nutraceutical compounds toward treating diabetes [84].

The most recently approved medication for diabetes is Afrezza (insulin human) Inhalation Powder which was approved by the FDA in June 2014. It is used to treat both forms and is administered with meals and is an ultrarapid-acting inhaled insulin which improves glycemic control in adult diabetics. Current medications for treating type 1 diabetes involve different types of insulin listed below.

1. Insulin-rapid acting:
   a. Insulin Lispro: onset (O)-15 min; peak (P)-1 to 1.5 h, duration (D)-2 to 5 h
   b. Insulin Aspart: O-10-20 min; P-1 to 1.5 h; 2−5 h
   c. Insulin Glulisine: O-10-20 min; P-1 to 3 h; 3−5 h
2. Premixed insulin: which involves mixing two types on insulin together and they are also called biphasic insulin.
   a. Insulin lispor protamine
   b. Insulin aspart protamine

   Oral medications are used to primarily treat type 2 diabetes.
3. Sulfonylureas: these stimulate the pancreas to release more insulin and are only effective in the presence of pancreatic β cell activity.

   First generation sulfonylureas are:
   a. Acetohexamide
   b. Chlorpropamide
   c. Tolbutamide
   d. Tolazamide
   e. Tolinase

   Second generation sulfonylureas are:
   a. Amyryl
   b. Diabeta
   c. Glucotrol
   d. Diamicron

e. Glimepiride
f. Glyburide
g. Glipizide
h. Gliclazide

The other classes of oral drugs and modes of action are given below:

3. Biguanides inhibit hepatocyte stimulated glucose production while elevating insulin-receptor binding and stimulating tissue uptake of glucose. Since it does not cause hypoglycemia or weight gain, patients suffering from obesity and with type 2 diabetes are normally given Biguanides as starters.

   Metformin is an example of this type of drug.
4. α-Glucosidase Inhibitors retard the activities of intestinal enzymes that are responsible for the digestion of carbohydrates, thus reducing the rate of carbohydrate digestion after a meal, lowering postprandial blood glucose elevation in diabetic patients. Some examples of this class of drugs include:
   a. Glyset
   b. Precose
   c. Miglitol
   d. Acarbose

   Thiazolidinediones/Glitazones increases the body's sensitivity to insulin thereby minimizing the amount of insulin that is needed to reduce BGLs
   a. Actos
   b. Avandia
   c. Pioglitazone
   d. Rosiglitazone
   e. Troglitazone
5. Meglitinides work by stimulating the pancreas to release insulin in response to a meal.
   a. Prandin
   b. Starlix
   c. Repaglinide
   d. Netaglinide

Other MDs of noteworthy concern include: Acid Lipase disease, Barth Syndrome, Farber's Disease, Central Pontine Myelinolysis, Hurler Syndrome, Krabbe disease, and many more [85]. Acid lipase disease occurs when there is a buildup of fats (lipids, waxes, oils, and cholesterol) in the body's cells because the enzyme lysosomal acid lipase which is needed to break down the fats is missing. Barth syndrome is unique to males and is caused by a mutation of the tafazzin gene which affects lipid metabolism. Also caused by an imbalance in lipid metabolism is Farber's Disease, concomitant with an excess buildup of lipids. Central Pontine Myelinolysis and Krabbe are disorders of myelin where the former is brought about by nutritional or electrolyte stress and the latter results in the breakdown of myelin. Hurler syndrome however is a disorder of sugars whereby persons with the condition are unable to break down long chains of glycosaminoglycans.

16.5 ROLE OF MICROBIOME IN GI TRACT

Our gut is the home to many microbes, over 100 trillion, and together these organisms influence the human physiology, metabolism, nutrition, and immune function [86]. Disruption of this homeostatic environment has been the cause of many diseases such as irritable bowel syndrome, e.g., Crohn's disease, as well as obesity.

Crohn's disease is also known as Crohn's syndrome and regional enteritis. It is one of the main types of irritable bowel syndrome that not only affects the small and large intestines but other areas of the gastrointestinal tract, including the mouth, esophagus, stomach, and anus. Common symptoms associated with the condition are abdominal pain, intestinal bleeding, weight loss, and diarrhea. The disease affects mainly persons in the developed countries since approximately 3 per 1000 persons in Europe and North America are affected while much lower occurrences are evident in Asia and Africa [87]. CD also places an enormous financial burden on individuals, families, and societies since an estimated $2 billion in expense per year was accounted for in Canada alone [87,88].

The onset of CD remains unknown though it is believed that individuals with certain genetic predispositions are more likely to experience CD when exposed to certain dietary and environmental factors [89]. Nonetheless, researchers

have explored the gut microflora across different populous. As human beings we heavily rely on the presence of microorganisms which span Archaea, Bacteria, and Eukarya, for encoding 150-fold more unique genes than our own [90]; immune system maturation; nutrition; maintenance of intestinal barrier permeability and function, in addition to the prevention of pathologic species [90]. These gut microbiota which are difficult to culture and are 10-fold more than our own cells [91] lie in close proximity to the immune system.

Peppermint oil (PO) is one of the most commonly used over-the-counter remedies for CD. Its bioactivity is believed to occur through its antispasmodic action through the movement of calcium ions across the cell membrane [92]. PO offers relief against abdominal pain and distension of functional dyspepsia/IBS, mostly in subjects with flatulence [93]. Research on plant metabolites toward the treatment of CD remains quite limited since mainly biologics, adalimumab, certolizumab pegol, and infliximab [94], are currently used and researched.

16.6 FERTILITY AND REPRODUCTION

The human reproductive system allows for the production of offspring and some scientists say that the organs involved in reproduction are among the most important as without them our species die [95]. A number of conditions can result in compromised function of these organs which ultimately affects the fertility of a couple. When a couple is unable to produce an offspring within a year of trying, they are deemed to be infertile. Nearly 20% of reproductive-aged couples are affected with infertility [96,97]. Of all infertility cases, roughly 40% are due to men, another 40% to women, with 20% due to complications with both [98].

Diseases that affect the reproductive system and so increase infertility in couples can be categorized as shown in Fig. 16.5. Some conditions are more relevant to males than females and vice versa. Those conditions that affect both genders include cancer, chemicals/toxins, and microorganisms.

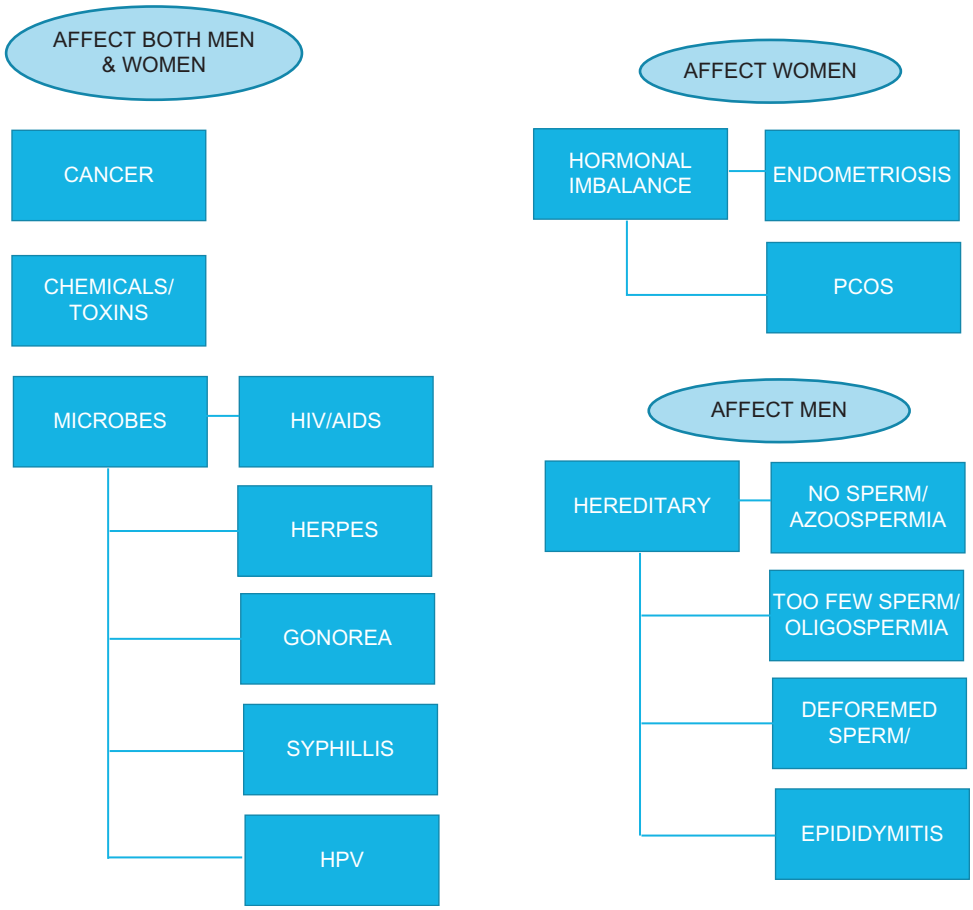


FIGURE 16.5 Diseases that affect fertility in men and women.

16.6.1 Cancer

Treatments from chemotherapy and radiation can reduce sperm count and quality in men and egg quality, quantity, and release in women. In many instances, the treatment of cancer is a combination of surgery, chemotherapy, and radiation. Despite the type of cancer, chemotherapy and radiation compromise fertility in both genders but such compromise is even more evident in cases of breast, ovarian, and prostate cancers, where the sex organs are directly affected and surgery can result in the removal of some or all of these organs.

16.6.2 Chemicals/Toxins

Many chemicals/toxins can and do affect a couple's fertility and also a woman's ability to give birth to healthy babies. Alcohol consumption was found to affect males' potency and nicotine has been confirmed to affect the sperm morphology and sperm count [99]. In many instances, exposure of the fetus to toxic chemicals results in spontaneous abortion or birth defects. Used early in the pregnancy, cocaine decreases uterine and placental blood flow by inhibiting the reuptake of norepinephrine (acts as a hormone and neurotransmitter), which causes arterial vasoconstriction [100]. Nicotine is the main addictive compound in tobacco smoke, it is also a strong vasoconstrictor (narrows blood vessels) as it reduces uterine and placental blood flow [101]. Further, tobacco smoke contains other toxic components, such as carbon monoxide, which decreases the fetus' access to oxygen as it binds to the oxygen carrier, hemoglobin, and cyanide, which diminishes vitamin B12, an essential cofactor for fetal growth and development.

16.6.3 Microbes

Microbes that play a role in infertility are usually transferred through sexually transmitted diseases, of which some are highlighted in Fig. 16.5. It is also important to note that many experts in the field argue the direct association of some of these microorganisms and infertility. Case in point, the HIV virus. There are still concerns as to whether compromised fertility occurs because of the direct presence of the virus, the other conditions brought about because of the virus (reduced immune system function, weight loss, and stress) or the antiretroviral themselves. In any event, what is certain is that when a person contracts this virus they are prone to infertility be it directly or indirectly.

The following are some of the conditions that arise from these microorganisms and have resulted in infertility in both men and women.

- Affect a females' menstrual cycle (missed periods or long intervals in between cycles)
- Affect the release of fertility hormones (estrogen and progesterone) in women and (testosterone) in men
- Cause early menopause
- Inflamed testicles
- Decreased sex drive
- Decreased sperm quantity and quality
- Reduced sexual activities
- Miscarriage
- Infant death

16.6.4 Female Infertility

Hormonal imbalance is another area that affects both men and women but is the leading cause of female infertility [102]. External factors like birth-controls and stress can heighten infertility as well as other disease conditions like hypothyroidism

Some hormonally associated conditions in both men and women include:

- Hypothyroidism (low thyroid function)
- Luteal
- Phase defect (low progesterone levels)
- Hyperprolactinemia (high male hormone levels)

Two of the main hormonally induced infertility conditions that occur among women are polycystic ovarian syndrome (PCOS) and endometriosis. The multiple growth of cysts on the ovaries associated with PCOS is the direct cause of hormonal imbalance. Such imbalance is associated with irregular periods, reduced egg quality and quantity, elevated

levels of the male sex hormone, androgen, and others. Roughly one in 15 women of child bearing age suffers from PCOS and as many as 5 million women in the United States may be affected. It can occur in girls as young as 11 years old [103]. Women with PCOS are 75% more likely to develop anovulatory infertility [104].

Endometriosis occurs when the cells of the uterus, also referred to as the endometrium, grow outside the uterine cavity. Given the influence of the endometrial cells outside of the uterus to hormonal changes, they therefore respond in a similar manner to those inside the uterus. The details of the condition are still unclear as egg quality and quantity have not necessarily been associated with the condition but other avenues likely to be affected are fertilization and implantation. The condition is mainly diagnosed in reproductive years, however, girls as young as 8 years old have been identified as having the condition. Roughly 4−10% of women are identified as having endometriosis and the association of the condition to infertility is well established [105] as 40−50% experience infertility [106].

16.6.5 Male Infertility

Azoospermia, oligozoospermia, and deformed sperms are all conditions associated with hereditary factors which can also influence hormonal imbalance. However, oligozoospermia is also mostly associated with exogenous factors such as infectious agents, environmental pollutants, age, and obesity [107]. Azoospermia, also termed sterility, affects 1% of the male population of which 20% suffer from infertile situations [108,109].

On the other hand, men with oligozoospermia do have some sperm, more than those with azoospermia but significantly less than their fertile counterparts. Also, this condition is associated with deformed sperms [2].

16.6.6 Natural Products Used to Treat Infertility

The current modern day approaches to treat infertility include fertility drugs, which are primarily hormonal-based, aimed at elevating sperm count and health in men and increase or regulate egg maturation in women. The following are current fertility drugs given to women to stimulate their ovaries to produce more mature eggs.

- Clomiphene: Clomid and serophene
- Gonadotrphins: Pergonal, Repronex, Fertinex, Follistim, Gonal F, Novarel, Ovidrel, Pregnyl, Profasi, and Menogon and Puregon (available only in Europe)
- Bromocriptine: Parlodel

The first natural supplement that comes to mind when you think of fertility is folic acid. This vitamin B complex found in fortified cereal can help to prevent birth defects of the baby's brain and spinal cord if taken before and during pregnancy. Also, folic acid is crucial to the production of red blood cells and it helps the baby's neural tube develop into her/his brain and spinal cord. In a review written by Nantia et al. [110] a number of medicinal plants were highlighted as having antiinfertility properties. These include extracts of *Panax ginseng, Panax quinquefolius*, and *Lepidium meyenii* showed positive effects on sexual desire; whereas extracts of *Astragalus membranaceus, Asparagus racemous, Withania somnifera, Andrographis paniculata*, and *Acanthopanax senticosus* improved sperm parameters.

16.7 NEURODEGENERATIVE DISEASE

The brain and the spinal cord are a part of the central nervous system (CNS) which are responsible for information assimilation, in that the information the brain receives is organized to impact all parts of the human anatomy. Communication within the brain is facilitated via neurotransmitters that travel through synapses responsible for connecting the building blocks of the CNS and neuronal cells, to each other. Progressive loss of function or structure of neurons including their death leads to neurodegeneration. Unlike other cells in the body that are replaced if they become damaged or die, neurons cannot reproduce or replace themselves. As a result, problems with movement also referred to as ataxias or mental functioning also called dementias arise ultimately leading to disease conditions associated with mortality since there are currently no cures [111].

Throughout the development of the nervous system, approximately one and a half times the number of adult neurons are generated. Essential to brain development, is the death of the additionally created cells which undergo apoptosis. The death of other neuronal cells later on in life can be as a result of traumatic injury, environmental toxins, cardiovascular disorders, infectious agents, genetic diseases, or inflammation. These factors can be a cause of apoptotic or necrotic cell death which is when the death is random, irreversible, and uncontrollable [112]. However, oxidative stress

is a primary cause of most if not all neurodegenerative diseases [113]. High levels of reactive oxygen species released and reduced activity of antioxidant mechanisms leads to neuronal cell death [114,115].

While *Alzheimer's* is a disease common among the aged population and was previously discussed, it is also a neurodegenerative disease and the plant extracts discussed previously are efficacious toward this condition. Some, primarily because of their antioxidant capacity, are also effective against other neurodegenerative conditions like ALS and Huntington (discussed more below).

16.7.1 Amyotrophic Lateral Sclerosis

ALS also called Lou Gehrig's disease is a terminal adult-onset neurodegenerative disease whose onset is still not clearly understood though current research has made some progress into its etiology. ALS has an incidence of 1−4 per 100,000 persons each year and a prevalence of 4−6 per 100,000 worldwide [116]. The condition is characterized by degeneration of the upper and lower motor neurons leading to loss of limb and bulbar function [117]. Such loss generally starts focally and then spreads throughout the body leading to paralysis and ultimately death within a few years of diagnosis [118,119].

To date, only 10% of ALS cases have been linked to hereditary causes of which 20% have been associated with mutations in the gene superoxide dismutase 1 (SOD1) [120]. Such genetic variations also vary from one ethnic group to the next [121]. SOD are enzymes that participate in dismutation (a redox reaction that simultaneously oxidizes and reduces a species to form two different products) reactions of the superoxide (O_2^-) radical to produce O_2 or H_2O_2. Superoxides are known to confer damage to cells and so their conversion to other safer forms, although H_2O_2 is known to contribute to cell damage but to a lesser extent is deemed important in the antioxidant process. Hence, SOD are important antioxidant enzymes in nearly all living cells comprising of three forms depending on location; SOD1 (cytoplasm), SOD2 (mitochondria), and SOD3 (extracellular). Mutations in SOD's expression therefore interferes with their antioxidant capacity and so the linkage of ALS to mutations in SOD1 indicates that antioxidants are likely effective toward treating this condition. The challenge however is sourcing entities that possess these properties while being able to also cross the blood−brain barrier, as many are hydrophilic.

Neurodegenerative diseases arise when there is above normal death of neuronal cells primarily because of reactive species and inflammation

This challenge seems to have been overcome by a recent formulation which was reported in a recent article. This article reports the Deanna protocol [122] that was created by orthopedic surgeon Vincent Tedone for his daughter who suffered from ALS. The protocol used a combination of natural supplements with a main ingredient of arginine alpha ketogluturate (AAKG), others include: nicotinamide adenine dinucleotide (NADH), coenzyme Q10 (CoQ10), ubiquinol (a CoQ10 formulation), Gamma-aminobutyric acid (GABA), and glutathione [123]. Together it is believed that these molecules defend against free radicals while they mitigate other processes responsible for ALS predisposition and onset. An observed amelioration of Danna's condition was observed while on the treatment.

16.7.2 Huntington's Disease

HD was first described by an American physician, George Huntington, in 1872 after he studied several affected individuals and also noted observations made by his father and grandfather [124]. HD was previously called Huntington's chorea, and is a rare neurodegenerative disorder of the CNS characterized by undesirable choreatic movements, behavioral and psychiatric disturbances, and dementia. HD usually manifests itself around mid-life (30s−40s) and if it shows earlier onset symptoms like in the early 20s, it is then classified as juvenile Huntington's disease (JHD). The disease has been found to be more prevalent among persons of Western European descent than those of Asian or African ancestry. Its occurrence in the Caucasian population is roughly 5−10 per 100,000 persons. Despite its incidence variability among individuals, the disease has an average life span of 15−18 years after diagnosis [124].

HD is caused by a genetic defect in the short arm of chromosome 4 concomitant with the expansion of a CAG trinucleotide repeat belonging to a novel gene. The expansion of the CAG repeats is approximately 36, sometimes more. In instances where there are more, the symptoms of the disease are usually detected earlier and so in JHD, repeats usually

exceed 55. Such genomic alterations are responsible for the progressive degeneration of the nerve cells which manifests themselves by retarding a person's functional abilities, their movement, and cognitive function [125].

Research by Bjorkqvist [126] showed evidence of widespread innate immune activation throughout the course of HD carriers with a mean of 16 years before the predicted onset of clinical symptoms. Further, they showed that monocytes from HD subjects were pathologically hyperactive in response to stimulation which inferred that the mutant protein could be turning on the cell-autonomous switch. Corresponding patterns were noticed in macrophages and microglia from HD mouse models. Concurrently, the cerebrospinal fluid and striatum of HD patients displayed atypical immune activation which suggests that immune dysfunction plays a role in brain pathology. Altogether, their data suggested a similar CNS and peripheral pathogenic pathways of immune activation in HD. While the exact biomarkers that play a role in innate or adaptive immune activation, individually or collectively, in HD are still unknown, research has shown a correlation between the HD gene and an upregulation of the IL-6 release through the IκB kinase/NF-κB signaling pathway which may play a role in neurotoxicity [127]. Also, previously implicated in the pathogenesis of HD are microglias [128,129].

Currently, there are no cures for HD; however, medications offer relief of the symptoms that accompany disease progression. The dopamine pathway inhibitor, Tetrabenazine (TBZ), was recently approved by the FDA for the treatment of chorea in patients with HD. TBZ alleviates motor deficits, diminishes striatal cell loss, and controls choric movement in HD mice [130]. Recent clinical studies show that PBT2, a metal protein-attenuating compound may reduce metal-induced aggregation of mutant Huntington and prolonged survival rates were seen in mice models with HD [131]. According to a recent review article [132], there have been several identified current potential therapeutic agents which are now undergoing in vivo research and these include: memantine, tetrabenazine, minocycline, treahalose, C2−8, creatine, coenzyme Q10, ethyl-EPA, cysteamine, HDAC inhibitors, and mitramicycin. They act on improving motor and/or cognitive dysfunction mostly in the R6/2 and N171-82Q mouse lines. A few are undergoing clinical trials: Riluzole (an inhibitor of glutamate neurotransmission in the CNS) [133,134]; creatine, coenzyme Q10; minocycline; cysteamine; memantine; and ethyl-EPA [132].

It is also believed that practicing a proper holistic way of life which comprises of nutritional support, societies, and home care services may prolong a patient's life and also improve quality of life [125].

16.7.3 Antidepressant and Drugs of abuse

Drug entities and natural products that act on the CNS are often times categorized as antidepressants and substance of abuse. Depression is usually connected to a deficit in the levels of the neurotransmitter norepinephrine in the brain. Such deficiencies are associated with a number of conditions as listed below.

Antidepressants are used alone or in combination with other medications to treat:

- Dysthymia
- Anxiety disorders
- Obsessive compulsive disorder
- Eating disorders
- Chronic pain
- Neuropathic pain
- Dysmenorrhea
- Snoring
- Migraines
- Attention-deficit hyperactivity disorder (ADHD)
- Substance abuse
- Sleep disorders

Therefore, mechanisms to treat depression should ultimately elevate the levels of norepinephrine, as such antidepressant drugs achieve one or more of the following stimulation type mechanisms:

- Increase release of norepinephrine:
  - Amphetamines and electroconvulsive therapy act by this mechanism. Amphetamines mimic norepinephrine.
- Prevent inactivation of norepinephrine:
  - Monoamine oxidase (MAO) inhibitors are believed to act as antidepressant agents partly by preventing the breakdown and inactivation of norepinephrine.

- Prevent the reuptake of norepinephrine:
  - The action of norepinephrine at the receptor site is terminated by the re uptake of norepinephrine.

The main classes of antidepressants include:

- Selective serotonin reuptake inhibitors (SSRIs)
- Serotonin–norepinephrine reuptake inhibitors (SNRIs)
- Tricyclic antidepressants (TCAs)
- Monoamine oxidase inhibitors (MAOIs) [135]

Drugs of abuse that are relevant to this section are:

- Cocaine
- Hallucinogens
- Heroin
- Marijuana
- Steroids
- Tobacco

These fall under psychoactive drugs and are discussed in more detail in Chapter 17, Psychoactive Drugs. Many of these are alkaloids and their effects on the body are heavily due to their similarity to neurotransmitters. They can either mimic or block the effects of the body's innate neurotransmitters or cause fluctuations in their normal levels leading to many physiological and psychological effects. Let us take for example cocaine which is isolated from the cocoa plant native to South America. Cocaine also called crack depending on its purity affects the CNS by elevating the levels of the neurotransmitter dopamine in the brain. Dopamine regulates pleasure and movement, as such sustained elevated levels result in the "high," characteristic of dopamine along with increased energy, talkativeness, and possibly dangerous physical effects like elevated heart rate and blood pressure.

16.8 CONCLUSION

When the body's innate homeostatic balance is affected, many disease conditions can and do arise, conditions that not only affect the disease bearer but the caregivers as well. Such conditions have steered the directional approach of the research community for centuries. Such direction has resulted in the genesis of many drugs, natural and synthetic, though most are only effective on 30–50% of persons who take them. As such, there is a current transitioning of the existing way of thinking and doing, among research communities and drug industries, from the lock and key paradigm to a synergistic one like network pharmacology. Such an area is very important given the numerous points of impact among various disease conditions, areas that would all need to be addressed to not only achieve efficacious outcomes but safer ones as well. While natural products have been known to be relatively safer than their synthetic counterparts, as research continues to evolve and new discoveries are made, other paradigms reveal further expanses of needed attention.

16.9 SELF-EVALUATION QUESTIONS

1. What is homeostasis?
2. Discuss the genesis of diseases.
3. Why do persons need drugs?
4. Are all natural products safe? Discuss.
5. What are the points of impact when treating a HIV patient?
6. Discuss the lock and key approach and give an example.
7. You are the President of the Global Research Health Institute, one that is geared toward improved health care around the world. Suggest a thorough and reasonable frame work that you could implement in order to ensure that each individual has a fair chance of survival and quality of life.

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Chapter 17

Psychoactive Drugs

J. Widelski and W.A. Kukula-Koch
Medical University of Lublin, Lublin, Poland

Chapter Outline

Learning Objectives

- To define and give examples of psychoactive ingredients
- To identify the main plant sources of psychoactive drugs and their points of origin
- To have a comprehensive understanding of *Amanita muscaria*, *Myristica fragrans*, and *Cannabis sativa*; their traditional use; their confirmed biological activities and active ingredients connected to these; their adverse effects

17.1 DEFINITION

Psychoactive drugs in general affect the central nervous system (CNS). Many are obtained from plant material, either in extract form or isolated compounds that can excite and enhance mental alertness and physical activity without altering consciousness; reduce fatigue and hunger (stimulants); while they can also repress mental activity; awareness; physical performance (depressant); and cause changes in mood, space and (or) time perception, visions, illusions (hallucinations).

17.2 EXAMPLES

Hallucinogens occur in different genera of fungi such as *Amanita*, *Psiolcybe*, *Conocybe*, and higher plants. Plants with hallucinogenic properties are present in most botanic families, e.g., wormwood (*Artemesia absinthium*; *Asteraceae*), diviner's sage (*Salvia divinorum*; *Lamiaceae*), deadly nightshade (*Atropa belladonna*; *Solanaceae*), peyote (*Lophophora williamsii*; *Cactaceae*), iboga (*Tabernanthe iboga*; *Apocynaceae*), and many others.

17.3 PLANT SOURCES

17.3.1 Amanita Muscaria

The fly agaric (*Amanita muscaria*) is a poisonous mushroom with a characteristic red or orange cup, often covered with white flecks. Various species occur in many continents and usually grow in deciduous wood, especially beech and birch as well as coniferous ones. In some parts of northeastern and western Siberia, the local tribes (e.g., chuckchee) use the

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fly agaric as an intoxicant. These inhabitants of Siberia ingest the mushroom alone, either sun-dried or toasted slowly over the fire. They may also take it as reindeer milk or with juice of wild plants, like those of a genus Vaccinium. The symptoms start after 20–30 min and usually end within 2 h. A small dose (up to four mushrooms) can cause dizziness, nausea, tiredness, a feeling of weightlessness, visual and auditory hypersensitivity, space distortion, unawareness of time, and colored hallucinations [1]. A larger dose gives more pronounced symptoms of poisoning with spasm and more vivid hallucination. Aggressive attitudes have not been reported. As a result of the poisoning, dryness in the mouth and mydriasis (dilation of the pupils) can occur followed by a period of drowsiness, then a deep sleep with vivid dreams, usually 2 h after. After the deep sleep, which generally lasts 8 h, the poisoning ends [2].

For a long time, it was believed that the intoxicating effects of *A. muscaria* was due to the alkaloid, muscarine (Schmiedeberg O, Koppe R. Das Muscarin, das giftige Alkaloid des Fliegenpilzes (in German). Leipzig, Germany: F.C.W. Vogel. OCLC 6699630;1869), but the concentration of this ingredient is in such minute concentrations (up to 3 mg/kg of fresh mushroom), that it could not act as the inebriant. It is now recognized that, in the drying or extraction of the mushrooms, ibotenic acid forms several derivatives. The most important is muscimole (formed through decarboxylation of ibotenic acid), the main pharmacologically active principle. Other compounds, such as muscazone, are found in lesser concentrations and may contribute to the intoxication. Ibotenic acid (α-amino-3-hydroxy-5-isoazoloacetic acid) as well as muscazone can be regarded as amino acids, while ibotenic acid and muscimole are oxazol derivatives.

Most bioactive ingredient in *Amanita muscaria*: muscimole

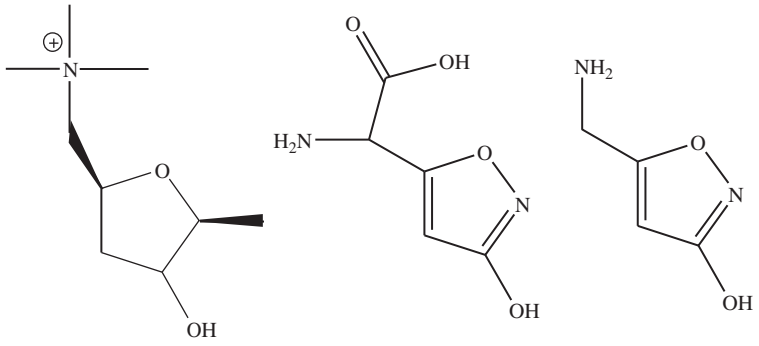
The mushroom is given the name fly agaric because of its age-old use in Europe as a fly killer. The mushrooms were left in an open dish, flies were attracted to and settled on the mushrooms, the flies were subsequently stunned resulting in the mushroom being deemed as having insecticidal properties.

Fly agaric = fly killer, an observation made in Europe years ago

17.3.2 Bioactivity

In vivo research confirms that biochemical changes develop 30 min after peritoneal injection of aqueous extracts of *A. muscaria* into male rats. Such changes included a decrease of acetylcholine esterase activity, liver glycogen, and blood urea nitrogen, together with an increase of blood glucose levels. Serum transaminase activities were not affected and all values returned to normal within 6 h [3]. The latter data demonstrated that the poisoning was not detrimental as vital organs like the liver and kidneys were not affected.

Fig. 17.1 shows the chemical structures of isozasol derivatives (ibotenic acid and muscimol) present in *A. muscaria* which are similar to those of glutamic acid and GABA (γ-aminobutyric acid), products of their enzymatic decarboxylation. Similarities in the structures of these compounds are thought to contribute to their ability to bind and activate receptors of endogenous [4].



(+) Muscarine Ibotenic acid Muscimol

FIGURE 17.1 Psychoactive ingredients: ibotenic acid and muscimol.

Behavioral changes, like ataxia and sedation, induced by muscimol (agonist of $GABA_A$ receptor and partially $GABA_c$ receptor) in mice depend on high-affinity binding of this compound to a distinct subtype of $GABA_A$ receptor in the cerebral cortex [5]. Moreover, mucimol inhibits GABA uptake by neurons and astrocytes and is a substrate to GABA transaminase [4,6,7]. For this reason the structure of muscimol was used as a template for the design of GABA uptake inhibitors and GABA agonists [7].

It is assumed that the two main compounds responsible for the hallucinogenic properties of *A. muscaria* are ibotenic acid and muscimol and the mechanism of their activity is connected to ligation of glutamate and GABA receptors, respectively [4]. However, the studies concerning the connection between brain activity involved with paradoxical sleep appearance and various paradoxical sleep-associated phenomena (called pedunculopontine tegmental nucleus) seem to negate hallucinogenic properties, both GABA and glutamate agonists.

The injection of glutamic acid and muscimol into pedunculopontine tegmental nucleus in rats resulted in induction or suppression of one of the paradoxical sleep hallmarks [8]. Influence of both toxins on the sleep architecture seem to be due to GABA and glutamate-dependent mechanisms that modulate activities of cholinergic neurons within pedunculopontine tegmental nucleus [4,9]. Muscimol, dose-dependently, affected encephalogram in experimental animals clearly differently from other typical hallucinogens such as LSD and mescaline. Particularly, electroencephalogram pattern caused by muscimol showed spikes, characteristic of convulsing activity [4,10]. Injected intravenously, muscimol also potentiates analgesic effects of opiates (morphine) in rats and mice and these effects are disrupted by GABAergic system [11,12].

17.3.3 Adverse Effect

It is important that the intake of fly agaric (*A. muscaria*) does not cause any damage to organs (liver, kidneys) and subsequent gastrointestinal disorders with vomiting are inconstantly reported [13]. Nevertheless the active components of *A. muscaria* may induce in vivo brain lesions. Regular consumption of the mushroom would probably be harmful, even though the vast majority of human poisoning cases do not report any after-effects [1].

17.4 MYRISTICA FRAGRANS

Nutmeg (*Myristicae semen*) is the kernel of the dried, ripe seed of *Myristica fragrans* Houtt, belonging to family Myristicaceae. The nutmeg tree grows to 10−20 m in height, with a natural origin in the Moluccas, but it has been cultivated in Indonesia, Malaysia, Sri Lanka, and the West Indies. *M. fragrans* is a dioecious tree, bushy and evergreen. In plantation the number of male trees is reduced to roughly 10% in total. The fruit is a one-seeded fleshy drupe, yellow and pear-shaped. During fruit ripening, the aromatic, orange pericarp splits, disclosing the black seed surrounded by a red, net-like aril, which is separated and dried to give the crude drug mace. The seed, after removing the red aril is dried in high temperature (in the oven) until the kernel shrinks and rattling can be heard in the testa. Seed shell (testa) is crushed after and separated from the kernel, which is the proper nutmeg drug. Nutmeg contains 30−40% of fats and about 10% of essential oils [14], which is mostly composed of terpenes (α-pinene, camphene, p-cymene, sabinene, β-phellandrene, γ-terpinene, myrcene), terpene derivatives (linalool, geraniol, terpineol), and phenylopropanes (myristicin, elmicin, safrole) [15]. Mace oil has a similar composition, but it contains higher levels of terpenes. Both nutmeg and mace are the two major primary products of *M. fragrans* that are commercially used as spices.

17.4.1 Bioactivity

Various extracts and essential oils of nutmeg seeds have been reported with antimicrobial activities against gram-positive and gram-negative bacteria, as well as a variety of fungi. Ethanolic extract of nutmeg seeds demonstrated antimicrobial activity against enterohemorrhagic *Escherichia coli*, which was found to be highly sensitive to β-pinene [16]. Another research reported potent antibacterial activity of chloroform extracts, against both gram-positive and gram-negative bacteria and trimyristin and myristic acid proved to be the chief antibacterial principles isolated from *M. fragrans* Houtt [17].

The nutmeg extracts demonstrated antifungal activity against *Candida albicans* and *Aspergillus niger*. Earlier studies have proposed that one of the mechanisms of the antifungal effects involved the inhibition of various cellular processes, followed by an increase in plasma membrane permeability and finally ion leakage from the cells [18]. Methanol extract of *M. fragrans* Houtt., containing lignans, has AChE (acetylcholinesterase) inhibition activity. Different AChE

inhibitors have been shown to significantly improve the cognitive function in Alzheimer's disease (these compounds enhance the signal transmission in nerve synapses by prolonging the effect of acetyl choline) [19].

It has been reported that myristicin, present in the volatile oil of *M. fragrans*, is a potential chemopreventive agent, by way of its ability to induce the activity of the detoxifying enzyme system, glutathione *S*-transferase [20]. In addition, extracts of nutmeg strongly suppressed the growth of human lymphoid leukemic cells (Molt 4B) [21]. Moreover, the dihydroguaiaretic acid from *M. fragrans* mace suppressed the viability of several cancer cell lines, leukemic, colon, and lung [22]. Myristicin, as well, induced apoptosis via the mitochondrial pathway and downregulated genes belonging to the DNA damage response pathway in human leukemia cells [23].

In vivo research examined the antimutagenic potential of nutmeg in male wistar rats. The plant extract showed significant bioactivities as there was a decrease in the mutation index in a dose-dependent manner. This was due to the antioxidant activities of the present phytochemicals that scavenged the active oxygen radicals [24].

17.4.2 Adverse Effects

Consumption of nutmeg seeds in large quantities cause a hallucinogenic effect, which is followed by unpleasant side effects such as facial flushing, tachycardia, hypertension, dry mouth, feelings of euphoria, unreality, and delirium. Several cases of nutmeg seed ingestion have been reported in adolescents who attempted to achieve a euphoric state at low cost [25]. For the psychoactivity of nutmeg to be experienced, the metabolic conversion of the two components of nutmeg essential oil, myristicin and elmicin into compounds similar to amphetamine has to take place. As a result of the metabolism of elemicin, 3,4,5- trimethoxyamphetamine is produced and metabolism of myristicin leads to 3-methoxy-4,5-methylenedioxy amphetamine [26]. Moreover, myristicin, as a weak inhibitor of monoamine oxidase, could be responsible for some symptoms of circulatory disorders [27].

17.5 CANNABIS

Cannabis (Indian hemp, *C. sativa* herba) consists of the dried, aerial parts of *C. sativa* L. belonging to family Cannabidaceae (also Cannabaceae). It is an annual diecious, wind-pollinated herb, with male and female flowers that develop on separate plants. The leaves and bracts on both types of plants have unicellular covering hairs with a pointed end and wide base. They also have glandular hairs which secrete a resin rich in cannabinoids. The plant occurs naturally in India, Bangladesh, and Pakistan and is grown in numerous countries with tropical climates suitable for fiber and seed production. The stem of the plant, which can grow up to 10 cm in diameter, contains long and tough fibers (the most durable fibers of natural origin), which are used for the production of ropes, carpets, etc. Although both male and female plants produce cannabinoids, female plants produce larger amounts of the resin and this is the reason they are preferred.

17.5.1 Cannabinoids

Cannabinoids are a group of C_{21} compounds occurring in resin produced by glandular hairs of *C. sativa* L. Among the over 420 known constituents of cannabis, more than 60 belong to cannabinoids, which chemically belong to the terpenophenols. Cannabinoids (phytocannabinoids) are accumulated in the glandular hairs, which account for more than 80% of the subcuticular secretion. Generally, they are present in all plant parts, except the seeds.

There are no qualitative differences in cannabinoid content among particular plant parts, only quantitative. The highest concentration of cannabinoids can be found in the bracts of the flowers and fruits while the other parts of the plant (foliage leaves, stem, and roots) possess lower amounts of the active phytocannabinoids.

The most important representative is Δ^9−tetrahydrocannabinol (THC), which has hallucinogenic properties. The other principal components are: cannabinol (CBN), cannabidiol (CBD), Δ^8−tetrahydrocannabinol, cannabigerol, and cannabichromene (CBC).

Most of the cannabinoids have an acid analog, where the only difference is the presence of a carboxyl group (acidic cannabinoids are regarded as being the primary compounds). In the fresh plant material they may occur in larger amounts compared to their neutral counterparts. The main cannabinoids (THC, CBD, CBN, and CBC) are usually detected in each breeding strain or cultivar of *C. sativa*. For the cannabinoid profile of a plant, storage and breeding conditions play a significant role along with variations during preparations of the medicine, mixing with other components (e.g., tobacco), and heating. Findings confirm that cannabinoids, except those produced from biosynthetic

pathways (acidic cannabinoids), leaving mainly the neutral cannabinoids (the majority that result from decarboxylation) are products of degradation (oxidation and isomerization) and are called artifacts. For example, cannabinoids of the CBN type are not formed as by-products of plant metabolism, but rather oxidative degradation of THC and CBD types. Also the Δ^8−tetrahydrocannabinol is the product of isomerization of THC.

17.5.2 Hallucinogenic Effects of *C. sativa*

The main reason why people almost all over the world use cannabis is to get "high." Cannabis users understand this term, as an experience of euphoria, relaxation, perceptual alternation, and the intensification of ordinary sensory experiences, such as eating, watching films, and listening to music. The "high states" may be accompanied by excessive laughter and talkativeness. Cognitive effects (including short-term memory and feeling of associations), motor skills, and reaction time are impaired. Since cannabis, specifically THC, lowers the psychological inhibitions comparable to alcohol, it may be perceived that sexual impulse and libido are heightened. The perception of senses like touch, smell, hearing, taste, and so on are sharpened and hence the sexual stimulants that lead to sexual arousal can be perceived to be enhanced. For that reason cannabis "products" are also used as aphrodisiacs. Body perception may become distorted after smoking a certain dosage of marijuana as spatial-temporal perception may alter in a dose-dependent manner. Time is perceived to pass slower or sometimes faster.

The tactile perception may become more intense. Visual hallucinations occur, about which the subject is aware that these are the acute effects of THC, not the reality. Hallucinations can appear as bright and colorful light flashes. After the ingestion of cannabis (including as a main compound THC), the following symptoms can occur: lowered skin temperature, increased heart rate and blood pressure, analgesia, sedation, slowed speech, slow reaction time and coordination disorder, challenges with concentration and memory, feelings of extreme pleasure, giggling and laughter, different feelings of senses (music may seem more distinct and subtle colors more brighter), a strong desire for food, impaired time perception, and feeling of being separated from reality. Less frequently occurring symptoms are delusions, seeing and hearing, anxiety, panic, attack of paranoia (feeling of being scared or suspicious without reason). Long-term effects include short-term memory impairment, difficulty in learning and problems solving, breathing problems, reproductive system problems, decreased motivation, and low energy [28−33].

17.5.3 The Endocannabinoid System

Natural cannabinoids (phytocannabinoids) are substances acting on the endocannabinoid system (ECS), which regulates numerous physiological processes. There are two types of cannabinoids' receptors: CB1 and CB2. The CB1 receptors have been shown to be highly concentrated in neuronal cells of the CNS, especially those placed in the cerebral cortex, hippocampus, lateral caudate-putament, substantia nigra pars reticulate, and cerebellum [34,35]. This location explains documented effects of cannabinoids on cognition and brain function. Agonists of CB1 receptors also exhibit analgesic properties reflective of the role presence of CB1 receptors on pain pathways in the brain and spinal cord and at the peripheral axons of primary sensory neurons. CB1 receptors are present at low levels in neurons located in peripheral tissues, including heart, bladder, vascular smooth muscle cells, lung smooth muscle cells, and intestine [36].

Within the CNS, endocannabinoids and their receptors modulate neuronal signaling and play very important roles in the regulation of movement (coordination of motor function, posture, balance), sleep, emotion, appetite, body temperature, memory storage, and pain perception.

The second type of cannabinoid receptors (the CB2 receptor) are found preferentially in the periphery. They are located in the cells of the immune and hematopoietic system, but have been found to also be present in the brain and other tissues. The presence of the CB2 in the lymphoid organs (tonsils, thymus, spleen) is prerequisite, that in addition to their psychoactive effects in the CNS, the ECS has a role in modulating the immune system. Indeed, cannabinoids have profound influence on cell mediated immunity by inhibiting the proliferation of T cells, cytokine secretion (proinflammatory agents), and the humoral responses from B cells. Such bioactivities demonstrate the therapeutic potential of cannabinoids as antiinflammatory agents [37].

The two best studied endogenous agonists of cannabinoid receptors are: anandamide (*N*-arachidonoyloethanolamide) and its glycerol ester 2-AG (2-arachidonoyl glycerol). The former acts as an endogenous ligand for the aCB_1 receptor, but has a very low affinity for the CB_2 receptor. 2-AG exhibits agnostic patterns to both receptors.

17.5.4 Bioactivity

17.5.4.1 Parkinson's Disease

Parkinson's disease (PD) is overwhelmingly a chronic, progressive, and neurodegenerative disease caused by the degeneration of dopamine-containing neurons of the substantia nigra, which innervate the striatum. Termination of dopaminergic neurotransmission subsequently interferes with the function of the basal ganglia decisive to coordination of motor function. Therefore PD characteristic symptoms are bradykinesia (slowness of movement), akinesia (postural immobility), muscular rigidity, resting tremor, and postural instability [37,38].

Several cannabinoid receptors, representing CB_1 type in the basal ganglia suggests that cannabinoids could play a therapeutic role in the treatment of movement disorders associated with PD [37].

In general there are three phases in PD development:

1. Early presymptomatic phase characterized by neuronal malfunctioning rather than neuronal damage (death), associated with downregulation or desensitization of CB_1 receptors [39,40].
2. Intermediate and advanced symptomatic phase, when the most important process is neuronal death. The PD characteristic is upregulation of CB_1 receptors, which is caused by adaptive responses and is also compatible with the akinetic profile of these patients [40,41]
3. The presence of CB_2, receptors that are characteristic of immune function, as basal ganglia structures show activation of glial elements during pathological processes. The activation of astrocytes and microglia, linked to neuronal injury in lesioned structures in PD is associated with upregulatory responses of CB_2 receptors that are located in cells, which play a role in the protection of neurons [40,42].

Different experimental models of PD exhibit elevated levels of activity of the ECS as the basal ganglia is increased. Such elevated activity is manifested by increased CB_1 activity, anandamide (endocannabinoid) levels, and decreased cannabinoid clearance [43]. Despite the upregulation of the CB system that is noticed at intermediate−late stages of the disease process, in the earlier, presymptomatic phase of PD, CB_1 receptors are desensitized, which may render the basal ganglia more vulnerable to the cytotoxic environment of the cranial activity associated with PD, which promotes excitotoxity according to the loss of CB_1-mediated presynaptic inhibition of glutamate release [44,45]. As a result of their ability to inhibit glutamate release and so mitigate glutamate-mediated toxicity, cannabinoids may prove useful as potential therapeutic targets against PD [34]. Given the fact that CB receptors promote hypokinesia, antagonists of the CB_1 receptors confirmed by preclinical studies should be likely potentials for the treatment of PD in order to counteract the consequences of an upregulation of the cannabinoid system that is common at the advanced stage of the disease.

Despite the undesired effects of the hypokinetic profiles of CB agonists (some phytocannabinoids (Fernández-Ruiz J. The endocannabinoid system as a target for the treatment of motor dysfunction. Br J Pharmacol. 2009;156:1029−40), some have shown neuroprotective properties which would aid in halting the neurodegenerative aspect of the disease. Preclinical studies have indicated that cannabinoids may attenuate neurodegeneration in animal models with PD. This is believed to be attributed to the antioxidative action of THC and CBD responsible for the neuroprotection observed against 6-hydroxydopamine, an inducer of neurotoxicty in the animal models [46]. Further, THC exhibited neuroprotective effects toward human neuroblastoma cells exposed to several PD-relevant toxins, however, neuroprotection was not blocked by CB_1 receptor antagonists [47].

Although many cannabinoids demonstrate neuroprotective effects in several models of PD where effects appear to be mediated by a CB-receptor-dependent mechanism, the same is also true for CB-independent mechanisms. This includes antioxidant effects, reduced microglia activation, and modulation of glial−neuron interactions [45]. Phytocannabinoids are capable of reducing oxidative damage by acting as scavengers of reactive oxygen species (ROS) and by enhancing endogenous antioxidant defenses [46].

Observational and uncontrolled studies suggest that cannabinoids may improve motor symptoms associated with PD. A survey was conducted in the Czech Republic to investigate the use of Cannabis and its effects on PD so patients who suffered with PD were examined. Findings indicated that 25% of the respondents reported using cannabis and 46% of them noticed some benefits; 31% reported improvement of rest tremor; 45% reported improvement of bradykinesia; and 14% reported improvements of Levodopa-induced dyskinesia [48]. Improvements in rigidity, tremor, bradykinesia, and pain were also reported in another, small ($N = 22$) open-label trial that assessed motor symptoms 30 min after smoking cannabis [49].

17.5.4.2 Cancer

Numerous recent studies have linked associations between cannabinoids and cancer. Firstly, the role of cannabinoids or cannabis smoking to cancer initiation and/or development; secondly, the role of cannabinoids as potential anticancer therapies; and lastly, the role of cannabis and cannabinoids in the palliation of common cancer-associated symptoms [50].

One of the primary concerns associated with the medical use of cannabinoids, especially inhaled cannabis, is their carcinogenic potential. Most of the studies that have investigated a connection between marijuana smoking and cancer have been case-controlled in which patients with cancer were compared with persons without the disease. Noteworthy is that tobacco smoking was found to be an important confounder [51]. Although one case−control study showed a link between marijuana smoking and incidence of head and neck cancer [52]. For lung cancer, a case−control study found no connection with marijuana smoking (even for those smokers who used more than one marijuana cigarette per day for 30 years) after adjustment for confounders (tobacco smoking) [53].

A systematic review, concerning a correlation of lung cancer and cannabis smoking, evaluating 19 studies from 1966 to 2006 have not confirmed associations among cannabis smoking and lung cancer development despite clear evidence of precancerous histopathologic changes of respiratory mucosa [50,54]. One study found no increased risk of lung, colorectal, melanoma, or breast cancers in current and former smokers of marijuana versus never smokers or experimenters (very rare use of cannabis) [55]. This could be due to the in vitro effect of THC and other cannabinoids on cell metabolism, DNA synthesis, and cell division, events that halt cell division rather than lead to cancer [56].

Research has shown that THC and other phytocannabinoids are mutagenic in standard microbial assays though such findings warrant further investigations [57]. There are no published studies addressing oral marijuana ingestion and vaporized ingestion to cancer risk.

17.5.4.3 Anticancer Effects of Cannabinoids

Evidence suggests that THC, naturally occurring cannabinoids (e.g., CBD, CBN), synthetic cannabinoid agonists, as well as endocannabinoids exhibit antineoplastic effects in vitro against lung carcinoma, gliomas, lymphomas, skin carcinomas, uterine carcinoma, and neublastoma [58].

Other studies have demonstrated in vitro and in vivo tumor growth inhibition of glioblastoma multiforme, breast, prostate, thyroid, colon, skin, pancreatic, leukemia, and lymphoma models [59]. The antitumor effects of these phytochemicals was found to occur via the suppression of proliferative cell signaling pathways, the inhibition of angiogenesis and cell migration, the stimulation of programmed cell death (apoptosis), and/or induction of autophagy [50]. For example, in gliomas, the use of THC (natural agonist cannabinoids receptors) induced cell death by downregulating the P13K/Akt and MAPK-signaling pathways that induced apoptosis through the activation of pro-apoptotic Bcl-2-associated death promoter protein [60]. Colon cancer cells exposed to phytocannabinoids experienced tumor necrosis factor-α-mediated, ceramide-induced apoptosis in vivo and in vitro [61]. Apoptosis was induced through ceramide by THC (2 μM and 15 mg/kg/d) in pancreatic tumor cells (Panc 1 and MiaPaCa2) [62]. Additionally decreased expression of the vascular endothelial growth factor one the most important proangiogenic factors was observed in glioma and skin cancer models treated with CB2 receptor selective agonist [63]. Cannabinoid agonists also directly inhibited angiogenesis induced by basic fibroblast growth factor in vitro and in vivo in a CB1-dependent manner [64].

Interestingly, the anticancer activity of CBD is probably completely independent of cannabinoid receptor activation. In bladder, CBD induced apoptosis of cancer cells via the activation of the TRPV2 channel protein, whereas CBD induced apoptosis in breast cancer cells independent of both cannabinoid and vanillin receptors [65,66]. Cannabinoid receptors have been found in higher concentrations in tumor cells than in corresponding normal tissue with variations from cancer to cancer. A good example of this fact is that CB2 receptors are expressed in 91% of HER2-positive breast cancers, in 35−72% of HER2-negative breast cancers, and only in 5% of normal breast tissue [50,67]. In addition to cannabinoid agonists, inhibitors of endocannabinoid transport or degradation have been shown to inhibit tumor growth and progression in numerous types of cancers, enhancing the levels of endocannabinoids in the cells [68].

Also, cannabinoids can selectively cause inhibition in the growth of tumor cells while ideally not affecting healthy tissue. A good example is that glioma cells that were exposed to cannabinoids underwent apoptosis (ceramide-induced) while astrocytes were protected from oxidative stress by the same cannabinoids [50,69].

Although the use of cannabinoids-related drugs for medicinal purposes could be limited by concerns of their psychotropic effects, they have shown to exhibit a reasonable safety profile, especially in comparison to current chemotherapeutics which all have more or less serious toxic adverse effects.

Despite the numerous collected evidence on the therapeutic potential of cannabinoids and related drugs in several types of cancers, only a single pilot clinical study has been performed thus far. This phase I/II clinical trial was aimed at evaluating the safety profile of THC administration and its antitumor activity in a cohort of nine terminally ill patients affected by recurrent glioblastoma multiforme, an aggressive primary brain tumor with poor prognosis (6–12 months survival) and no efficacious treatment. THC decreased tumor cell proliferation, and also induced apoptosis; however, it had only a slight impact on the overall median survival of the cohort (24 weeks) [59,69].

17.5.4.4 Cannabinoids in Cancer Therapy as Palliative Agents

The cannabinoids are emerging as valuable adjunctive agents for optimizing the management of multiple symptoms of cancer and the treatment of therapy-related side effects. In fact, while much remains unknown about the pathophysiological mechanisms of the ECS, available data support a broad spectrum of palliative properties, including appetite stimulation, inhibition of nausea and emesis associated with chemotherapy or radiotherapy, pain relief, mood amelioration, and relief from insomnia [59,70].

In the United States, two medicinal cannabis products (approved by FDA) are available: Marinol, a synthetic form of THC, and Cesamet, a synthetic THC analog. Both are currently approved for chemotherapy-induced nausea and vomiting (CINV) in patients who have failed to respond adequately to conventional antiemetic compounds. Dronabinol (synthetic THC; Marinol) is also approved for the treatment of anorexia associated with AIDS. A third medicinal cannabis product, Sativex (a combination of THC and CBD isolated from *C. sativa* in ratio 1:1) is already approved and marketed in Canada as an adjunctive treatment for the symptomatic relief of neuropathic pain in multiple sclerosis [71].

One of the earliest recognized medical indication for cannabinoids was CINV. Approximately one-half of cancer patients will suffer from these side effects of cancer treatment. This may lead to discontinuation of therapy because of noncompliance. To address this problem, antiemetic drugs are routinely given before and after chemotherapy. There is evidence that cannabinoids act on CB1 receptors in the dorsal–vagal complex of the brainstem region controlling the vomiting reflex, and that endocannabinoids and their inactivating enzymes are present in the gastrointestinal tract and might have a physiological role in the control of emesis [59].

Dronabinol and prochlorperazine were tested alone and in combination in a randomized, double-blind, parallel-group, multicenter study. The results of the study showed that a combination was significantly more effective than was either single agent in controlling CINV [72]. Another experiment confirmed a significant increase in appetite and a decrease in nausea in most patients after treatment with dronabinol [73].

Using THC, synthetic cannabinoids, and smoking cannabis, numerous clinical trials showed that the antiemetic effectiveness of cannabinoids is almost same to that of conventional antiemetics, such as dopamine D2-receptor antagonists, 5-HT3 receptor antagonist, and NK1 receptor antagonists [70]. Currently, the efficacy of cannabinoids as first-line treatment is challenging because of the psychoactive aspects and risk for emergence of dependency and tolerance even though they have the potential to target chemotherapy or radiotherapy-induced nausea and vomiting. Therefore, it is considered as second-line treatment in intractable cases and also can be coadministered with other first-line pharmacotherapeutic agents (such as 5-HT3 receptor antagonist) for additive or synergistic effects [74].

Other serious challenges associated with cancer are anorexia and cachexia, the cancer anorexia–cachexia syndrome is an important risk factor for morbidity and mortality in people with cancer. Numerous studies confirmed that THC and other cannabinoids have a stimulatory effect on appetite and increase food intake in animals [75]. The orexigenic effect (appetite stimulation) occurs through the inhibition of leptin at the hypothalamic level [76], because endocannabinoids in the hypothalamus may tonically activate CB1 receptors to maintain food intake. Anecdotal information from cannabis smokers and numerous clinical trials support the appetite-stimulating properties of THC. In fact, the synthetic cannabinoid dronabinol is approved by the FDA for treatment of anorexia associated with weight loss in AIDS patients.

17.5.5 Adverse Effects

Many of the beneficial (for therapy of different diseases) effects of cannabinoids rely on CB_1 receptor-mediated mechanisms (sometimes CB_2 or receptor-independent mechanism too). The high expression of CB_1 receptors in the CNS, like cerebellum and hippocampus, means that therapeutic doses of phytocannabinoids are causing often unwanted effects. Volunteers intoxicated with Δ^9-THC exhibited 3D inversion illusion, which has similarities to a neuropsychological cognitive impairment in the regulation of perception seen in patients with schizophrenia [77]. Some of the more

common adverse effects of phytocannabinoid administration are sedation (result of CNS depression), perception disorders, motor function disorders (like ataxia, incoordination), deficit in short-term memory (cognition disorders), and psychosis [78–80].

In cases where cannabinoids have been used in clinical trials for nausea and vomiting caused by chemotherapy, the most common adverse effects were somnolence, dry mouth, ataxia, dizziness, and dysphoria [81]. Despite the presence of adverse effects from cannabinoids that are usually acceptable in comparison with those caused by other drugs.

Like other intoxicants, marijuana can impair driving skills and increase the risk of motor vehicle accidents as well as accidents caused by the use of dangerous equipment at the workplace (e.g., used during construction work) [82]. Some studies showed that women who used marijuana during pregnancy were more likely to have a still birth [83]. The frequent use marijuana during pregnancy has also been linked to adverse neurobehavioral effects in the offspring [84].

Long-term effects of the administration of cannabinoids include disorders of the respiratory system (bronchitis), cardiovascular system (tachycardia, postural hypotension, aggravation of heart disease), and reproductive system (decreased sperm counts) [37,80]. Marijuana smoking can cause injuries in the large airways and increase the symptoms of chronic bronchitis. However, these effects cease after discontinuing the use of marijuana and there is no clear evidence for connections between marijuana smoking and development of chronic obstructive pulmonary disease [51].

17.6 CONCLUSIONS

Hallucinogenic plants have been used by mankind for thousands of years. Different species with hallucinogenic properties were and still are an important part of culture and religion of primitive tribes as well as well-developed civilizations. Later, hallucinogens have become a part of popular culture and serve as illegal and often dangerous entertainment. Their extensive use as stimulants cause many social problems and an interest in the world of science, firstly because of their adverse effects. Numerous experiments have demonstrated that the active ingredients of hallucinogenic plants, like THC, have different activities and most of them can be used in therapies against major diseases of concern, such as cancer, PD, AD, and sclerosis multiplex. Their influence on the human organism and especially the CNS provides an avenue for further exploration towards the creation of new promising drugs.

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Chapter 18

Marine Metabolites: Oceans of Opportunity

W. Gallimore
University of the West Indies Mona, Kingston, Jamaica

Chapter Outline

Learning Objectives

- To gain an understanding of the importance of marine natural products chemistry in drug development
- To be able to map the process involved in drug development from marine natural products
- To gain an appreciation of the range of biological activities associated with compounds isolated from micro- and macroorganisms
- To identify the marine-derived drugs which are undergoing clinical evaluation

18.1 INTRODUCTION

Over 75% of the earth's surface is covered by vast expanses of ocean. Its inhabitants are diverse with 15 of the 34 phyla occurring exclusively in the oceans with only one phylum (Onychophora) being reported as present on land only [1]. The marine environment provides an array of structurally unique and diverse constituents produced by an equally diverse consortium of marine organisms living on our coral reefs and in benthic communities. The marine organisms are highly variable in species, color, and morphology and belong to several phyla including Porifera (sponges), Ascidiacea (sea squirts), and Octacorallia (soft corals). The metabolites of marine origin emanate from a variety of parts of the plants and animals and are thought to be produced as a form of chemical communication, defense, or to ward off potential predators [2–12] (Figs. 18.1–18.3).

Of the 34 animal phyla recorded, **15** are ONLY found in the ocean with only 1, Onychophora present on land only.

The pioneers in the field, Paul Scheuer of the University of Hawaii at Manoa and D. John Faulkner of *Scripps* Institution of Oceanography, University of California at *San Diego*, La Jolla, set the foundation with active research programs sourcing marine compounds from a wide range of species including algae, sponges, coelenterates, ascidians, and bryozoans. Bioprospecting efforts over the last 40 years have yielded over 20,000 compounds of marine origin with

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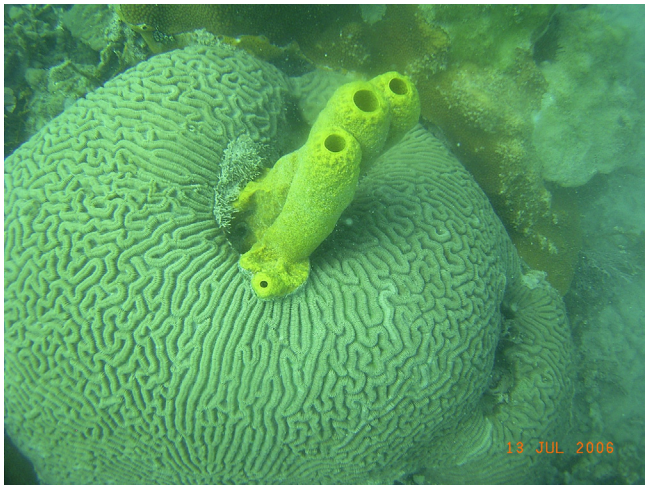


FIGURE 18.1 Tropical sponge growing on a brain coral, Port Royal, Jamaica.

FIGURE 18.2 Purple ascidian growing on soft coral, Drunken Man's Cay, Port Royal, Jamaica.

FIGURE 18.3 Diverse assemblage of marine species growing on a mangrove root.

the potential for a range of applications including anticancer, antibacterial, antiviral, antiinflammatory, antimalarial, antituberculosis activity, as well as pharmacological and industrial applications. The classes of compounds manufactured by marine organisms include alkaloids, terpenoids, shikimates, peptides, and polyketides [2–15].

18.2 COLLECTION, EXTRACTION, AND ISOLATION OF MARINE NATURAL PRODUCTS

The decision regarding which marine organisms to collect may be based on different considerations including the nature of the collection location and the novelty of the organisms being collected. It has been reported that specimens collected in different geographical locations have different secondary metabolite profiles and factors such as salinity, temperature, light intensity, pollution levels, as well as predation pressures, and the nature of bacterial symbionts may play a part in the occurrence and concentrations of specific secondary metabolites [16–18].

Organisms to be collected, depend on:
1. Collection environment (salinity, temperature, light intensity, pollution levels, predation pressures, nature of bacterial symbionts)
2. Novel organisms
3. Nonfouled species

Other specimen collections are made based on what may be observed with the organism. For example, if there is an organism which appears not to be fouled by other species which are competing for space in the marine habitat, it could be surmised that the organism may be producing secondary metabolites to ward off the competition, thereby preventing fouling. Such an organism may therefore be selected for investigation on the premise that ecologically bioactive compounds may also have therapeutic or industrial potential [19]. Organisms which were not previously investigated have a significant likelihood of yielding novel metabolites boasting unique biological activity (Fig. 18.4).

18.2.1 Collection

Marine organisms are collected by *snorkeling* or *wading* in water for specimens in shallow locations while scuba diving is required when procuring specimens in deeper waters (2–30 m). NITROX diving can facilitate the collection of specimens up to 80 m in depth (Figs. 18.5 and 18.6).

The use of the *remotely operated vehicle* (ROV) has revolutionized the study of the ocean and its organisms. The ROV can be deployed to depths unsafe or inaccessible to scuba divers. In fact, the photographing and collection of specimens including uncommon and new species at depths of up to 12,000 m has been facilitated by this technology. Consequently, many previously unexplored sites across the world's oceans are now being opened up to chemists, biologists, archeologists, as well as oil exploration teams. Sensors on the ROV are capable of retrieving data on the water

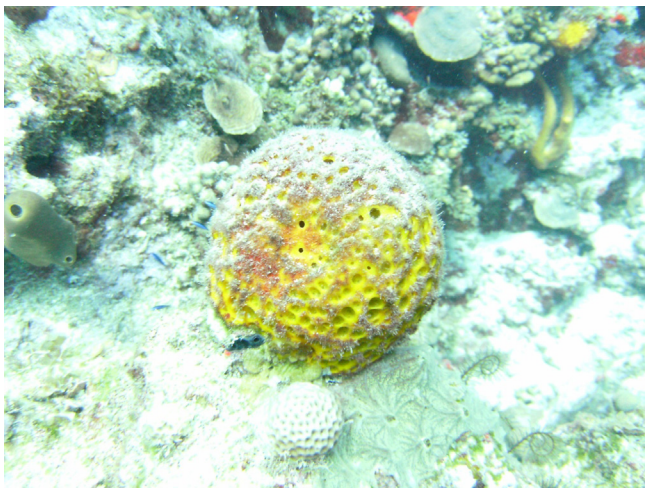


FIGURE 18.4 Orange ball sponge fouled by red algal species.

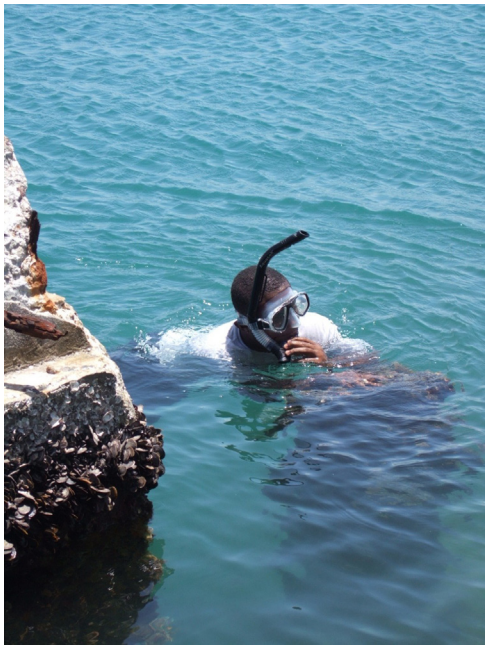


FIGURE 18.5 Student snorkeling to collect marine specimens.

FIGURE 18.6 Divers collecting sponge specimens.

temperature, salinity, pH, and dissolved oxygen concentrations in the water, thereby providing useful information to facilitate environmental studies [20,21].

Caution should always be exercised in the collection of marine species. Gloves should be worn in the collection and subsequent handling of specimens. Scuba divers should be clad in wet suits to protect against the possible deleterious effects of chemicals being exuded into the water by the organisms being collected. The personal unfortunate experience (author's) of hours of severe discomfort and rashes as a result of collecting the sponge *Neofibularia nolitangere* from a reef in Discovery Bay, Jamaica, provides clear evidence regarding the level of respect which should be accorded to

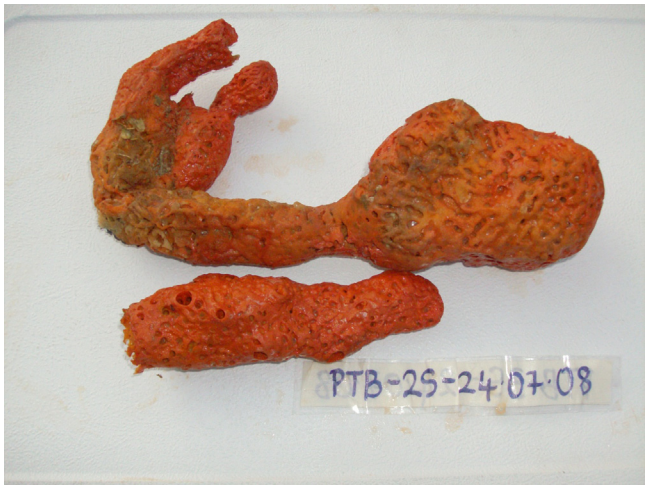


FIGURE 18.7 Coding of collected sponge specimen.

FIGURE 18.8 Boats are often used to access collection locations.

marine organisms whose chemistry is yet to be investigated. Records are made of the depth, habitat, global positioning system coordinates (latitude and longitude), color, morphology, and associated organisms. An appropriate coding system should be employed to distinguish specimens. Where possible, the specimens are photographed in situ as well as by the dockside (Figs. 18.7 and 18.8).

A voucher specimen of each organism is usually preserved in 70% aqueous ethanol for the purpose of taxonomic identification. Ascidians are usually preserved in seawater containing menthol crystals with more long-term storage in 10% formalin solution [22].

It should be noted that the recollection of organisms has proved to be a challenge in some instances. An ascidian species, e.g., found to be thriving on the mangrove in the summer of one year could all but disappear from the ecological landscape 6 or 12 months later, while a healthy bed of algae may be short-lived if there are dynamic factors involved in their growth. For example, the occasional nutrient runoff or groundwater seepage event could provide the ideal environment for the growth of selected algal species. Environmental factors are key in the marine landscape and often provide a source of frustration to the specimen collector.

18.2.2 Extraction

Prior to extraction of the collected organism, the specimens may be frozen, air-dried, freeze-dried, or could be retained in the fresh state. The majority of the marine organisms are extracted fresh or frozen while the remaining specimens are

lyophilized or dried in air before extraction [22]. In some instances, dried algal species are ground to a powder prior to extraction as described by Sansom and coworkers who isolated an antiproliferative bis-prenylated quinone from the alga *Perithalia capillaris* [23]. The extraction of marine organisms may be carried out using a range of organic solvents including hexanes, dichloromethane, acetone, ethyl acetate, as well as more polar solvents such as ethanol and methanol. In many instances, a mixture of polar and medium polarity or nonpolar solvents is utilized in the extraction protocol. For example, the extraction of the Madagascar sponge *Monanchora dianchora* was achieved in CH_3Cl:MeOH (1:1) to yield two polycyclic guanidine alkaloids [24]. Extractions are usually exhaustively performed over several days with at least three aliquots of the solvent being used. The solvent is then removed in vacuo by rotary evaporation. Solvent partitioning is another strategy employed in the extraction of the organisms. This involves single one-step or two-step partitioning systems usually involving an aqueous phase portioned with a solvent immiscible with that phase. The Kupchan and modified Kupchan procedures are often employed in natural products as was described in the isolation of a diterpene from an *Axinella* species [25]. In this procedure, the concentration of the aqueous layer is progressively adjusted to afford three or four different fractions. Complex partitioning procedures are also employed, albeit rarely so. Simple partitioning has been most commonly employed with Kupchan schemes being utilized with less frequency [22].

18.2.3 Isolation and Component Identification

Chromatographic methods of separation include gravity column chromatography, flash column chromatography, and vacuum liquid column chromatography utilizing silica gel as the packing material. With silica gel, the components of the marine extract are separated on the basis of polarity of the compounds. As the polarity of the eluting solvent increases compounds of increasing polarity are eluted from the column with hydrocarbons, e.g., eluting before alcohols. The elution of the components of a column is monitored by using thin layer chromatography (TLC) plates which are spotted to show the sequence of elution of the compounds (Figs. 18.9 and 18.10).

Bonded reverse phase silica is employed in instances where the constituents of the marine extract include polar metabolites. Bonded phases include ODS (C_{18}), C_8, cyano, and diol columns.

Separation of constituents may also be effected using gel permeation chromatography which effects separation of constituents on the basis of the size of the compounds. In this regard, Sephadex LH-20 is commonly utilized in marine natural products isolation work [26]. Resins such as BioBeads, Amberlite, XAD-2, and XAD-4 are also utilized in separating components of relatively high polarity. The use of XAD-2 in the separation of antiviral trisulfated triterpene glycosides from the sea cucumber *Staurocucumis liouvillei* is one such example in marine natural products isolation work [27].

Chromatographic methods
1. Gravity column chromatography
2. Flash column chromatography
3. Vacuum liquid column chromatography
4. Gel permeation chromatography
5. High-performance liquid chromatography (HPLC)
6. Medium pressure liquid chromatography (MPLC)
7. Recycling HPLC

The use of HPLC employing a reversed phase stationary phase system is commonplace in marine natural products isolation work with C_{18} and C_8 semipreparative and preparative columns being used. MPLC and recycling HPLC techniques are related techniques for purification of a range of metabolites including alkaloids, peptides, and terpenoids.

Tandem systems such as liquid chromatography-mass spectrometry systems are also employed to assist with dereplication efforts. Unusual MS peaks in the profile suggest that novel components are present in the fraction or extract being evaluated. Those fractions with unusual constituents may then become the focus of the research efforts. Solid-phase extraction methods are also employed in separating compounds.

The structural identification of compounds isolated from the range of marine sources is facilitated by the use of spectroscopic techniques such as 1D and 2D nuclear magnetic resonance (NMR) spectroscopy and infrared (IR) spectroscopy. X-ray crystallographic techniques are also important in aiding in the determination of the stereochemistry of the compound. The identification of nanogram quantities of a novel compound is becoming increasingly more facile with the use of the cryoprobe, capillary probe, and Mans probe [15].

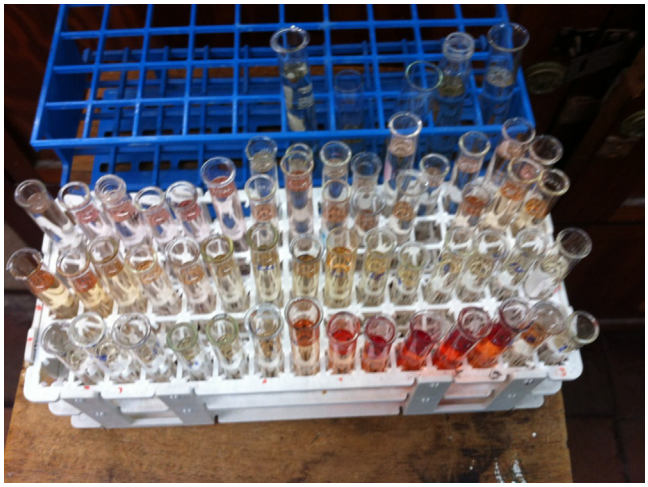


FIGURE 18.9 Test tube fractions from a silica gel chromatography column on a marine extract.

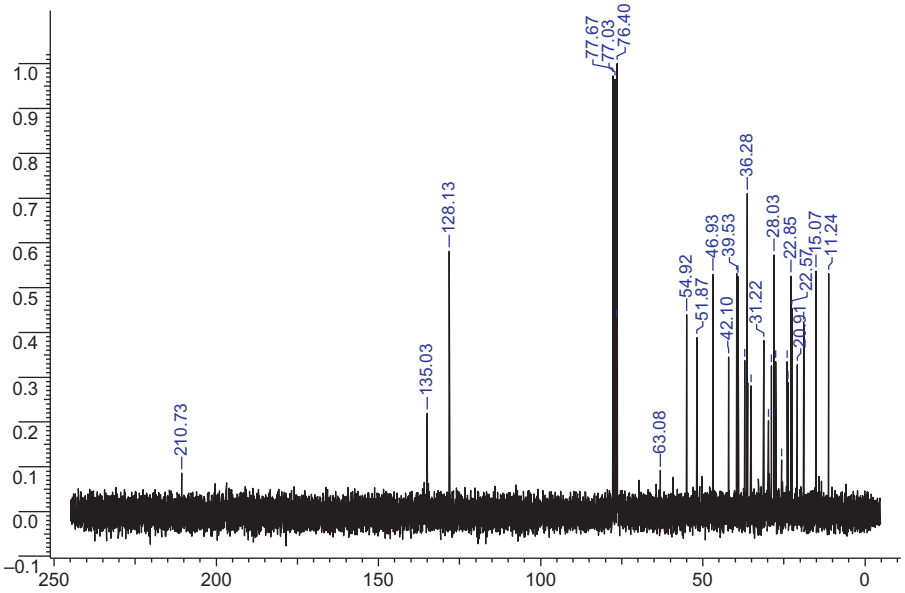


FIGURE 18.10 NMR spectrum of a compound isolated from a sponge.

18.3 IN VIVO AND IN VITRO BIOACTIVITY OF METABOLITES FROM MACROINVERTEBRATES, MACROALGAE, AND MICROORGANISMS

In vitro activities of marine metabolites have been investigated for a diverse range of cell systems including antiinflammatory, antimicrobial, and anticancer activities. Crude extracts, fractions from crude extracts, as well as pure compounds are typically evaluated for biological activity. The in vitro biological evaluation of the isolated compound may be performed using cell lines from human subjects or animals. Brine shrimp, fish, and sea urchin are among the organisms employed in the evaluation of compounds or extracts for ecological and therapeutic importance (Figs. 18.11 and 18.12). A summary of the biological activity of some of the organisms discussed in this section is presented in Table 18.1.

18.4 EVALUATION OF MARINE EXTRACTS

Preclinical trials are an essential component of the process of evaluation of the therapeutic potential of a compound. These trials often include animal models such as rats, dogs and monkeys. The major sources of biologically relevant

FIGURE 18.11 Student with sea urchin specimen.

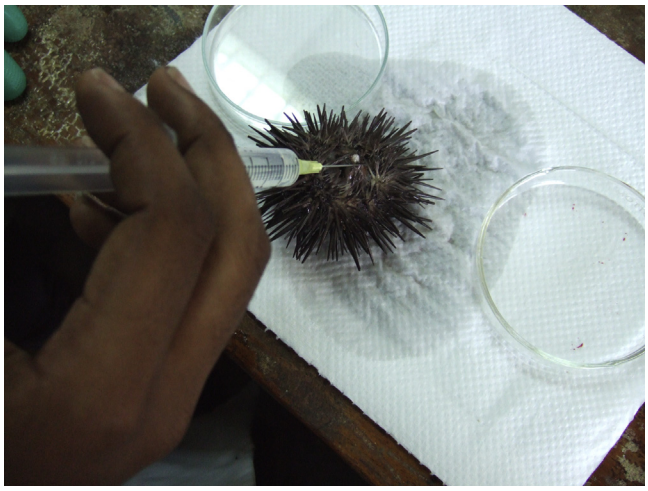


FIGURE 18.12 Injection of sea urchin with salt solution to obtain eggs and sperms for biological evaluation.

compounds have been found to be from sponges, coelenterates, algae, echinoderms, ascidians, molluscs and microorganisms [14].

18.4.1 Metabolites From Macroinvertebrates

Macroinvertebrates include sponges, ascidians, and soft coral. It has been found that the vast majority (75%) of novel compounds obtained from the marine environment have been sourced from the Porifera and Coelenterata (Cnidaria) phyla [15]. Scheme 18.1 shows representative structures of compounds isolated from macroinvertebrates.

Macroinvertebrates include:
1. Sponges
2. Ascidians
3. Soft coral

TABLE 18.1 Representative Macroinvertebrates, Macroalgae, and Microorganisms Investigated to Obtain Biologically Active Metabolites

Source	Species	Biologically Active Compound	Reported Biological Activity
Sponge	*Halichondria okadai*	Halichondrin B	Anticancer
	Agelas mauritianus	Agelaspin	Anticancer
	Agelas nakamurai	Agelasine D	Antimicrobial
	Discodermia dissoluta	Discodermolide	Anticancer
	Mycale sp.	Mycalamide A	*Herpes simplex* virus inhibitor
	Jaspis sp.	Jasplakinolide	Antimicrobial
	Monanchora arbuscula	Batzelladine alkaloids	Antibacterial
Soft coral	*Cespitularia taeniata*	Cespitulactam K	Anticancer, antimicrobial
	Clavularia inflata	Dolabellane diterpenes	Anticancer
Ascidian	*Trididemnum solidum*	Didemnin B	Antiviral
	Didemnum guttatum	Cyclodidemniserinol trisulfate	Antiviral
Macroalgae	*Dictyota* sp.	8α,11-dihydroxypachydictyol A	Antimalarial
	Stypopodium zonale	Zonaquinone acetate	Anticancer
	Laurencia undulatea	Polyphenols	Antiinflammatory
	Halimeda monile	Phenols	Antioxidant
Microorganisms	*Pseudoalteromonas*	3,3′, 5,5′-tetrabromo-2,2′-diphenyl diol	Antibacterial
	Corallospora pulchella	Melinacidins and gencidin	Antibacterial
	Marinospora sp.	Lynamicins A-E	Antibacterial

Sponges (Porifera) are sedentary, filter feeding metazoans which utilize a single layer of flagellated cells (choanocytes) to pump water current through their bodies in a unidirectional manner. There are over 5000 species of sponges accounting for much of the epifaunal biomass. Extracted fresh or freeze-dried, sponge extracts are an important source of biologically active compounds. These isolates exhibit an impressive array of biological activities, some of which are described here.

One sponge which has gained a place in history due to the promising biological activity being displayed is *Halichondria okadai*, the producer of halichondrin B, which underwent evaluation as an anticancer agent. Okadaic acid, also from *H. okadai*, exhibited inhibitory activity against phosphatase-1 and phosphatase-2A [28] (Fig. 18.13).

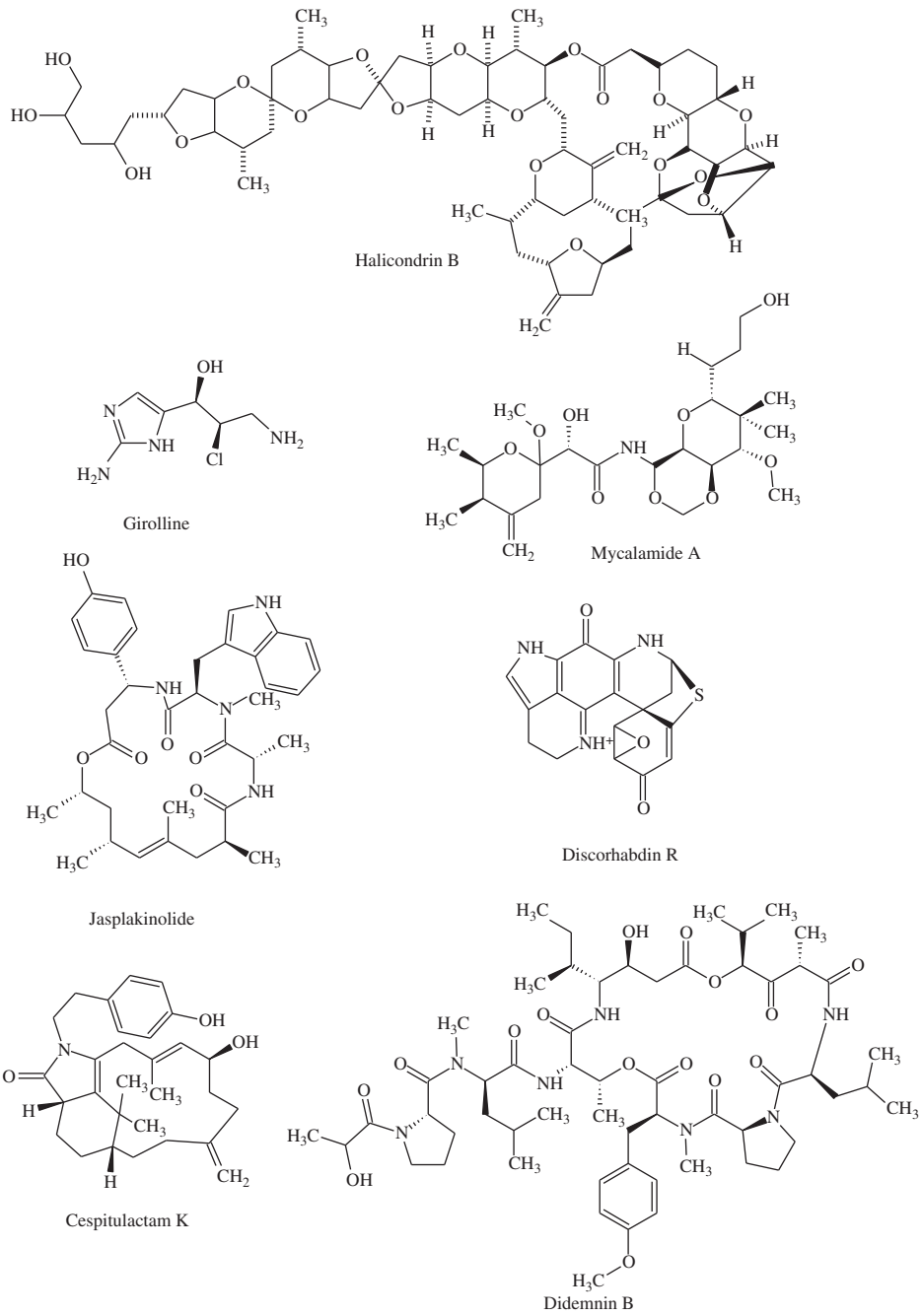
Agelaspin, an antitumor glycosphingolipid obtained from the marine sponge *Agelas mauritianus*, demonstrated antitumor activity in vivo against murine B16 melanoma. This compound was also found to stimulate the immune system. A derivative of agelaspin, KRN-7000, underwent clinical investigations for cancer immunotherapy [28]. More recently, the extracts of another *Agelas* sp., *A. nakamurai*, contained the compound agelasine D which exhibited high antibacterial activity [29].

The deep water sponge *Discodermia dissoluta* produced discodermolide, a polyhydroxylated lactone which exhibited anticancer activity, as well as immunosuppressive activity. It was found to stabilize microtubules in a manner similar to the drug taxol and underwent evaluation for use in tumors resistant to taxol [30,31]. *Dysidea arenaria* was found to contain arenastatin A which showed potent activity against KB cell lines ($IC_{50} = 5$ pg/mL) [32].

Girolline is a substituted imidazole isolated from the sponge *Pseudaxinyssa cantharella* which functions by inhibiting the termination step in eukaryotic protein synthesis. Having entered Phase 1 clinical trials, it was withdrawn due to its adverse hypertensive effects seen in treated patients [28].

Mycalamides A and B are protein synthesis inhibitors isolated from the New Zealand sponge *Mycale* sp. In vivo activity against A59 coronavirus was observed in mice when treated with a 2% mycalamide mixture at a dosage of 0.2 μg/kg daily with 100% survival over a two-week period. Pure mycalamide A inhibited the *Herpes simplex* virus 1 and Polio virus type 1 at a concentration of 0.005 μg/disk. Mycalamide B was found to exhibit more potent antiviral activity and cytotoxicity than mycalamide A [28]. The baculiferins I, J, L, and M from the marine sponge *Iotrochota baculifera* have been found to inhibit human immunodeficiency virus-1 (HIV-1) with IC_{50} values between 0.2 and 7.0 μm [33].

Jasplakinolide, the first example of a cyclodepsipeptide isolated from a sponge, is a 19-membered macrocyclic depsipeptide from the *Jaspis* sp. exhibiting in vitro antimicrobial activity at a minimum inhibitory concentration of



SCHEME 18.1 Representative bioactive compounds isolated from macroinvertebrates.

25 μg/mL against *Candida albicans*. With a topical administration of 2% jasplakinolide solution, an effect similar to that of miconazole nitrate was achieved in vivo [28].

Discorhabdin R is a novel pyrroloiminoquinone isolated from the southern Australian sponge *Negombata* sp. and Antarctic *Latruncula* sp. which was found to display antibacterial activity against both Gram-positive (*Staphylococcus aureus* and *Micrococcus luteus*) and Gram-negative bacteria (*Serratia marcescens* and *Escherichia coli*), respectively [34].

Antibacterial activity against a strain of the bacterial parasite *Plasmodium falciparum* was reportedly identified in *Monanchora arbuscula* with the active agents being the batzellidine alkaloids ($IC_{50} = 0.2-0.9$ μm) [35].

An important isolate from a *Spongia* sp. is the polyhydroxylated steroid, agosterol A, which functions by reversing multidrug resistance caused by the overexpression of two kinds of membrane glycoprotein in cancer cells [36].

From the phylum Cnidaria the genera *Sinularia* and *Briareum* have proven to be prolific sources of novel compounds. Cembranoids, 5,8-epidoxysteroids, sinulaflexiolides, and africanenes have been isolated from *Sinularia* species [1] (Fig. 18.14).

Examples of other species of soft corals include the Taiwanese soft coral *Cespitularia taeniata* which was extracted with ethanol to yield a group of verticillene diterpenoids including cespitulactam K. The compounds were evaluated against human epidermal carcinoma and murine L1210 leukemia cell lines. Cespitulactam K exhibited activity against the cancer cell lines (3.7−5.1 μg/mL) and also showed marked antimicrobial activity against *M. luteus* and *Cryptococcus neoformans* [37].

The methanol extract of the octocoral *Muricea austera* showed in vitro activity against chloroquine-resistant *P. falciparum* and was found to contain a range of different classes of compounds including tyramine derivatives, steroidal pregnane glycosides, and sesquiterpenoids [38].

Cytotoxic dolabellane diterpenes were isolated from the Formosan soft coral *Clavularia inflata* var *luzoniana* and bioactivity against P388 cell lines with ED_{50} values between 0.5 and 3.6 μg/mL was observed [39].

Tunicates, sea squirts, or ascidians belong to the subphylum of Tunicata (Urochordata). They are so named because of their cellulose-containing protective tunic surrounding the organism. Tunicates attach to a substratum, usually a marine solid surface such as a mangrove root, rocks, jetties, or even algal species (Fig. 18.15).

FIGURE 18.13 Color morphs of a mangrove sponge.

FIGURE 18.14 Dried specimen of a soft coral (Phylum Cnidaria).

FIGURE 18.15 *Ascidia curvata* growing on a mangrove root, Port Royal, Jamaica.

Much like sponges and soft corals, ascidians have also been found to be a good source of bioactive agents. Didemnin B, isolated from the tunicate *Tridemnum solidum*, is one such bioactive compound, showing remarkable antiviral and cytotoxic activity. Didemnin B demonstrated activity against P388 and L1210 murine leukemia cell lines. It was advanced into preclinical and clinical trials 1 and 2, but had to be withdrawn due to its harsh toxicity [30]. Aplidine, formally known as dehydrodidemnin, an isolate from the Mediterranean tunicate *Aplidium albicans*, is one such bioactive compound. Being structurally related to didemnin B, aplidine was found to be up to 10× more active and less toxic than didemnin B. It entered into Phase 1 clinical trials in 1999 under investigation for the treatment of solid tumors and non-Hodgkin's lymphoma. Broad spectrum activity was displayed in vitro and in vivo against leukemia, melanoma, breast, ovarian, colon, and lung (nonsmall cell) cancer. Having advanced to Phase 2 clinical trials, aplidine affects protein synthesis through GTP-dependent inhibition of elongation factor 1-α [30].

The extract of the Palauan ascidian *Didemnum guttatum* afforded the sulfonated serinolipid cyclodidemniserinol trisulfate which exhibits an antiviral effect by inhibiting HIV-1 integrase, an attractive target for antiretroviral chemotherapy [30].

18.4.2 Metabolites From Macroalgae

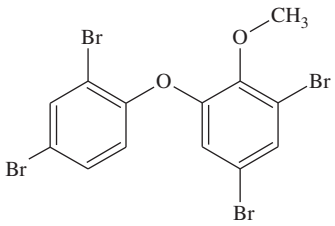
Macroalgae belong to three main phyla: Rhodophyta (red algae), Chlorophyta (green algae), and Phaeophyta (brown algae). Biological activities identified in extracts and metabolites of algal origin include anticancer, antiobesity, neuroprotective, and antioxidant activity and Scheme 18.2 shows chemical structures of representative bioactive compounds isolated from the macroalgae. A wide range of algal species are utilized in fresh or dried forms as food particularly in Asian countries where folklore traditions govern their industrial and medicinal usage [40].

Macroalgae are the source of agar, carrageenan, and alginate, which are all of importance in the food industry. The range of compounds isolated from algal sources has been variable. Representative examples of bioactive constituents from macroalgae are mentioned below.

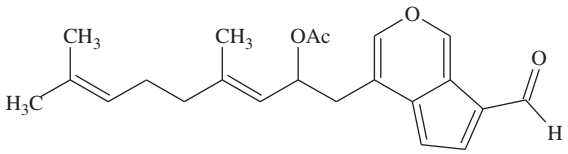
Cytotoxic activity has been identified in 8α,11-dihydroxypachydictyol A, a diterpenoid compound from a *Dictyota* sp. collected on Bangsaen Beach in Thailand. Antimalarial activity was also found in the diterpene isolated from this extract when the compound was tested with malarial parasites [41].

Stypolactone, an isolate from the brown alga *Stypopodium zonale*, was found to exhibit weak cytotoxic activity in vitro when evaluated with A-549 and H-116 cell lines [42]. Zonaquinone acetate, obtained from Jamaican populations of *S. zonale*, displayed in vitro activity against breast and colon cancer cell lines [43]. Specimens of *Taonia atomaria* produced atomarianones A and B which were reportedly found to be cytotoxic against NSCLC-N6 and A-549 cell lines [44] (Fig. 18.16).

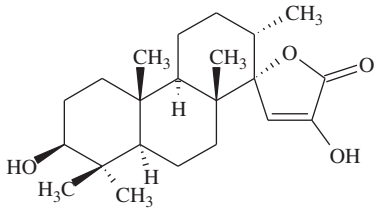
Crude extracts of algal species have been found to exhibit a range of biological activities. For example, aqueous extracts of *Gracilaria corticata* and *Sargassum oligocystum* exhibit bioactivity against cancerous human leukemia cells [45,46] while a methanol extract of *Plocamium telfairiae* was observed to display bioactivity against HT-29 colon cancer cells [47].



2- (2, 4'-dibomophenoxy) -4, 6-dibromoanisol



Halitunal



Stypolactone

SCHEME 18.2 Representative bioactive compounds isolated from macroalgae.

FIGURE 18.16 Specimen of the brown alga *Stypopodium zonale*.

Antiinflammatory activity was found in the green alga from which 2-(2,4′-dibromophenoxy)-4,6-dibromoanisol was isolated. This activity was identified using a snake toxin-induced mouse limb model [48].

Also exhibiting antiinflammatory activity is a mixture of phytosterols obtained from *Dunaliella tertiolecta*. When administered in a sheep model of inflammation-induced cytokine production, an inhibitory effect was observed [49].

Polyphenolic extracts from the red alga *Laurencia undulatea* displayed antiinflammatory activity in vivo. These extracts served to inhibit asthmatic reactions in mice sensitized and challenged with ovalbumin which was used to induce murine allergic reactions in test subjects [50].

Antiinflamatory agents floridoside and D-isofloridoside from the South Korean alga *L. undulatea* were found to inhibit free radical oxidative stress at IC_{50} values between 22 and 43 μm [51].

Biologically active compounds have been isolated from the brown seaweed *Dictyota cervicornis* from which was obtained sulfated polysaccharides with powerful anticoagulant activity [52].

Antioxidant activity, evaluated using the DPPH method, was reported in phenolic isolates of *Halimeda monile* when liver injury was induced in a rat model. The phenolic fraction was administered over a 20-day period and led to protective effects against chemicals harmful to the liver [53].

With IC_{50} values between 0.5 and 2.9 μm, potent antimalarial activity against the human malarial parasite *P. falciparum* was identified in new macrolides bromophycolides J, M, N, O, P, and Q from the red algae *Callophycus serratus* [54] collected in Fiji.

The marine alga *Halimeda tuna* was studied by Koehn and coworkers, leading to the isolation of halitunal, a diterpene displaying in vitro antiviral activity against murine coronavirus A59 [55].

Ecologically important roles are played by some compounds from alga sources. For example, halimedatrial, a diterpene isolated from *Halimeda lamouroux*, exhibited toxicity toward reef fishes and appeared to be a feeding deterrent. Antimicrobial activity was also reported from this compound [56].

18.4.3 Metabolites From Microorganisms

Almost 20% of all bioactive marine compounds currently being studied are obtained from marine microorganisms [15]. These microbes are found in swabs from the surfaces of marine plants and animals, suspended in the water from geothermal vents and deep water environments, or on sediment surfaces. They thrive in a variety of environments including locales characterized by high pressures of up to 600 atmospheres, high temperatures, and high salinities. Efforts at culturing some of the microorganisms have met with varying degrees of success. The ability to propagate these microorganisms in an economically feasible way will be of great significance as potent bioactive metabolites are discovered [57] (Figs. 18.17–18.19).

Marine microorganisms are found
1. On the surface of marine plants and animals
2. Suspended in water
3. On sediment surfaces

Historically, terrestrial microbes have been a potent source of pharmaceutical agents with the seminal discovery of penicillin. The discovery of new antibacterial agents is a serious priority because of the development of potent resistance to current antibiotics on the market.

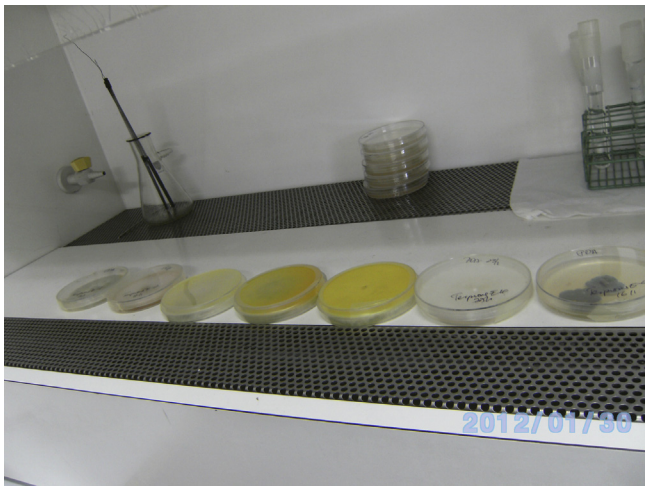


FIGURE 18.17 Plates of fungi isolated from a sponge species.

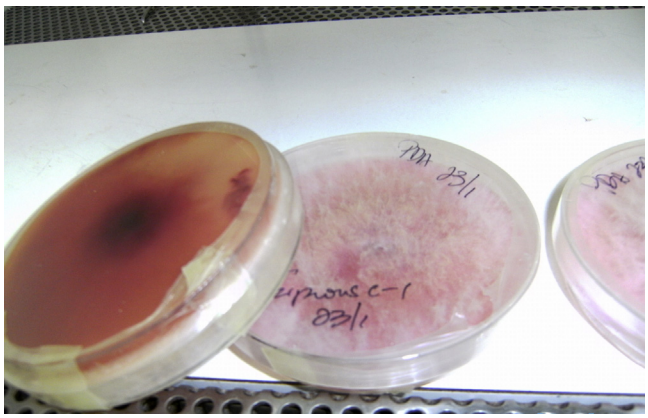


FIGURE 18.18 Pink unidentified fungus isolated from a mangrove sponge.

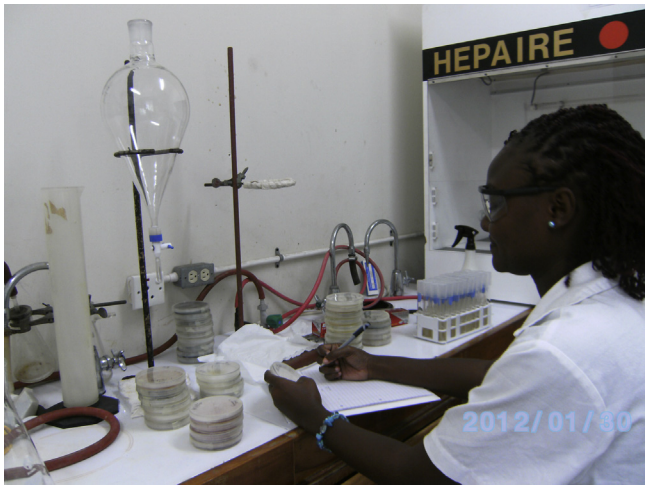
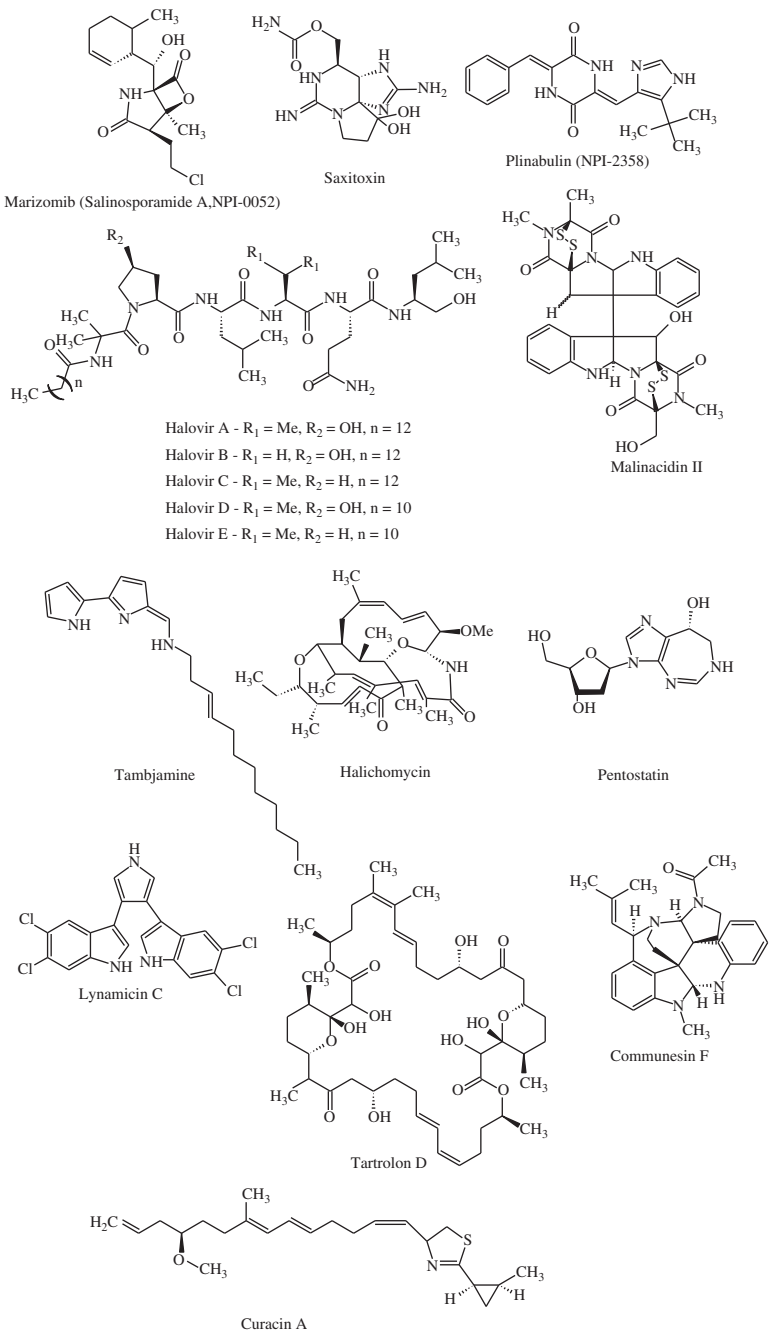


FIGURE 18.19 Student examining fungal isolates from a sponge.

Marine bacteria produce a wide variety of secondary metabolites for the purpose of defending themselves against other microbes. Scheme 18.3 shows structures of representative compounds from microorganisms associated with marine specimens. Marine bacteria which produce compounds of biological significance include *Pseudoalteromonas* species which was found to produce 3,3′, 5,5′-tetrabromo-2,2′-diphenyl diol, an inhibitor of methicillin-resistant *S. aureus*. The class of 4-methoxypyrrole-containing compounds, the tambjamines, isolated from *P. tunicata*, was found to be active antifungal, immunosuppressive, and antimicrobial agents. Biologically active compounds from marine bacteria also include *Streptomyces* species from sediment and fish gut from which anticancer (e.g., halichomycin and δ-indomycinone) and antibacterial agents (e.g., phenazines) have been obtained [58–60]. *Vibrio* species obtained from sponge specimens have produced phenolic and trisindole compounds with antibacterial activity [61,62]. A *Micromonospora* sp. obtained from a soft coral produced thiocoraline, a compound exhibiting anticancer activity [63].

Marine fungi have also been known to produce compounds with a range of bioactivities including antiviral, antifungal, enzyme inhibition, and anticancer and antibacterial activities. The isolation and cultivation of fungi from the marine environment is of critical importance for propagation of the microbes from which biologically relevant compounds may be obtained. Protocols have been established for this work [64]. Fungal species which have produced antibacterial compounds include *Corallospora pulchella* isolated from sand. This species produced melinacidins and gencidin [65].

Anticancer activity has been reported from metabolites of *Aspergillus* sp. (including the aspergillamides and fumiquinazolines) and *Penicillium* sp. sourced from a marine alga which was found to contain pentostatins and communesins among other compounds [66,67]. Antiviral activity, attributable to the presence of halovirs, was identified in a *Scytalidium* sp. collected from a seagrass species. Potent antiviral activity against *H. simplex* virus (type 1) was observed and may be acting by binding directly to the virus [68].



SCHEME 18.3 Representative bioactive compounds isolated from microorganisms.

Actinomycetes have been the source of a wide range of antimicrobial agents, the most common of which include tetracycline and streptomycin. Other bioactive compounds originating from actinomycetes include antitumor and antimicrobial agents. A *Marinospora* sp. produced a group of bisindole pyrroles, lynamicins A−E, which exhibited biological activity against Gram-positive and Gram-negative species. Importantly, activity was also shown against drug-resistant pathogens including methicillin-resistant *S. aureus* [69].

Anticancer activity against lung, colon, and breast cancer cell lines was exhibited by isolates from the fermentation of a *Streptomyces* sp. (MBG-04-17-069). Tartrolon D was found to be the bioactive agent [70].

Microalgae are found in seven phyla. These include Chlorophyta, Phaeophyta, Rhodophyta, Crystophyta, Cryptophyta, Eugelophyta, and Pyrrhophyta. The blue-green algae, Cyanophyta, are cyanobacteria which have been

found to share characteristics with eukaryotic algae. These microalgae produce compounds with a high degree of structural diversity and species, such as *Lyngbya majuscula*, have produced a vast array of biologically active compounds [71]. Curacin A, e.g., isolated by Gerwick and coworkers in 1994 [72], was found to function by disturbing microtubule assembly, thereby functioning as a lead compound in chemotherapy. *Microcystis aeruginosa* is the source of potent protein phosphatase-1 and phosphatase-2A inhibitors identified in microcystins [73].

Other microalgal species under examination include dinoflagellates which produce an array of bioactive toxins including saxitoxin and maitotoxin which function by blocking or activating sodium/calcium channels. Challenges exist with respect to the culturing of these organisms due to relatively low proliferation rates and the large quantities of culture required to obtain small amounts of bioactive compounds. Diatoms, microscopic unicellular colonial algae, grow at a faster rate and are amenable to culturing but few bioactive metabolites have been identified from these microalgae [28].

Some marine compounds sourced from microbes are of clinical significance, undergoing evaluation as potential pharmaceutical agents.

18.5 DRUGS IN CLINICAL TRIALS

The marine-derived drug pipeline, almost nonexistent in decades gone by, now has a range of candidates at various stages of development as shown in Table 18.2. Representative structures of marine-derived compounds in clinical trials are shown in Scheme 18.4.

Drugs in Phase three clinical trials include tetrodotoxin, a guanidinium alkaloid under the trademark name Tectin obtained from the Pufferfish [34]. Affecting the sodium channels, this drug is being investigated for the treatment of chronic pains (Scheme 18.5).

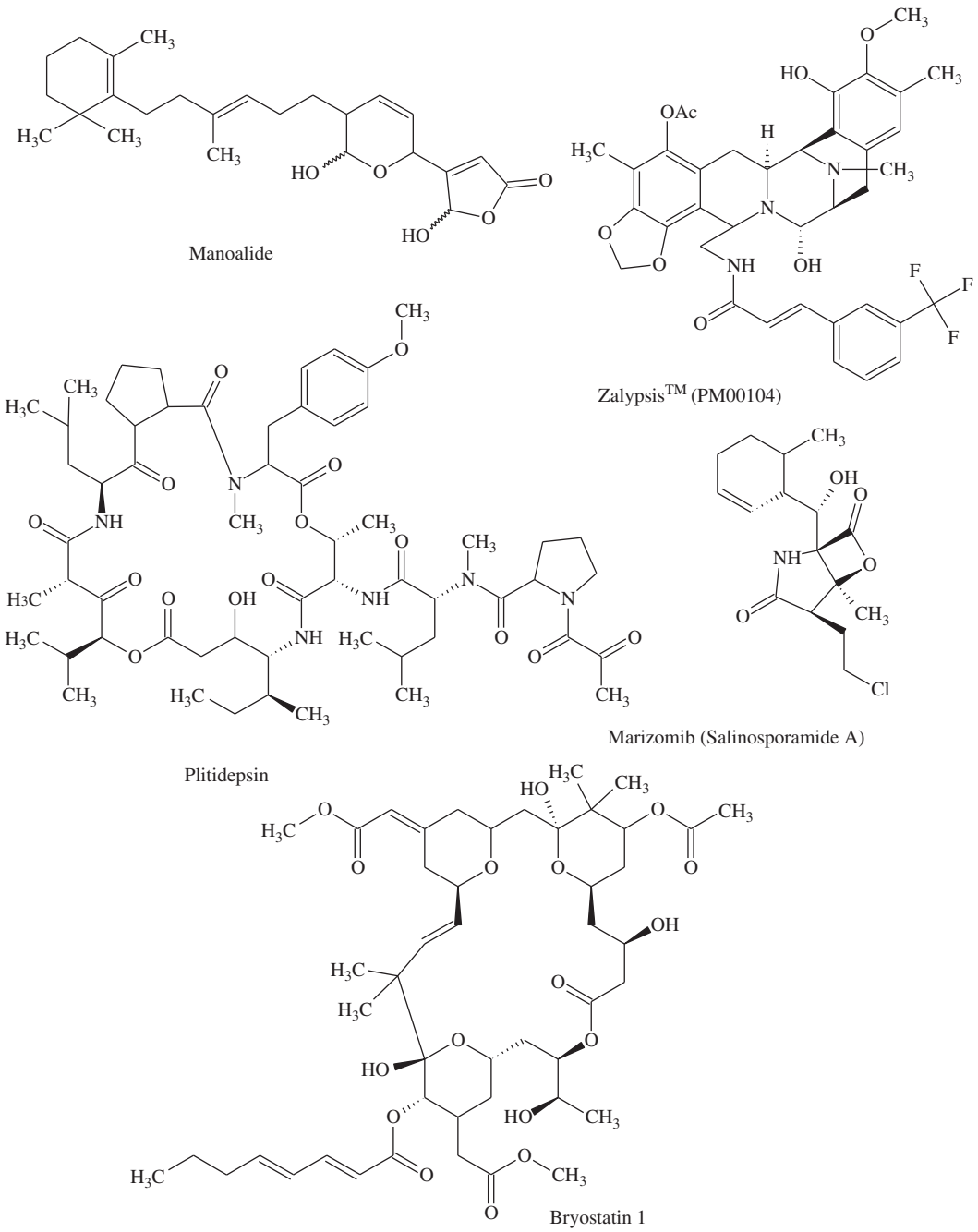
A depsipeptide from a tunicate, plitidepsin, is being tested by Pharmamar in the treatment of a variety of cancers, namely leukemia, multiple myeloma, and lymphoma. Another drug under evaluation by Pharmamar for cytotoxic activity is Zalypsis (PM00104) sourced from a mollusc which targets the DNA-binding capacity of diseased uterine, lymphoma, cervical, and endometrial cancer cells. The alkaloid-derived compound PM01183 is another drug candidate from Pharmamar being evaluated for its efficacy against a range of cancers including ovarian, breast, lung, acute leukemia, and endometrial cancer [74,75].

Bryostatin I, from the bryozoan *Bugula neritina* has been involved in a battery of clinical trials being investigated for its potency against cancer. It is currently under phase I evaluation as a treatment for Alzheimer's [74]. In the early years, the challenge associated with the supply of the drug was underscored by the fact that, in order to obtain 18 g of a cGMP quality bryostatin I, 13 tonnes of *B. neritina* had to be collected in Californian waters [13,76]. The gene cluster of the uncultivated microbial symbiont of *B. neritina, Candidatus endobugula sertula* has been successfully identified, thereby opening the potential for the supply of the compounds [77].

Kahalalide F, a cyclic depsipeptide, was found in the mollusc *Elysia rufescens* as well as the green algae *Bryopsis* sp. on which it feeds. This compound is currently in Phase I/II trials as a treatment against prostate cancer [74] (Fig. 18.20).

TABLE 18.2 Representative Marine-Derived Compounds in Clinical Trials

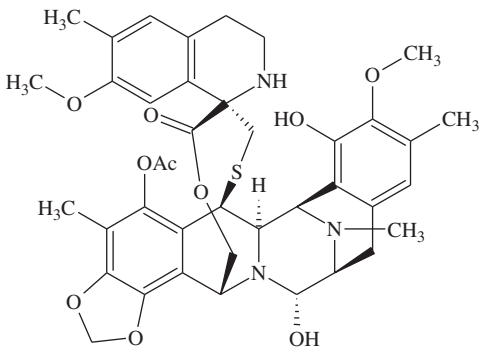
Trial Stage	Marine Source	Compound	Molecular Target	Main Disease Target
Phase III	Ascidian	Plitidepsin	JNK/Rac1 activation	Cancer (lymphoma/leukemia/multiple myeloma)
	Pufferfish	Tetrodotoxin (Tectin)	Sodium channels	Chronic pain
	Mollusc	PM00104 (Zalypsis)	DNA-binding	Cancer (uterine/cervical/endometrial)
	Worm (Annelid?)	DMXBA (GTS-21)	α7 Nicotinic acetylcholine receptor	Alzheimer's disease, schizophrenia, attention deficit hyperactivity disorder (ADHD)
	Ascidian	PM01183	DNA	Cancer (ovarian/breast/lung/endometrial)
Phase I	Bacterium	Marizomib (Salinosporamide A)	20S Proteasome	Cancer (nonsmall cell lung/pancreatic/melanoma/multiple myeloma)
	Bryozoan	Bryostatin	Protein kinase C	Cancer (prostate/pancreatic/kidney/lung/fallopian tube)



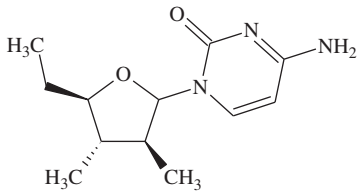
SCHEME 18.4 Representative marine-derived compounds in clinical trials.

DMXBA [(3-(2,4-dimethylxybenzylidene)]-anabaseine is a derivative of anabeseine, an alkaloid found in marine worms. Found to improve cognition in animal models, DMXBA and other related compounds have demonstrated neuroprotective activity in both in vitro and in vivo screens. Thought to have an effect on macrophage 7 receptors, antiinflamatory activity was also observed in animal models. Phase I evaluation of healthy males and schizophrenics have shown that DMXBA has led to marked improvements in cognitive function [74].

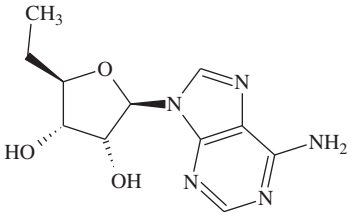
There are several marine compounds sourced from microbes which are of clinical significance. Clinical trials are being conducted on Plinabulin (NPI-2358), a vascular disrupting agent obtained from a marine fungal extract with potential for activity against multidrug resistant tumor cells. Marizomib (Salinosporamide A, NPI-0052), an isolate from a marine bacterium *Salinospora tropica*, is a novel proteasome inhibitor which is currently under investigation for



Trabectidin (ET-743)



Cytarabine (Cytosar-U)



Vidarabine (Vira A)

SCHEME 18.5 Representative marine-derived pharmaceuticals.

FIGURE 18.20 Collection site in Hawaii for the mollusc *Elysia rufescens*.

its efficacy against solid tumor models. The compound exhibits low cytotoxicity to normal cells and has significant potential for oral and intravenous administration [74].

18.6 DRUGS OF MARINE ORIGIN TO TREAT DISEASES

The ultimate goal of many marine natural products and synthetic chemists is that the isolated or synthesized molecule possesses therapeutic applications.

There are several Food and Drug Association (FDA)-approved drugs of marine origin obtained from sponges, a fish, a cone snail, a mollusc, and cyanobacterium species, while Yondelis (Trabectidin) obtained from the ascidian

Ecteinascidia turbinata, has been approved in the European Union. The antitumor effects of aqueous ethanol extracts of *E. turbinata* were observed from 1969. In vitro trials had been carried out on a 60 human cancer cell panel by the company developing the drug, Pharmamar, and the National Cancer Institute. Aquaculture of the ascidian proved to be the initial strategy used to obtain sufficient quantities for evaluation of the efficacy of the compound. Semisynthetic procedures involving the fermentation of *Pseudomonas florescens* are now currently employed in the pharmaceutical preparation of the drug which is sold in over 80 countries, including South Korea and Russia, under the trade name Yondelis. Yondelis is also used in patients with relapsed platinum-sensitive ovarian cancer. This drug is currently under evaluation in phase II for breast, prostate, lung, and pediatric cancers.

The sponge *Tethya crypta (Cryptotethia crypta)* was the original source from which the drug Cytarabine was developed. Cytarabine is a synthetic analogue of the nucleoside which was originally isolated from the sponge. Sold under the trade name Cytosar-U, this cytotoxic agent inhibits deoxyribonucleic acid (DNA) polymerase and DNA synthesis. Acute lymphocytic leukemia, non-Hodgkin's lymphoma, and acute myelocytic leukemia are among the conditions being treated by this drug approved by the FDA in 1969 [74].

Produced by fermentation of *Streptomyces griseus*, cytarabine has limited bioavailability but improvements in the delivery system have been made [78]. A slow-release liposomal form of cytarabine (Depo Cyle) has been approved in the United States and Europe for the prolonged administration/exposure in cerebrospinal fluid.

A related drug, Vidarabine (Vira-A), was developed from spongouridine and found use as an antiviral treatment for epithelial and superficial keratitis caused by the *H. simplex* virus types 1 and 2. Viral DNA polymerase and DNA synthesis of herpes are inhibited by this drug which was discontinued over 10 years ago. This drug is still in use in Europe for ophthalmological challenges.

Prialt (Ziconotide) was obtained from a peptide ω-conotoxin MVIIA isolated from the cone snail *Conus magus.* With a unique mode of action, this drug acts by reversibly blocking N-type calcium channels in some specific nerves in superficial layers of the spinal cord. This drug is used for the management of severe and chronic pains in patients suffering from cancer and Acquired immunodeficiency syndrome who are unable to use or are unresponsive to other drugs such as morphine.

Ziconotide had to be synthesized using solid-phase peptide synthesis due to the insufficient quantities supplied by the cone snail, *C. magus* [79]. The blockage of the spinal cord induced by this drug prevents the release of neurotransmitters responsible for pain from specific neurons. Related *Conus* peptides are undergoing evaluation in human clinical trials [80].

Brentuximab vedotin (SGN-35) is being marketed under the trade name Adcetris by Seattle Genetics and has gained repute for the treatment of Hodgkin and systemic anaplastic large cell lymphoma [81]. This drug is an analogue of dolastatin 10, a compound isolated from the sea hare *Dolobella auricularia*, which was later found to be produced by diet-associated cyanobacteria *Symploca hydnoides* and *L. majuscula.*

Preliminary phase I and II clinical trials of dolastatin 10 and a related analogue were largely unsuccessful. Antibody-drug conjugates function by selectively delivering the drug to the cancer cell by linking the dolastatin 10, e.g., to an antibody that targets a cell membrane protein on the surface of Hodgkin's lymphoma cells. This technology has proven to be a seminal development.

Omega-3 fatty acids from fish oils are being marketed under the trade name Lovaza by GlaxoSmithKline. Used in the treatment of hypotriglyceridemia, the drug controls ethyl esters of eicosapentaenoic acid and docosahexaenoic acid and functions by lowering triglyceride levels. [81].

Eribulin mesylate (E 7389), with the trade name Halaven was formulated from the macrolide halichondrin B sourced from the sponge *H. okadai.* Studies related to the anticancer activity of simpler analogues of halichondrin B showed that the efficiency is retained leading to the development of eribulin mesylate which is more water soluble than the parent macrolide. Now approved for use, potent and irreversible inhibition in cancer cells medicated by this drug resulted in the death of the cells by apoptosis. In the absence of tubulin, cell growth grinds to a halt. Related compounds are currently being evaluated in Phase II trials [81].

One of the more recent formulations on the market is Carragelose, an antiviral nasal spray which functions by creating a physical antiviral barrier in the nasal cavity. The company Marinomed Biotechnologie GmbH, utilized iota-carrageenan, sulfated polysaccharides found in the Rhodophyceae seaweed as well as other seaweeds. The product is effective against the early symptoms of the common cold [81].

It should be noted that, in addition to the pharmaceutical applications of marine-sourced therapies, a range of cosmetic applications also exist and are thriving industries. The foray into cosmetic applications was led by Estee Lauder with the antiaging skin care remedy Resilience which contains an extract from the Caribbean Sea whip *Pseudopterogorgia elisabethae*. The active antiinflammatory and analgesic agents are the pseudopterosins, tricyclic

diterpene glycosides, which have been found to inhibit PLA2 and 5-lipoxygenase. Derivatives of the pseudopterosins underwent phase I and II trials to examine wound healing efficiency but the lipophilic and insoluble nature of the compounds have served to limit its potential as an effective drug. Compounds from this group of tricyclic diterpene glycosides also underwent preclinical evaluation as antiinflammatory drugs [81].

Abyssine is marketed as a product used to soothe and reduce irritation in skin sensitive to ultraviolet B light as well as chemical and mechanical attack. It consists of an extract from an *Alteromonas* species and contains a high molecular weight polymer with two different oligosaccharides (exopolysaccharide), while Seacode represents another exopolysaccharide which occurs as a mixture of extracellular glycoproteins and other glucidic exopolymers produced by fermentation of a *Pseudoalteromonas* sp. This product has been found to improve skin roughness after up to four weeks of administration.

RefirMAR, a recent product to be introduced, was obtained from an intracellular extract from a fermentation of a new *Pseudoalteromonas* sp. Isolated from a deep (2300 m) hydrothermal vent in Portugal's Exclusive Economic Zone, extraction of the cultured biomass afforded a mixture of macromolecules which inhibit muscle contraction. The hydrating and antiaging potential of the product has been evaluated in vivo and in topically applied formulations [81].

18.7 DISCUSSION

The area of marine natural products chemistry has clearly developed leaps and bounds as evidenced by the relatively large number of marine-derived drugs undergoing evaluation as potential therapeutic agents. Buoyed by the potential for the development of natural products from the sea, research work continues to advance with the discovery of new bioactive compounds and new applications for previously isolated molecules [2–15].

The supply issue, however, remains one of critical importance as it relates to the development of drugs from a marine organism. For example, (+) spongistatin 1 has been reported to be highly cytotoxic. It has been deemed to be the most active of all natural and synthetic compounds investigated by the National Institute of Cancer (USA). Three tonnes of the sponge yielded 0.8 mg of the compound. Another collection and processing of 400 kg of the sponge afforded 10 mg of the compound. This isolation work facilitated structure elucidation work. The IC_{50} value for this compound was evaluated at 10^{-6} M in colon cancer cells and 10^{-12} M for breast cancer cell lines [82]. Synthetic approaches to the compound have been presented by research groups including Petit and coworkers [83,84].

Total synthesis of biologically active marine compounds is often fraught with its attendant challenges due to the length of multistep synthetic procedures and the general complexity of the structural motifs which must take into account stereochemical considerations. Propagation through mariculture and aquaculture are also being studied to determine the viability of using these approaches to deal with the challenges associated with procuring sufficient quantities for clinical trials and subsequent formulation into drugs [85].

The timeline from discovering the drug, leading to the entry into the market typically spans a 20- to 30-year period during which time the capital injection is considerable, often necessitating support from the large pharmaceutical entities which are sometimes hesitant about making investments which may not yield significant financial rewards [86]. The Caribbean region, being an important source of marine species with which much research work has been carried out, is not likely to become the recipient of the potential benefits to be derived from the development unless more research work in this area is undertaken in the region with support from the appropriate collaborators.

In the future, it is expected that new strategies will be employed to ensure the supply of large quantities of the target compounds. These include optimization or fermentation techniques for propagation of microbes, including mixed fermentation methods. Biotechnological approaches are likely to include whole genome sequencing, genome mining, genetic engineering, chemoenzymatic synthesis, and in vitro enzymatic synthesis in the hope that new therapeutic drugs will come from our seas [87].

18.8 QUESTIONS

1. If you were required to evaluate an extract for its potential as a drug, what approach would you adopt?
2. Silica gel chromatography is essential for the purification of organic compounds. Identify three methods of chromatography.
3. Design a form which could be used to document information when collecting a specimen.

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Chapter 19

Animal Metabolites: From Amphibians, Reptiles, Aves/Birds, and Invertebrates

K.-D. James
Florida Atlantic University, Davie, FL, United States

Chapter Outline

19.1 INTRODUCTION

The concept of metabolism and metabolites.
The potential sources of the substrates used in the synthesis and production of metabolites.
The enormous diversity of metabolites and host animals that produce them. The significance of symbiosis with respect to metabolite synthesis and production. The ecological and health significance of metabolites in nature, animals, and humans.
The significance of metabolites to the pharmaceutical company.

In general terms, metabolism may be defined as a set of chemoenzymatic transformations of one form of molecule into another, within living cells, for the purpose of sustaining life, and in order to maintain the overall homeostasis and reproduction, and propagation of life. Metabolites are therefore defined as the intermediate or end products of the normal process of metabolism. The mechanism by which metabolism occurs is made up of steps or pathways during which one or more molecules are transformed to a new one (metabolite) by cascades of enzymatic reactions along the pathways [1,2] (Fig. 19.1).

Metabolism is made up of two components—catabolism (breaking down nutrients and nonnutrient molecules) and anabolism (building up of new complex products). The overall result of the process of metabolism is the formation of metabolites from molecules such as proteins, lipids, carbohydrates, and other nutrients. Together with enzymes, metabolites play major roles in nutrient breakdown which provides energy that is needed to perform biochemical functions essential to maintain the general homeostasis of the body [1,2,3].

19.1.1 General Classification of Metabolites

Metabolites are generally formed as transient or stable products during the normal/natural process of metabolism which includes the biochemical processes of building (anabolism), degradation (catabolism), and elimination (excretion) of

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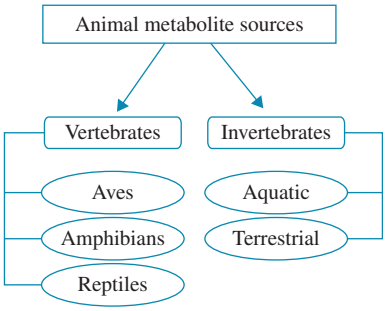

FIGURE 19.1 A flow chart of the sources of metabolites as described in the chapter.

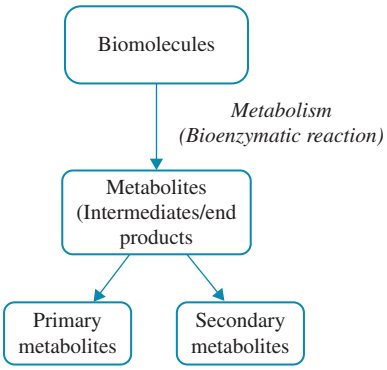

FIGURE 19.2 A flow chart of the metabolic products of biomolecule and types of metabolites as described in the chapter.

compounds in/from the body [4]. The mechanism by which metabolites are formed involves enzyme-induced cascade chemical reactions, in which the product of one reaction can stimulate/initiate the subsequent biochemical reaction (Fig. 19.2).

19.1.2 Metabolites Are Grouped Into Three Main Classes

i. Metabolomes
ii. Primary metabolites
iii. Secondary metabolites

> Primary metabolites are involved in embryogenesis, cell division, proliferation, differentiation, growth, development, and reproduction of the organism.
> Secondary metabolites participate in the overall homeostasis of the organism.

19.1.2.1 Primary Metabolites

Primary metabolites are molecules (of less than 900 Da molecular mass) that function at the cellular level. Physiologically, primary metabolites are directly involved/utilized in normal embryogenesis and overall homeostasis, i.e., cell division, proliferation and differentiation, growth, development, and reproduction of the organism [5]. Some examples of primary metabolites include ethanol, lactic acid, certain amino acids, lipids, protein, and carbohydrates [5–7].

19.1.2.2 Secondary Metabolites

Secondary metabolites that can be created from primary ones are not directly involved in the normal growth, development, and reproduction of the organism. They are molecules which are primarily involved in the overall maintenance/homeostasis of the organism. Secondary metabolites specifically modulate health-maintaining processes, including excretion of waste and toxic products from the body. That means, sustaining the overall health and functional status of

the cells within organ systems of the body, the principal function of secondary metabolites. As an illustration, one could cite the biotransformation of tryptophan, a primary metabolite, into Actinomycin, which is a secondary metabolite [8].

Though the production of metabolites is a natural chemical and bioenzymatic reaction that occurs during metabolism in the body of all organisms, metabolites may also be produced as by-products of the body's reaction to exogenous/external substances or stimuli, such as medications and/or antigens [9]. The network of metabolites, working with enzyme reactions during the entire process of metabolism, is called the *metabolome* [10]. The metabolome involves/implicates all the series of combinations of cascading reactions between enzymes and substrates in the steps of metabolism, and ending in the production of the primary and secondary metabolites.

19.2 EXTRACTION OF METABOLITES

Biologically and chemically active metabolites can be isolated from animals of either terrestrial and/or aquatic habitats, especially marine environments [11,12]. The isolation and characterization of secondary metabolites from animals (vertebrates and invertebrates), and/or their bacterial/algal symbionts, have been extensively described in the literature [13–19]. With biological assays (bacterial and tissue cultures) and HPLC-diode arrays detection, Gebhardt et al. [15] described the isolation and characterization of bacterial and bioactive metabolites, in addition to antifungal and antibacterial peptide antibiotics, from arthropod–bacilli endosymbionts. Other methods of metabolites' extraction and profiling include the application of HPLC-GC coupled with nuclear magnetic resonance-based, and mass spectrometry-based metabolomics as described by Lankadurai et al. [20]. Spectrophotometric challenge assay techniques to screen for metabolites have been described [14]. Normally, samples needed for the extraction and isolation of metabolites include isolated cells, tissues, entire body organs, whole organisms, or biofluids from the host organisms.

19.3 METABOLITES IN VERTEBRATES

19.3.1 Amphibians, Reptilians, and Aves

There are several biologically active metabolites identified and/or isolated from nonmammalian vertebrates, most of which are also found in other vertebrates and invertebrates. The major sources of secondary metabolites in both vertebrates and invertebrate animals are diverse and include both endogenous/internal sources (naturally produced during certain biochemical synthesis in the body within cells and tissues), and exogenous/external sources (derived from diets and other molecules in the environment and the habitat of the host animals) [4,21,22]. Studies of metabolism in several amphibian species have identified several metabolites and new metabolic activities. The presence of corticosteroids has been demonstrated in the amphibian, reptilian, and the Aves [23]. Corticosterone and ll-deoxycorticosterone, which are biosynthetic metabolites of progesterone, have also been isolated [24]. Narayan [25] and Haruki et al. [26] reported identification of pyrene metabolites, including pyrene-1-glucuronide, pyrene-1-sulfate (PYOS), and pyrene glucoside sulfate, in several species of amphibians. The analysis indicated interspecies differences among amphibians in pyrene metabolism and metabolites [26]. Brucker et al. [3] identified two metabolites, indole-3-carboxaldehyde and violacein, from certain bacterial symbionts on the skin of amphibians including the salamander. Further studies confirmed that the metabolites were from the antifungal (*Janthinobacterium lividum*) symbiont [3]. The host amphibian needs the metabolites to resist the deadly fungal infection [27]. This association highlights the significance of coexistence among host–symbiont relationships in the ecosystem (Fig. 19.3).

19.3.2 Amphibians

Amphibians are a source of biologically significance metabolites, many of which have been identified in the skin secretions of poison dart frogs, especially *Dendrobates* and *Phyllobates* [28]. Over 100 toxins/metabolites have been identified in the skin secretions of poison dart frogs, especially the genus *Dendrobates* and *Phyllobates*. These are also known as poison arrow frogs. These toxins may be derived from the metabolism of chemicals obtained from the frogs' diet [28]. Some of the known metabolites produced/secreted by the *Phyllobates* include *batrachotoxin*, which is a potent steroidal alkaloid neurotoxin that has been used by the native South American tribes for hunting purposes to paralyze and capture animals. Some amphibian species produce *tetrodotoxin*, a metabolite which has the same mechanism of action as *batrachotoxin*. Other bioactive metabolites including epinephrine and norepinephrine have been identified in some toads. These biogenic amines have effects on the cardiovascular system. Toads of the family Bufonidae produce

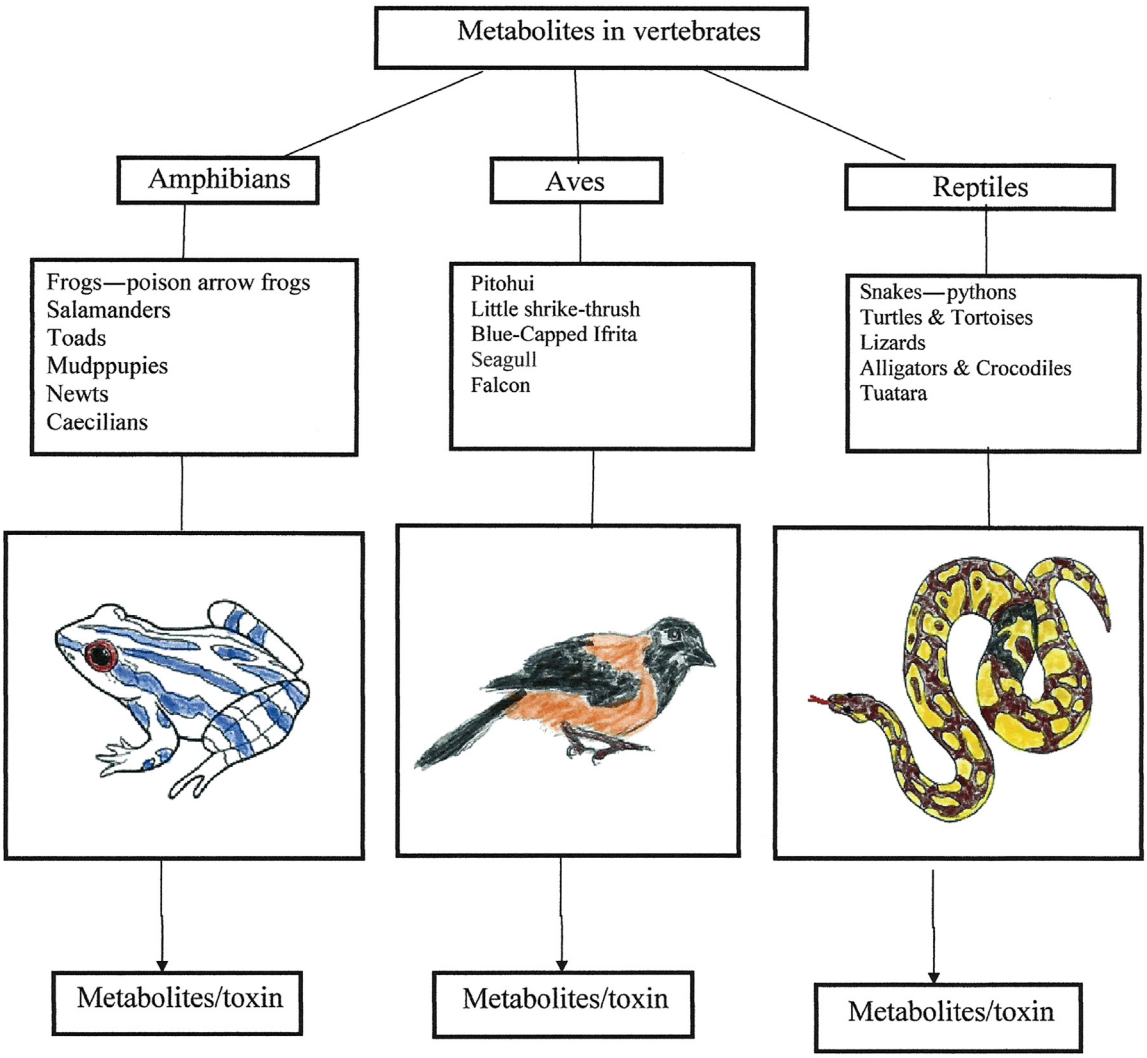

FIGURE 19.3 A flow chart of the metabolic products/metabolites from vertebrates-amphibians/reptiles and aves.

bufotoxin, which has effects similar to digitalis, and *bufotenin* (an indolealkamine), which is a potent hallucinogen. Some of these toxins including salamander toxins can be lethal to animals and humans.

19.3.3 Reptiles

Several bioactive metabolites have been isolated in the venom and/or blood plasma of reptiles, most of which are potently toxic. Hunter et al. [29] have reported several metabolites in the plasma of the ball python (*Python regius*) and the blue and gold macaws (*Ara ararauna*). The venom of snakes and most other reptiles is modified saliva, containing a combination of proteins and various enzymes, constituting toxins of varying potencies [28,30]. Some snakes and other venomous reptiles produce *neurotoxins* affecting the central nervous system, *hemotoxins* affecting tissues and blood, *myotoxins* affecting the musculature, *cardiotoxins* affecting the heart, and many other venoms/toxins [28,30].

The usefulness of these venoms to the host reptiles for catching their prey, and the dangerous effects on humans are well documented [28,30]. However, medical uses of these metabolites as substrates for drug manufacturing in the pharmaceutical industry are also known. Such uses include manufacturing of various kinds of antivenin, blood clotting proteins found in Taipan venom that is used to stop bleeding during surgery, and enzymes derived from copperhead venom, used to treat breast cancer [30].

19.3.3.1 Aves

A wider range of secondary metabolites has been identified in Aves. In all cases, plasma and tissue concentrations of metabolites in birds vary significantly in response to the metabolic activities of the birds, which in turn depend on the physical, physiological, and metabolic states and activities of the birds, such as migration, breeding, and postbreeding activities or period [31,32]. Metabolites could be synthesized in response to stressors and other stimuli in the habitat of both vertebrate and invertebrate animals. Metabolic profiling in birds has revealed a range of diverse secondary metabolites in the plasma of both migratory and nonmigratory birds. Included in the documented plasma secondary metabolites in Aves are plasma B-hydroxybutyrate, triglycerides, and nonesterified fatty acid [33–35], just to list a few. General analysis of the secondary metabolites in the plasma, tissue/organ, and body fluids may reveal and provide significantly useful information on the overall physiological status and physical activities of Aves [2].

Avian species of the genus *Pitohui* in New Guinea have been found to contain a potent bioactive toxin in their feathers and muscle tissue previously known only from the skins of frog species in the genus *Phyllobates* including the neotropical poison arrow frogs (Dendrobatidae) [36–39]. The toxin is classified as neurotoxic steroidal alkaloid homobatrachotoxin [36]. It is postulated that the toxin is to ward off ectoparasites and bacterial infection rather than to defend against predators [38].

19.4 METABOLITES IN INVERTEBRATES

19.4.1 Aquatic and Terrestrial

In the animal kingdom, animals that do not have vertebral bones (lack of notochord) and therefore do not have a vertebral column are called invertebrates. In reference to location or habitation, invertebrates are broadly classified as either *aquatic* or *terrestrial* habitation. Reports from several ecological and zoological studies indicate that invertebrates make up the most abundant species of organisms on earth [40,41], producing the greatest diversity of secondary metabolites (Fig. 19.4).

Common examples of aquatic invertebrate groups include annelid worms, sea anemones, conchs, corals, octopus, crabs, jellyfishes, sea cucumbers, sea nettles, sea urchins, sea snails, shrimps, sponges, squids, and starfishes [42]. These are just a few of the aquatic invertebrate groups. There are many species within each group with different phenotypic characteristics, different territorial/aquatic locations or habitations, different nutritional requirements, and varying microbial/algal symbionts within and between the groups and species [42–46].

General examples of terrestrial invertebrates include spiders, scorpions, centipedes, grasshoppers, insects, land snails, millipedes, and nematodes. However, like the aquatic invertebrates, terrestrial invertebrate population is made of different groups/phyla of animals with enormous diversity of species within and between the groups: *Annelids*—leeches, earthworms; *Arthropods*—crustaceans, arachnids, insects; *Mollusca*—slugs and land snails; *Nematodes*—hookworm, roundworm, whipworm, and lungworms [47–50].

Secondary metabolites can be isolated from most of these invertebrates. The physiological urge or need for formation of metabolites is modulated by both the internal and external conditions of the host animal. Under certain specific conditions (either internal/endogenous and/or external/environmental), invertebrates synthesize varieties of biochemically active metabolites of physiological and ecological importance, not only to the host animal but also of medicinal value to humans. Both terrestrial and aquatic invertebrates are known to be rich sources of chemical and bioactive secondary metabolites with diverse chemical and biological activities between and within the species of invertebrates [51]. Metabolites are synthesized from molecules within the invertebrate hosts. The metabolic/biosynthetic origin of metabolites include *innate sources*—endogenously synthesized; *dietary sources*—synthesized from consumed nutrients; *environmental sources*—synthesized from materials absorbed from the habitat; and *symbiotic sources*—metabolites obtained from microbial/algal symbiotism with the host invertebrate [52–56].

19.4.2 Metabolites From Aquatic Invertebrates

Metabolites are extracted from these organisms alone or with their associated microbial–fungal symbionts [57,58]. Among the most common of the marine invertebrate sources of bioactive metabolites are the sponges that are marine diploblastic metazoans. They produce a wealth of biologically and chemically active secondary metabolites of ecological and therapeutic significance [58–61]. Actually, the bacterial/algal symbionts of the sponges are the major producers of the metabolites. Diverse secondary metabolites that have been extracted from various species of sponges with their symbionts include polyacetylenic alcohols, including petrocortyre-A purified from the marine sponge

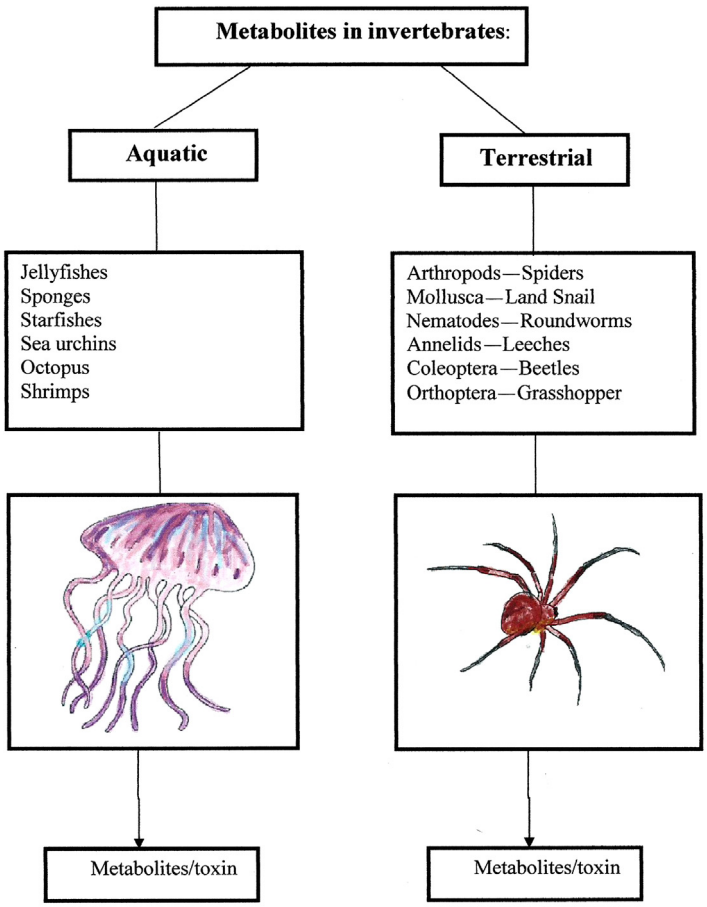

FIGURE 19.4 A flow chart of the metabolic products/metabolites from aquatic and terrestrial invertebrates.

Petrosia sp.; aeroplysinin and aerthionin and other dibromo- and dichlorotyrosine derivatives from the biometabolic activity of *Aplysina cavernicola*; bromoalkaloids (halogenated alkaloids); and terpenoids [62,63]. *Tedania ignis* and related species of the same genus have been reported to produce many secondary metabolites including the aryl carotenoid tedanin [64], atisanediol [65], tedanalactame [66], pyrazole [8], diketopiperazine [65], benzothiazoles, and diketopiperazines [1,67,68]. Others including dibromotyrosines, aerothionin, and homoaerothionine are derived from the *Aplysina* sponges [69] and their bacterial symbionts. *Dysidea herbacea* with its bacteria symbionts produces several types of secondary bioactive metabolites including sesquiterpenes spirodysin [70,71], herbadysidolide [72], brominated biphenyl ethers [73], and several kinds of hexachlorinated amino acid derivatives, such as 13-demethylisodysidenin [74] and the diketopiperazine [70].

Several bioactive secondary metabolites have been extracted from the homogenates of the sponge *Halichondria okadai* and its microbial symbionts. These latter metabolites (metabolic by-products) include okadaic acid [75] and norhalichondrine-A [76], which is a potent antitumor by-product; carotenoid (β-carotene, α-carotene, γ-carotene), which can serve as antioxidants [77]; and alteramide-A, which is known to have powerful cytotoxic properties against P388 leukemia, lymphoma L1210, and KB carcinoma [78]. In most cases, the concentrations and types of metabolites extracted from such microorganism−macroorganism symbiosis depend on the location or habitat of the host sponge [78]. Furthermore, the type of metabolites in the bacterial or algal symbionts may either be different or the same as the metabolites from the sponge host [79−81]. In other instances, the secondary metabolites are not present in either the independent host sponge or the associated symbiont [79]. This situation symbolizes true synergistic symbiosis in the synthesis of secondary metabolites, most of which are of physiological and ecological significance to the host−microbial symbiont, to the ecosystem, and to humans. Under the same or identical endogenous and environmental stressors or stimuli, other aquatic invertebrates also produce a wide diversity of secondary metabolites. Some of which can be same as or chemically and functionally related to other metabolites, produced by other invertebrates.

19.4.3 Metabolites From Terrestrial Invertebrates

Many terrestrial invertebrates could synthesize secondary metabolites especially in response to physical stress and other factors in their habitat. Some species of mollusks and arthropods may synthesize secondary metabolites including alanine, succinate, fatty acids, propionate, and acetate; these being the end products from the biotransformation of arginine phosphate, glycogen, and aspartate as the substrates [82,83]. Some species of gastropods metabolically transform arginine phosphate and glycogen to produce lactate, octopine, and strombine/alanopine as the main end product secondary metabolites [82,83]. Elevation of histidine, a bioactive metabolite, has been observed in two species of earthworms, *Eisenia andrei* and *Lumbricus rubellus*, in copper-rich environments [84]. Other metabolites extracted from *Porcellio scabbier* (Isopoda), *E. andrei* (Lumbricidae), and *Folsomia candida* (Collembola) species of invertebrates are 1-hydroxyp-yrene, pyrene-1-glucoside, and PYOS. These latter metabolites are identified metabolic products of pyrene biotransformation [85].

A wide diversity of bioactive metabolites has been isolated from invertebrates that live in symbiosis (ectosymbionts and/or endosymbionts) with arthropods [86,87]. Examples of reported metabolically bioactive by-products/metabolites from arthropod−endosymbiotic bacteria are alkanes, alkenes, phenols, cyanobenzene, actinidin, carboxylic acid and aromatic esters, peptide-like secondary metabolites, and a variety of peptide and polypeptide antibiotics [15,16,19,88].

19.5 IMPORTANCE OF METABOLITES

In terms of metabolite synthesis and production, symbionts (both ectosymbiosis and endosymbiosis) are important to the viability of both the host vertebrates and invertebrates [15,16,88]. Animals may physiologically respond to stressors and other obnoxious stimuli in their habitat by producing metabolites. Therefore, justifiably, metabolites may serve as biological markers or bioindicators of environmental pollutants and/or stressors in the ecosystem, and may be used for risk assessment to health and diseases in the ecosystem for humans, animals, and plants [20]. That is to identify the nature of stressors, inhibitions, and other environmental factors/objects that may impact survival in the ecosystem. Such may be used as management tools for risk assessment.

A wide diversity of secondary metabolite of ecological and medical significance have been isolated from both vertebrates (especially amphibians and Aves), and invertebrates. For instance, varieties of peptide and polypeptide antibiotics have been isolated as secondary metabolites from microorganisms living in symbiosis (ecto- and endosymbionts) with animals, especially invertebrates, as well as from free-living bacteria. Listed among the antibiotics isolated from endosymbionts and free-living bacteria are polymyxin M_1, gramicidin S, tyrocidin, and bacitracin [37]. Other bioactive secondary metabolites extracted from sponges and their microbial symbionts include okadaic acid [75]; norhalichondrine-A [76], which is a potent antitumor by-product [77]; and alteramide-A, which is known to have cytotoxic properties against P388 leukemia, lymphoma L1210, and KB carcinoma [78]. Additionally, metabolites of potential medical significance (drug development) include: swinholide—cytotoxic and antifungal [89−91]; onnamide A—antiviral [92]; and theonellamine B—inhibits Na/K-transporting ATPase [90,93]. Inhibition or interference of the Na/K channel could upset the electrolyte balance of the body fluid. Furthermore, Harris [94] demonstrated that treatment of the frog *Rana mucosa* with the metabolite violacein resulted in significant decline in morbidity and mortality rates of the population of frogs infected by the fungus *Batrachochytrium dendrobatidis*. Violacein is a metabolite produced by the antifungal bacterium *J. lividum* which are resident (symbionts) on several species of amphibians [94]. This highlights the synergistic significance of host−symbiont symbiotism and the potential role of metabolites in disease control in the ecosystem.

19.6 CONCLUSION

One of the main aims of this chapter was to delineate the enormous diversity of metabolites from the wide diversity of animals, focusing on amphibians, reptilians, Aves, and invertebrates (terrestrial and aquatic). It seems quite obvious that additional exploration of bioactive secondary metabolites from both vertebrates and invertebrates will create a new paradigm for studies and discovery of biomedical compounds for the pharmaceutical companies, thus encouraging continuous development/synthesis of new, economically viable drugs of commercial benefit, as earlier anticipated [13,80,95,96].

19.7 SELF-EVALUATION QUESTIONS

1. Metabolites could either be intermediate products or end products of metabolism. Briefly explain what this means.

2. What may account for the enormous diversity of secondary metabolites in nature?
3. Comment on the potential significance of host−symbionts in the synthesis of secondary metabolites.
4. Discuss the significance of metabolites risk assessment of health and diseases in the ecosystem.
5. Explain the potential benefits of secondary metabolites to:
   a. the host organism,
   b. the ecosystem,
   c. the health/medical industry.
6. In general, what are the material sources of metabolites synthesized/produced in the host organism?

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Chapter 20

Fungal Metabolites

D.K. Daley[1,3], K.J. Brown[2] and S. Badal[2]

[1]University of Technology, Jamaica, Kingston, Jamaica, [2]University of the West Indies, Kingston, Jamaica, [3]Biotech R&D Institute, Kingston, Jamaica

Chapter Outline

Learning Objectives

- Define endophyte, phytochemicals, and fungal metabolites.
- Types of transmission of fungi.
- Effects of fungal endophytes on plants.
- Production and extraction of fungal metabolites.
- Types of fungal metabolites and their applications.

20.1 INTRODUCTION

In the pharmaceutical industry, the search for novel compounds is endless as scientists look to nature for curing various ailments. Plants are traditionally used as medicines and have been and continue to be the source of phytochemicals with therapeutic properties. These phytochemicals are made by the plants as part of their survival strategy and have been shown to be beneficial to man. Microbes, such as bacteria, fungi, and to a lesser extent viruses, that invade and reside within the tissues of plants are called endophytes, which literally means "within plants." This ensuing symbiotic relationship can be described as mutualistic when both the microbe (endosymbiont) and the plant (host) share in benefit [1], while a commensalism relationship occurs when one, usually the endosymbiont benefits without affecting the host.

One of the subsequent benefits to the plant involved in this symbiotic relationship is the resulting unpalatable taste that it may now have because of the presence of the microbes, which in turn prevents consumption by the animals that would normally graze on it. The plant may also become resistant to other pests or strenuous environmental conditions that would otherwise cause the plant to die. Research has now shown how significant the effect can be when an endophyte is within a host plant, it may alter the reproduction strategies, growth and development, physiology, and also its internal biochemistry, thus contributing to the development of new compounds which this chapter will explore in more detail [2,3].

Endopytes are organisms that live within the tissues of plants (host), utilizing their machinery for survival. Endophytes may be bacteria or fungi and may have beneficial or deleterious effects to the host.

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Fungi are eukaryotic spore-producing organisms that reproduce rapidly once environmental conditions are favorable. Many fungi will survive and reproduce within the plant cell and thus make new compounds or instigate the plant's production of new compounds in response to its presence within the tissues. Some of the common fungi that are considered endophytes are found across the seven phyla of the fungi kingdom which include: Basidomycota, Ascomycota, and Glomeromycota. All the members of the Glomeromycota phyla are mutualistic with plant root tissues and improve the nutrient content of the surrounding environment for the plant, which in turn benefits their own survival. *Piriformospora indica* is a member of the phyla Basidiomycota and infects a wide variety of plants; it is mainly a root symbiont as it invades the roots of parsley, tobacco, barley, maize, and tomato and causes an overall improvement in the crop yield. *P indica* is also able to significantly enhance the growth of many grass species, which are often infected by fungi. Another similar example is *Claviceps* species of fungi.

Fungal endophytes may reside within the roots, stem, or leaves of the plant and tend to reproduce just before or during the senescence phase of the plant at which time the spores would be released [4]. These plant endophytes often produce compounds that may be harmful or beneficial to the plant and thus may be extracted for their therapeutic value or may be of agricultural significance. *Festuca* species (Poaceae) are also significantly affected by fungal−plant interactions. These endosymbionts may increase the water and nutrient uptake of the plants, which facilitates the production of new metabolites or an increase in the production of those the plant synthesizes. On the other hand, the presence of these host-specific endophytes causes observable physiological changes within the plant [5] and as such the plant may be identified as infected. For example, *Ustilago maydis* is a Basidomycotic corn pathogen which can significantly cripple the corn industry once plants become infected by this fungus. Other fungi that cause significant crop losses include *Magnaporthe oryzae* which attacks *Oryzae* (rice) species and causes rice blast disease [6].

Some fungal endophytes may improve the quality, quantity, and yield of the plant host, such as those within the Glomeromycota phyla, while other fungal endophytes may reduce the plant's physiology, function, and yield, e.g., *U. maydis*, which affects the *Zea mays* significantly.

Secondary metabolites, mainly alkaloids, may also improve the plant's resistance to pests and other pathogens, e.g., peramine, a secondary metabolite that prevents herbivory once produced in the host plant [7]. Secondary metabolites also may allow the plants to adapt to changes in climatic conditions, such as drought or extreme temperature changes, as some of the compounds produced include alkaloids. Many endophytic fungi help to improve the growth of *Zea mays* and other grass-like crops from the Poaceae family; these fungal endophytes often aid in improving development as they prevents herbivory from pests, insects, or mammals [1,3,8]. Sometimes the compounds produced can be toxic and as such the animals would avoid these plants, e.g., *Achnatherum robustum*, also known as sleepy grass, because of its effect on mammals. Grazing mammals, such as horses and other domesticated animals that consume this grass, would sleep for days before recovery. This is due to the presence of a toxic alkaloid, lysergic acid amide, that is produced when the grass is infected by a fungal endophyte, *Neotyphodium* [1]. Ergot alkaloids produced as fungal endophytes have also prevented animals from feeding on the host plant [9].

20.2 TRANSMISSION

Endophytes are often transmitted to the host plant vertically, that is, from generation to generation through seeds or vegetative propagation, such as seen with the *Neotyphodium* spp. These endophytes are usually sterile. However, a few fungi, like *Epichloe* spp., are able to adapt to the environmental change and reproduce sexually releasing spores that will undoubtedly infect a nearby host plant. On the other hand, during horizontal transmission, these endophytes produce spores via sexual or asexual reproduction for insect and wind dispersion. An endophyte may also be transferred horizontally via exposure to a nearby infected plant [10]. These endophytes then reproduce resulting in either beneficial or deleterious effects.

20.3 BIOACTIVE FUNGAL METABOLITES

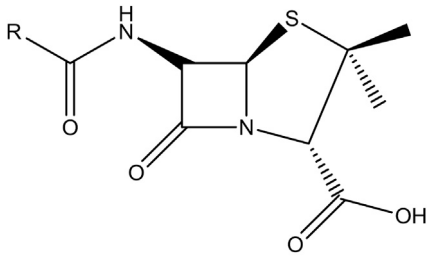
A metabolite is a compound produced by the plant for growth and development (primary metabolite) or as a part of their defense or survival mechanism (secondary metabolite), the latter usually synthesized from the former [11]. Secondary metabolites are unique to particular plant families, genera, or species and as such have been the source for

new drug isolation and subsequent identification and elucidation based on their chemical structures. These isolated organic compounds are referred to as bioactive metabolites, once their therapeutic effects have been determined by bioassays. The bioactive secondary metabolites then form the template for the creation of new pharmaceutical agents after structure modification and further bioassay. Research has shown the effectiveness of some of these metabolites as antidiabetic, antibacterial, antioxidant, antitumor, and even insecticidal agents. Bioactive metabolites have been isolated from many plants infected with bacterial and fungal endosymbionts. A significant number of these bioactive compounds have been isolated from plants infected with fungi and are called toxic fungal metabolites (mycotoxins). This chapter will explore these compounds in more detail.

20.4 EXTRACTION OF FUNGAL METABOLITES

Most fungal metabolites that are currently known can be easily isolated from their host plant tissue or culture media. These secondary metabolites, as mentioned before vary, from plant to plant and are relatively low molecular weight compound alkaloids [12]. One of the first fungal metabolites to be isolated was the antibiotic, benzlpenicillin, commonly called penicillin in 1928 (Fig. 20.1); this was isolated from *Penicillium notatum* as a compound in the observed "mold juice" which was able to kill different types of microbial colonies that were responsible for certain diseases in humans [13,14].

A series of tedious purification and collaborative efforts led to the tremendous discovery and production of an efficacious pharmaceutical item. Overall, the steps involved in the extraction processes (summarized in Fig. 20.2) of secondary metabolites from either plant, marine, or fungal sources are more or less the same; the differences lie in the solvent system used and the means of detection and structural elucidation, areas discussed in more detail in subsequent chapters. Because the fungi usually grow on a host organism, the first step involves retrieving the host and sterilizing it, usually with 70% alcohol and washing with appropriate buffers and sterile water. After which, the aim is to separate the fungi from the host, usually via agar plates containing isolation medium (that allows for growth of the fungi). This occurs after pressing/cutting/grinding of the host material. Identification and separation of the various fungal strains follow, after which they are cultivated. The subsequent sections involve the identification of the various secondary metabolites using one of more of the following: thin layer chromatography, vacuum liquid chromatography, size exclusion chromatography, and high performance liquid chromatography. The structures are then elucidated by one or more of the following: NMR spectroscopy, mass spectrometry, UV measurements, IR spectroscopy, and optical rotation.



Penicillin

FIGURE 20.1 The structure of penicillin.

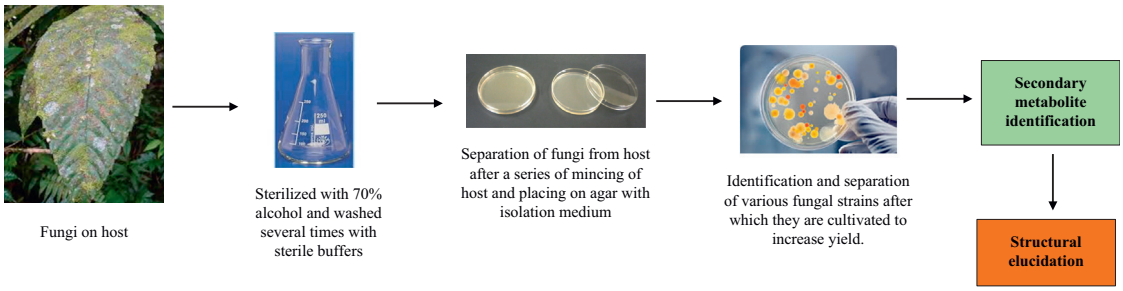


FIGURE 20.2 A scheme of the extraction of secondary metabolites from fungi.

Today penicillin is synthesized in the lab via solvent extraction with amyl acetate, methyl-cyclohexanone, or dimethylcyclohexanone [14] or via condensation of L-cysteine, L-valine, and L-α aminoadipic acid, followed by oxidative conversion of this tripeptide to an intermediate isopenicillin which is later reacted to form the various strains of penicillin. The natural production of penicillin is enhanced by the fact that the fungus is unable to grow due to stressful conditions and thus this initiates the formation of penicillin via acetyl coenzyme A and α-ketoglutarate [15,16]. There are many types of fungal metabolites that have been isolated and their therapeutic value determined using many technological separation and extraction techniques, such as supercritical fluid−carbon dioxide extraction among others.

Secondary metabolites are those phytochemicals made from the primary compounds within the plant that are used as a survival tool. Fungal metabolites are those made due to the presence of fungi within the plant tissues and may possess therapeutic effects. These compounds may be extracted as medicinal and pharmaceutical agents.

20.5 TYPES OF FUNGAL METABOLITES

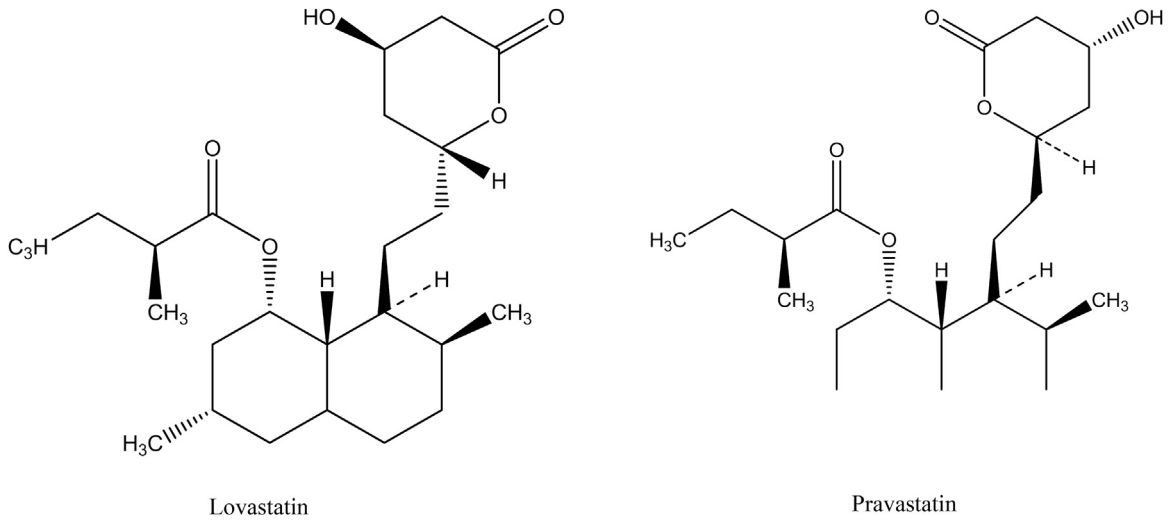
The production of fungal secondary metabolites commences once active growth ceases as with all secondary plant metabolites [17−19]. The synthesis of these compounds may be via various pathways within the plant that are generally divided into four classes according to chemical structure or biosynthetic origin: polyketides, nonribosomal proteins, terpenes, and indole alkaloids.

20.5.1 Polyketides

Polyketides are the most abundant fungal secondary metabolite group and are formed via the polyketide pathway by type I polyketide synthases (PKSs) through the condensation of acetyl-coenzyme A (CoA) and malonyl-CoA during condensation reactions. Included in this group are the aflatoxins and statins [12]. There is great diversity within this group due to a number of factors such as the number of reduction reactions they can undergo, the number of iteration reactions, the type of extender unit used, and the possibility of cyclization of the polyketide chain. These polyketide metabolites are mainly produced by the *Penicillium*, *Fusarium*, and *Alternaria* species of fungi [18]. Some secondary metabolites act as cholesterol-lowering agents. The polyketide lovastatin from *Monascus ruber* and *Aspergillus terreus*, as well as pravastatin, display cholesterol-lowering properties [5,17,20] (see Fig. 20.3).

20.5.1.1 Aflatoxins

These polyketides, also categorized as mycotoxins, are involved in a number of disease states in plants and animals due to their toxic nature [13,21]. Illudin S (Fig. 20.4) is a sesquiterpene from the mushroom *Omphalotus illudens* that



Lovastatin

Pravastatin

FIGURE 20.3 The structures of lovastatin and pravastatin.

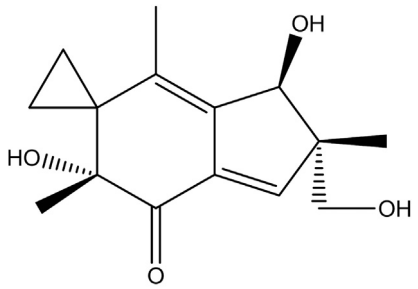
displays extreme toxicity due to its ability to alkylate DNA [22]. Aflatoxins are the most widely known mycotoxins, with the four major ones being aflatoxin B_1, B_2, G_1, and G_2. Of the four major aflatoxins, B_1 has been noted as one of the most toxic and carcinogenic compounds yet discovered [11].

20.5.1.2 Statins

Statins exhibit biological activity on the liver enzyme 3-hydroxy-3-methylglutaryl-CoA reductase which is responsible for cholesterol production. This activity results in the reduction and or removal of low-density lipoproteins from blood vessels. As such, these metabolites are referred to as hypocholesterolemic agents [20].

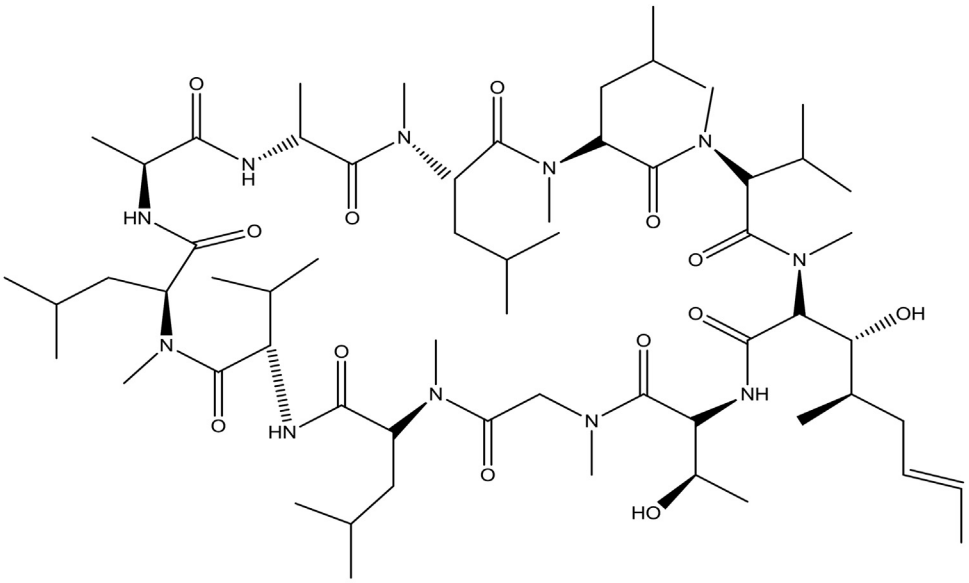
20.5.2 Nonribosomal Peptides

The nonribosomal peptides are formed from both proteinogenic and nonproteinogenic amino acids with the use of nonribosomal peptide synthases. Synthesis of this class of peptides is mRNA independent [12,17]. Examples include the immunosuppressive drug cyclosporine (Fig. 20.5) produced by *Tolypocladium niveum*, which is used to treat patients who have undergone organ transplant surgery.



Illudin S

FIGURE 20.4 The structure of Illudin S.



Cyclosporin

FIGURE 20.5 The structure of cyclosporine.

20.5.3 Terpenes

Gibberellins, trichothecenes, carotenoids, indole-diterpenes, and aristolochenes all belong to this category of fungal metabolites [12] and can be seen in Fig. 20.6. Terpenes are made up of isoprene units (C_5) as seen in Fig. 20.6 that can be arranged in a linear or cyclic format, be saturated or unsaturated, or modified in a number of other ways to give diversity in this group. They are classified according to the number of isoprene units they contain into hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), and tertaterpenes (C_{40}) [23]. In fungi, terpenes are synthesized using the mevalonic acid pathway which produces isopentenyl

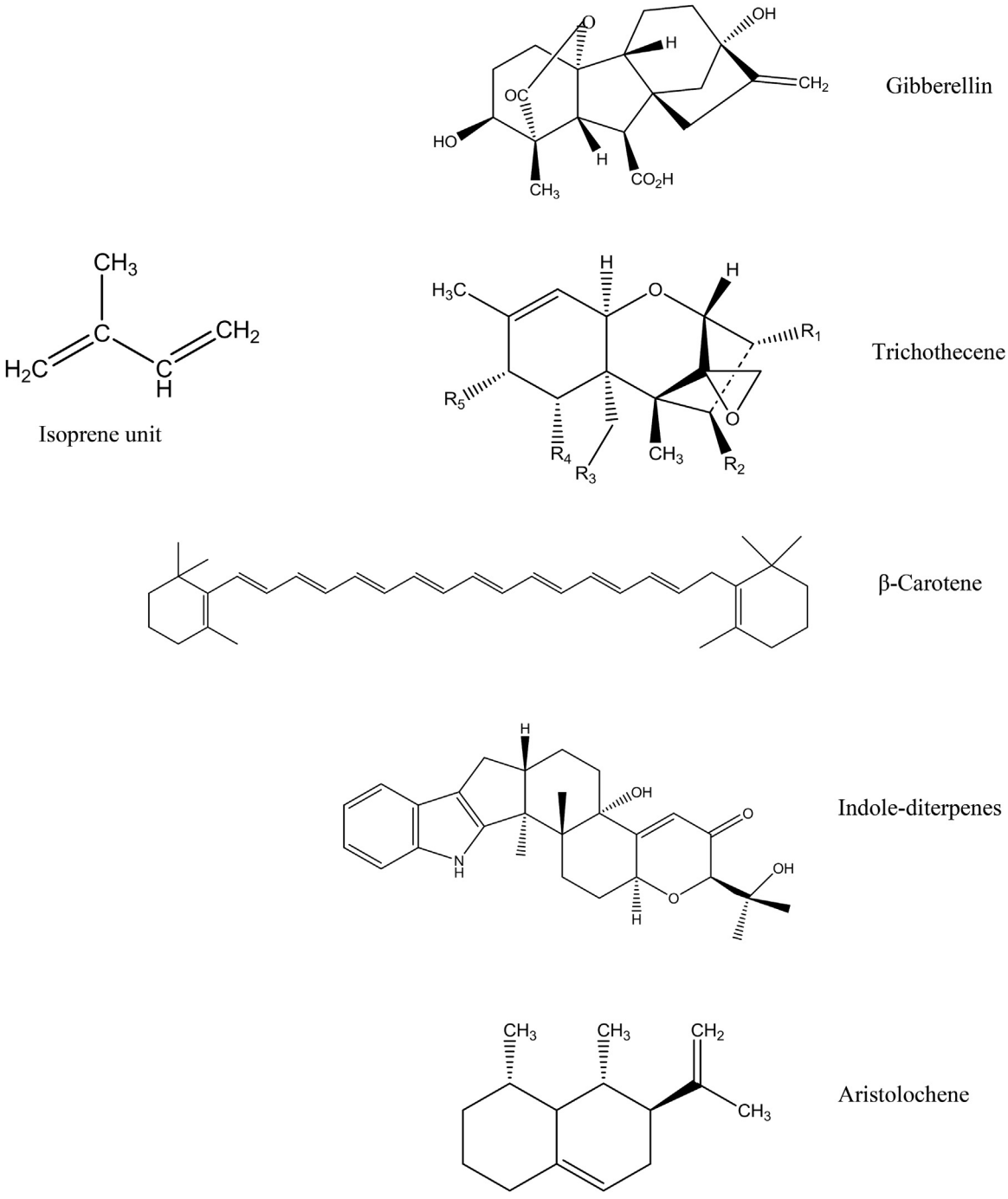
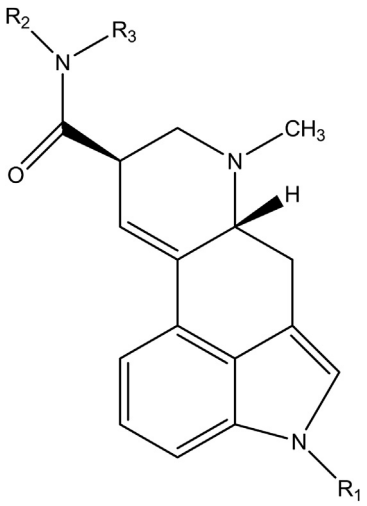


FIGURE 20.6 The structure of some common terpenes.



Ergoline

FIGURE 20.7 The ergot alkaloid, ergoline.

TABLE 20.1 The Application of Some Common Fungal Metabolites

Fungal Metabolite	Class	Applications
Aflatoxin B1	Polyketides	Carcinogenic
Cyclosporine	Nonribosomal peptide	Immunosuppressant
Ergots	Indole alkaloids	Hypotensive; induce contractions
Gibeberellins	Terpenes	Plant growth hormone
Penicillins	Nonribosomal peptide	Antibiotic
Statins	Polyketides	Hypocholesterolemic

diphosphate and its isomer dimethylallyl diphosphate. Diversity in structure and properties of this group could possibly be due to varied enzymatic modifications to include redox reactions, alkylation, decarboxylation, glycosylation, rearrangements, and cyclization.

20.5.4 Indole Alkaloids

Tryptophan and dimethylallyl phosphate are the main precursors from which indole alkaloids are synthesized. On occasion, amino acids other than tryptophan are used as precursors [12]. The most widely studied group of indole alkaloids are the ergot alkaloids that contain the indole ring and are found in *Claviceps purpurea* and its related species. The ergot alkaloids (Fig. 20.7) are able to reduce blood pressure by dilating blood vessels. They also inhibit noradrenaline and sclerotin through their action on the sympathetic nervous system. The ergots are also capable of inducing abortion as they are able to promote contraction of the uterine muscles. Fumigaclavines and fumitremorgens are also tryptophan-derived alkaloids synthesized by *Aspergillus fumigates* (Table 20.1).

20.6 APPLICATIONS OF SECONDARY METABOLITES

A number of secondary metabolites function as antibacterial, antifungal, or antitumor agents [12,24]. In 1995, there were approximately 12,000 known antibiotics and of this amount approximately 22% were produced by fungi [5,20]. In 2010, it was estimated that fungi accounted for 61% of the microbial metabolites [25]. Included are the broad-spectrum penicillins from *Penicillium* sp., and the cephalosporins form *Cephalosporium* sp. Griseofulvin is a broadly

used antifungal agent first isolated form *Penicillium griseofulvin*. It acts by inhibiting fungal mitosis and thus fungal growth and is commonly used as a topical antifungal agent [26]. Taxol, from the fungus *Taxomyces andreanae*, is an approved agent for the treatment of breast and ovarian cancer [5,20]. Cyclosporin A (from *Trichoderma polysporum*) was originally indicated as an antifungal agent but is now widely known for its powerful immunosuppressive activity [27,28]. It is used to prevent rejection in patients who have undergone organ transplant surgery.

Secondary metabolites may also function as hormones, e.g., the gibberellins from *Gibberella fujikuroi*, used in the production of seedless grapes [18], to increase vegetable yield, increase the rate of barley malting and improve malt quality, control flowering, seed germination, and stem elongation, as well as to lower the time required for reaping lettuce and sugar beet seed crops [29]. The estrogen zearelanone is also produced by *Gibberella* sp. (*Gibberella zeae*) and is used to increase growth and feed efficiency in sheep and cattle [5,20]. Secondary metabolites can have negative effects as is seen with the aflatoxins which cause a number of diseases. These include both chronic and acute conditions, such as growth retardation, immune suppression, cancer, and in severe cases, death [30,31].

Other industrial applications of fungal secondary metabolites include the use of pigments. The carotenoid astaxanthin from the yeast *Phaffia rhodozyma* gives crustacean shells and the flesh of salmonids their orange-pink color when boiled [5,20]. β-Carotene is also industrially produced in Russia from the fungus *Blakeslea trispora* [32] and functions as a pigment in plants such as carrots and also aids in photosynthesis and photoprotection. Polyunsatured fatty acids that form an important part of heart health and lower the levels of "bad cholesterol" can be accumulated through the use of fungal species like *Morteriella isabellina* and *Mucor circinelloides* [32].

Secondary metabolites have applications in agriculture, medicine, pharmaceutical, and manufacturing industries. Some metabolites, such as the ergot alkaloid, lysergic acid diethylamide (LSD), have been abused due to their intoxicative effects. Despite this, fungal secondary metabolites show great diversity and application in a number of industries where they play key roles.

Lysergic acid diethyl amide, commonly known as LSD, is a member of the ergot alkaloids and was accidentally discovered as a hallucinogen. Prior to its use as a recreational drug, LSD was used in psychiatry to treat schizophrenia.

20.7 CONCLUSION

The use of fungal metabolites have made significant strides in the fields of agriculture, medicine, pharmaceutical, and manufacturing industries, especially with the incorporation of technology. Research is always ongoing to unlock new secondary compounds with therapeutic value, and as such, the isolation and extraction from plants may continue to lead to the knowledge and role of fungal endophytes contributing to the development or production of these compounds. This allows the diversity of these metabolites and their application in various industries.

20.8 SELF-EVALUATION QUESTIONS

1. What are endophytes?
2. Describe how a fungal endophyte may affect plant production.
3. Explain how plants generally become infected with endophytes.
4. What is the difference between primary and secondary metabolites?
5. What are the major classes of fungal secondary metabolites?
6. Name the class of metabolites which:
   a. are most abundant,
   b. have the most diversity.
7. Name three important fungal metabolites and their use in the various industries.

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Chapter 21

Fats

C.S. Bowen-Forbes and A. Goldson-Barnaby
The University of the West Indies, Kingston, Jamaica

Chapter Outline

Learning Objectives

After reading this chapter, students should:

- Know that fats, a class of lipids, are glycerol esters of fatty acids and that triacylglycerols are the most abundant types of fats
- Know the general classifications of lipids, and various nomenclatures for naming fatty acids
- Be aware of the general composition of different animal fats, with the understanding that terrestrial animals possess higher amounts of saturated fatty acids than marine animals, the latter being good sources of long-chain omega-3 polyunsaturated fatty acids
- Have an understanding of the extraction and refining processes for animal fats
- Have an appreciation for the nutraceutical and pharmaceutical applications of selected animal fats and oils
- Have a clearer understanding of the complex and controversial relationship between different types of fats and health.

21.1 INTRODUCTION

Fats, small molecules present within living tissue, are comprised mainly of glycerol esters of fatty acids, triacylglycerols (TAGs) being the predominant components. Food fats and oils also usually contain traces of nonglyceride substances, including phospholipids, sterols, and pigments. Fats are commonly described based on their source, for example, fish oil, bird oil, and pork fat (lard), and belong to the general class of compounds called *lipids*, which may be classified based on their composition, origin, and nature. Lipids encompass a diverse range of compounds that are generally non-polar or water-insoluble, and are derived from living organisms [1]. It must be noted, however, that some compounds classified as lipids (albeit only a minute percentage) are in fact water-soluble. Lipids include glycerides (such as mono-, di-, and TAGs), phospholipids, prostaglandins, steroids, carotenoids, and waxes. Similar to carbohydrates and proteins, fats represent a significant proportion of the constitution of living organisms. Of the numerous structural classes of fats, TAGs represent the overwhelming majority in animals (and indeed in living organisms). Lipids are present in all organs of animals and are also present as depositions in various places within organisms. Larger amounts of fats are associated with connective tissue, adipose tissue, bone marrow, brain, liver, and about the kidneys. Fat, in addition to protein, is a major component of whole milk powder [2]. Fats may exist as liquids or solids at room temperature and

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may respectively be described as oils or simply as fats on this basis. Of the three basic foods, carbohydrates, proteins, and fats, the latter are the most energy-rich. The types of lipids that have the most significant impact on health are arguably fatty acids (especially in the form of TAGs). As such, these substances will be the main subject of focus in this chapter. Furthermore, fish oils account for the majority of lipidaceous substances that are consumed specifically for the promotion and maintenance of good health, and will therefore be discussed at length.

Fats, small molecules present within living tissue, are glycerol esters of fatty acids that belong to the general group, lipids. Lipids include glycerides (such as mono-, di-, and TAGs), phospholipids, prostaglandins, steroids, carotenoids, and waxes, of which TAGs are the overwhelming majority.

21.2 CLASSIFICATION OF LIPIDS

There are various classifications of lipids that exist in living tissue. One of the more straightforward was developed by Bloor in 1920 [3]. In this classification, lipids are divided into three groups: simple lipids (comprised of fats and waxes), compound lipids (inclusive of phospholipids and glycolipids), and derived lipids (inclusive of fatty acids, glycerol, and sterols, with cholesterol, bile acids, and vitamin D being examples of animal sterols). Upon hydrolysis, simple lipids directly yield two types of products per mole: fatty acids and an alcohol (usually glycerol). Simple lipids are further divided into neutral fats or acylglycerols and waxes. The term *neutral fats* is generally used to describe fatty acid esters of glycerol. These may be mono-, di-, or triesters of glycerol, the latter being the major ones found in nature. TAGs are also known as triacylglycerides and triglycerides, the latter being the least acceptable term, chemically, though very frequently used in nutrition literature. Animal (and plant) fats and oils are comprised mostly of this group of compounds. They are esters comprised of three fatty acid molecules attached to a glycerol backbone. Compound lipids give rise to at least three types of primary products upon hydrolysis. Phosphatides, sphingolipids, glycolipids, and sulfolipids are included in this group, of which phospholipids are the most abundant [4]. Fatty acids, phospholipids, and cholesterol are discussed further below. Waxes are discussed in Chapter 22, Waxes.

Lipids may be divided into three groups: simple lipids (comprised of fats and waxes), compound lipids (inclusive of phospholipids and glycolipids) and derived lipids (inclusive of fatty acids, glycerol and sterols such as cholesterol, bile acids and vitamin D).

21.2.1 Fatty Acids—Components of Acylglycerols

Fatty acids may be termed, short, medium, long, or very long-chained based on the number of carbons (2−4, 6−10, 12−18, and 20−24 carbon atoms, respectively). No strict convention exists for the classification of fatty acids based on chain lengths, and so several different versions of this classification can be found in the literature [5,6]. Naturally occurring fatty acids generally have an even number of carbons arranged in a straight chain with most having 14−24 carbons present. Fatty acids with odd numbers or branched chains are more characteristically found in microorganisms and dairy fats. Dairy fats and tropical oils possess significant amounts of short-chain fatty acids. Fatty acids may also be described as saturated (having no carbon−carbon double bonds) or unsaturated [7]. Those having one C = C double bond are called monounsaturated (MU), with those possessing two or more being described as polyunsaturated (PU). Double bonds in fatty acids naturally occur in the *cis*-configuration and are separated by a methylene (i.e., CH_2) group. Subsequently, if the position of the first double bond is known, those of all the others may be easily predicted, with the exception of conjugated *trans* fats such as conjugated linoleic acids (CLA) found in ruminant fats. Double bonds may be numbered starting from the carboxylic acid end or the methyl end (named omega or n- end; Fig. 21.1). Although fatty acids are formally named based on the number of carbons and double bonds present, they are often given common names based on their source.

Trans fats are uncommon in nature, with the exception of ruminant fat (including cows and sheep), which contains vaccenic acid as the main *trans* fatty acid, produced as a result of incomplete biohydrogenation of linoleic and linolenic acids by microorganisms in the rumen. Dairy fat contains 2−9% *trans* fatty acids. Industrial *trans* fatty acids are produced by partial hydrogenation of vegetable or fish oils. *Trans* fatty acids from industrial sources are known to lower high-density lipoprotein cholesterol (HDL-C), raise low-density lipoprotein cholesterol (LDL-C), and increase the risk of coronary heart disease (CHD). The major *trans* fat in partially hydrogenated vegetable oil products is elaidic acid,

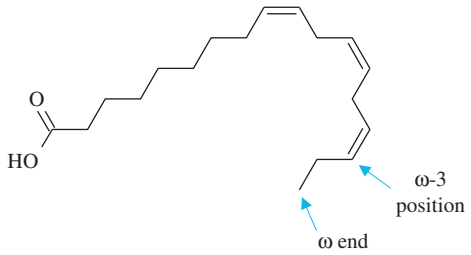

FIGURE 21.1 Structure of alpha linolenic acid (18:3n-3; or 18:3Δ9,12,15).

TABLE 21.1 Fatty Acids Commonly Found in Foods

	Common and Numerical Names	Systematic Names	Food Sources
Saturated			
	Caprylic (8:0)	Octanoic	Palm and coconut oil
	Capric (10:0)	Decanoic	Goat and cow butter
	Lauric (12:0)	Dodecanoic	Coconut oil and palm kernel oil
	Myristic (14:0)	Tetradecanoic	Coconut oil, dairy fat
	Palmitic (16:0)	Hexadecanoic	Palm oil, meat, and dairy fats
	Stearic (18:0)	Octadecanoic	Meat, poultry, fish, and grain products
Unsaturated			
Monounsaturated	Oleic (18:1n-9)	*cis*-9-Octadecenoic	Olive, canola, and sunflower oil
Polyunsaturated	Linoleic (18:2n-6)	all-*cis*-9,12-Octadecadienoic	Corn, safflower, evening primrose, and grape seed oil
	α-Linolenic (18:3n-3)	all-*cis*-9,12,15-Octadecatrienoic	Canola oil, walnuts, flaxseed, flax oil
	Arachidonic (AA) (20:4n-6)	all-*cis*-5,8,11,14-Eicosatetraenoic	Chicken, eggs
	Eicosapentaenoic (EPA) (22:5n-3)	all-*cis*-5,8,11,14,17-Eicosapentaenoic	Marine algae, fish oils
	Docosahexaenoic (DHA) (24:6n-3)	all-*cis*-4,7,10,13,16,19-Docosahexaenoic	Animal fats as phospholipid component, fish oils, dairy products

whereas *trans* isomers of C20:1, 20:2, 22:1, and 22:2 are found in partially hydrogenated products from marine origin. Although the effects of *trans* fatty acids from natural sources are less clear, some research suggest that all *trans* fats have a similar effect on plasma cholesterol levels. Research in this area is however limited and, in some cases, conflicting [8,9]. Fatty acids commonly found in foods are shown in Table 21.1.

Fatty acids may be termed, short, medium, long, or very long-chained based on the number of carbons (2–4, 6–10, 12–18, and 20–24 carbon atoms, respectively), and as saturated (having no carbon-carbon double bonds) or unsaturated (possessing at least one carbon-carbon double bond). Most of these double bonds occur naturally in the *cis*-configuration.

Trans fats are uncommon in nature, with the exception of ruminant fat. *Trans* fatty acids from industrial sources are known to lower HDL cholesterol, raise LDL cholesterol, and increase the risk of coronary heart disease. Some research suggest that all *trans* fats have a similar effect on plasma cholesterol levels.

Saturated fatty acids (SFAs) having 10 or more carbon atoms are solids at 25°C and are a more dominant feature in animal fats than plant oils, with unsaturated fats being more prominent in plant oils. Unsaturated fatty acids are liquids, as are all SFAs with less than 10 carbon atoms, and the higher the degree of unsaturation, the lower the melting point of a fatty acid. Long-chain omega-3 fatty acids are characteristically found in large amounts in fish oils compared to fats from terrestrial animals (Tables 21.2 and 21.3). Oleic acid and linoleic acid (LA) are the main fatty acids in most vegetable oils.

Palmitic, stearic, and oleic acids are dominant in terrestrial animal fats, while palmitic, oleic, eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids are among the dominant fatty acids in marine oils. Oleic and LAs are the main fatty acids in most vegetable oils.

Phospholipids are the most abundant type of lipid constituents in cell membranes, their chief role involving structural integrity of the membrane bilayer. They are glycerol esters in which (most of the times) two of the glyceride OH groups are linked to fatty acids while the other is attached to a phosphate group. The phosphate is then linked to a simple, polar organic molecule. The majority of phospholipids are comprised of a diacylglycerol, a phosphate group, and a simple organic molecule, such as ethanolamine or choline. As they possess an abundance of LA, phospholipids are susceptible to autooxidation [10]. Palmitic acid is also commonly present.

Phosphatidylcholine (PC), which is often referred to as lecithin, is the most abundant class of lipids in animal cell membranes, accounting for nearly half of the total. Similarly they are the major components of plant membranes. Lecithin serves as a surface-active agent in the production of emulsions. Commercially, egg yolk is the most important animal source of lecithin (with soy being the most important plant source). PC is the main plasma phospholipid and an important component of lipoproteins, especially HDL. It strongly influences the circulation of different classes of lipoprotein, more so the very-low-density lipoproteins (VLDL). PC is the biosynthetic precursor of phosphatidic acid, lysophosphatidylcholine platelet-activating factor, and phosphatidylserine. Additionally, it provides the choline for sphingomyelin (one of many sphingolipids) biosynthesis. Phosphatidylserine, phosphatidylethanolamine, and lysophosphatidylcholine are among the more abundant phospholipids. Table 21.4 provides information on their various functions, as well as those of sphingolipids [4].

Phospholipids are the most abundant type of lipid constituents in cell membranes, with PC (which is often referred to as lecithin) being the most abundant class. Commercially, egg yolk is the most important animal source of lecithin, with soy being the most important plant source.

21.2.2 Cholesterol

Cholesterol is the most dominant sterol in animal fats and oils, being present in vegetable oils in negligible amounts [5]. In addition to synthesis by the body (within the liver), it may also be obtained from the diet via the consumption of animal foods. Cholesterol has a number of important biological roles and is required for human life and health. It resides mainly in membranes, the brain being the most concentrated source of cholesterol among animal organs. Due to its water insolubility, cholesterol has to be combined with water-soluble proteins in order to be transported in the body, thereby forming lipoproteins [5]. These lipoproteins (e.g., HDL and LDL) have been associated with much controversy as it relates to their relationship with cardiac health. High levels of cholesterol in the blood can lead to the development of atherosclerosis, and has been associated with a myriad of health problems. For this reason, cholesterol is considered “bad” by the masses. Functions of cholesterol however include modulation of membrane fluidity and permeability, maintenance of the structural integrity of membranes, development and functioning of central nervous system, sperm development, and embryonic development. Additionally, it is a precursor in the biosynthesis of vitamin D, steroid hormones, and bile acids. The latter facilitate the digestion and absorption of lipids and the prevention of cholesterol buildup in the bile. This is owing to their strong emulsifying properties.

21.2.3 Vitamin D

The fat-soluble vitamin D (cholecalciferol) is known mostly for its role in calcium and phosphorus metabolism, and, by extension, bone development and the prevention of rickets. Humans and other animals naturally produce this vitamin

TABLE 21.2 Fatty Acid Composition (% wt) of Fish Oils and Other Marine Sources

	Myristic 14:0	Palmitic 16:0	Stearic 18:0	Palmitoleic 16:1	Oleic 18:1	Cetoleic 22:1	Eicosenoic 20:1	EPA 20:5	DPA 22:5	DHA 22:6	SFA	MUFA	PUFA	Omega-3	Omega-6
Fish Oils															
Capelin	7	10	NA	10	14	14	17	8	NA	6	17	55	14	14	
Norway pout	6	13	NA	5	14	12	11	8	NA	13	19	42	21	21	
Mackerel	8	14	NA	7	13	15	12	7	NA	8	22	47	15	15	
Sardine/ Pilchard	8	16	NA	10	11	3		18	NA	9	26	30	27	27	
Horse mackerel	8	18	NA	8	11	8	5	13	NA	10	26	32	23	23	
Anchovy	9	19	NA	9	13	2	5	17	NA	9	28	29	26	26	
Menhaden[a]	8	29	4	8	13	2	1	10	1.5	13	41	24	24.5	23	1.5
Blue whale[a]	5	8	0	1	1	14	22	2.5	1.5	3	13	38	7	5.5	1.5
Seal[a]	4	7	1	16	28	7	12	5	3	3	12	63	11	8	3
Cod liver[b]	4	13	3	6	17	8	9	10	1	13	20	45	24	23	1.2
Shark liver[b]	4	32	9	7	22	0	2	1	2	5	45	31	8	6	2
Fish Oil Capsules[c]															
Enriched fish oil	0	1	1	0	8	NA	4	41	4	33	2	12	78	74	4
Pure fish oil	9	16	3	9	9	NA	2	22	3	16	28	20	41	38	3
Salmon oil	7	24	4	12	8	NA	2	17	3	16	35	22	36	33	3
Cod liver oil	5	19	3	8	14	NA	9	13	2	16	27	31	31	29	2
Krill oil	11	21	5	8	8	NA	3	19	ND	16	37	19	35	35	ND

NA, not analyzed.
[a]*Belitz, HD, Grosch, W, Schieberle, P. Food Chemistry. 5th ed. Springer, Berlin, 2009.*
[b]*Nunez, GC. Quality and stability of Cuban shark liver oil: comparison with Icelandic cod liver oil [dissertation]. [Iceland]: The United Nations University; 2007.p. 38.*
[c]*Adapted from Raber, G, Laoteng, K, Francesconi, KA. Identification and characterization of fish oil supplements based on fatty acid analysis combined with a hierarchical clustering algorithm. Eur J Lipid Sci Tech 2014;116:795–804 [16].*

TABLE 21.3 Fatty Acid Composition (%wt) of Terrestrial Animal Fats

	Myristic 14:0	Palmitic 16:0	Stearic 18:0	Palmitoleic 16:1	Oleic 18:1	Linoleic 18:2	Linolenic (18:3)	SFA	MUFA	PUFA	Omega-3
Beef tallow	3	26	20	4	40	5	0	49	44	5	0
Sheep tallow	2	21	28	3	37	4	0	51	40	4	0
Lard	2	24	14	4	43	9	1	40	47	10	1
Goose fat	1	21	7	3	58	10	2	29	61	12	2
Emu fat[a]		22	8	4	48	11	2	30	52	13	2

[a]*Abdominal fat.*
Source: Wang, YW, Sunwoo, H, Sim, JS, Cherian, G. Lipid characteristics of emu meat and tissues. J Food Lipids 2000;7:71–82 [30]. Adapted from Belitz, HD, Grosch, W, Schieberle, P. Food chemistry. 5th ed. Springer, Berlin, 2009.)

TABLE 21.4 Phospholipids and Sphingolipids and their Functional Properties

Phospholipid	Locale	Function	Sources
Phosphatidylcholine	Cell membrane Pulmonary surfactant	Structural element of biological membranes Cell signaling Biosynthetic precursor of sphingomyelin	Egg yolk Soybean
Phosphatidylserine	Cell membrane	Component of cellular membranes Precursor for other phospholipids Essential cofactor that binds to and activates a large number of proteins Blood coagulation process in platelets Regulation of apoptosis Key component of the lipid-calcium-phosphate complexes that initiate mineral deposition during bone formation	Bovine brain Cabbage Soybean
Lysophosphatidylcholine	Cell membrane Blood plasma	Proinflammatory properties Cell signaling	Oats, egg yolk, soybean
Phosphatidylethanolamine	Sarcolemmal membranes, nerve tissue	Membrane fusion Secretion of lipoproteins	Egg yolk
Sphingolipids	Biological membranes	Signal transmission Cell recognition	Dairy, eggs, soybean

Source: Jing L, Xuling W, Ting Z, Chunling W, Zhenjun H, Xiang L, et al. A review on phospholipids and their main applications in drug delivery systems. Asian J Pharm Sci. 2015;10:81–98.

(from cholesterol) in their skin upon exposure to sunlight. It may also be obtained from the diet (only small amounts are naturally present in most foods) or dietary supplements. Good food sources of vitamin D include fatty fish, such as salmon, sardines, and herring, beef, eggs, and fortified foods, such as cereal and milk.

21.3 EXTRACTION OF ANIMAL FATS

Over the last decade the total production of oils and fats has grown by over 50%. This, however, is mainly attributed to plant oils. The production of animal fats, which involves more complex and costly processes, has grown by 12%. Unlike oilseeds which are cultivated for the oils they produce, animals are not generally reared for their fats, but for other products, such as eggs, milk, and meat. Less than 5% of commercial animal fats are used for human consumption, the major applications being for fuel, animal feed, soap, oleochemicals, and pet food. There are many types of animal fats, the most commonly used for culinary purposes being pork lard. The only animal fat that has significant application for the promotion or maintenance of health is fish oils. Menhaden, anchovy, capelin, sardines, and jack mackerel may

be caught specifically for their oil and fishmeal. Animals reared on pasture rich in omega-3 acids as opposed to feeds high in omega-6 oils are, for some communities, the next best source of long-chain PUFAs which are usually obtained by consuming fish [11]. Animal fats are usually extracted using heat, via rendering.

21.3.1 Rendering

Depending on the physical state of the animal fat to be extracted (dried or wet), dry or wet rendering is applied, respectively. The latter is normally used for edible oils. In wet rendering, the raw material is first crushed and minced, followed by melting using dry or moist heat at 70−90 °C. Three phases are produced: solid, aqueous, and melted fat. The fat is obtained by separation of the liquid phase via a decanter, which utilizes centrifugal force. The solid phase is drained, pressed, and dried. In dry rendering, the dried raw material is crushed, cooked, and dried (usually contact drying). Disc dryers and evaporation towers are often used. Pressing of the dried material produces fat and solids. The fat obtained from the wet or dry rendering process is cleaned using different methods, often in combination. The fat may be sieved to remove extraneous fibers and plastics. Sedimentation may also be carried out, where the fat is separated from water and solids by decanting after storage in a conical-bottom tank. Centrifugal force is applied in the use of decanters and separators, the former being horizontal equipment. Water or acid may be added to improve the cleaning of the fats. Physical adsorption on activated carbon may be applied for removing contaminants, including dioxins, furans, polychlorinated biphenyls, and polynuclear aromatic hydrocarbons (PAHs), from fish oils intended for the nutraceutical, animal feed, aquaculture, or pet food market [11−13].

21.3.2 Oil Refining

Extracted crude food oils are a complex mixture containing several classes of compounds including glycerides, free fatty acids, sterols, phospholipids, pigments, and at times, toxic substances [14]. Oil refining is therefore required to remove impurities to produce edible oils. It is not without its shortcomings, though, as some neutral oils are lost, and it involves the use of alkalis which are unfriendly to the environment [15].

Degumming (via addition of water) serves mainly to remove phospholipids and other substances such as resins from the oil. Neutralization, which follows, removes free fatty acids, which form insoluble soaps and are removed with the water fraction. This results in a decrease in oil acidity. Washing with water removes remaining soaps and residual caustic, in addition to oxidation products and trace metals. The oil is then dried and bleached. Bleaching removes pigments that cause undesirable colors or promotes oxidation. Residual free fatty acids, phospholipids, and toxic contaminants are also removed during the bleaching step. Winterization, which follows, involves storing the oils at low temperatures, which results in the crystallization of waxes and the more saturated TAGs, which have higher melting points. This yields oils with a greater percentage of PUFAs. Deodorization, the finishing step, effects the removal of volatile compounds with objectionable odors. This step is particularly important in crude fish oils, in order to reduce the fishy odor and improve sensory quality [11]. It however involves the use of temperatures above 180 °C, which can cause PUFA degradation, further resulting in the formation of undesirable products such as *trans* fatty acids and polymers [16,17]. Alternative methods for the elimination of odorous compounds from fish oils have been recommended. These include silica gel column treatment subsequent to vacuum steam distillation at low temperatures, treatment with diatomaceous earth, or adsorption on a resin. New technologies are being explored for the refining of fish oils involving supercritical fluid extraction together with membrane and enzymatic processes. High-quality oils have been obtained from these methods [18].

Animal fats are usually extracted using heat, via rendering. Crude animal oil is refined via several steps which include degumming, neutralization, bleaching, winterization, and deodorization.

21.3.3 Extraction of Marine Oils

Global fish oil production has been fairly constant, being around 1 million tonnes yearly. Fish oils are produced mainly in South American countries (Peru and Chile), Europe (Norway, Denmark, and Iceland in particular), and Japan. Continued increasing demands compounded by severe shortage in supply propelled the prices of fishmeal and fish oil to a record high in the latter part of 2014. The majority of fish oils are obtained from the flesh or body of the fish, with menhaden being the major source. Small amounts are obtained from the liver and head of some fish. In sharp contrast

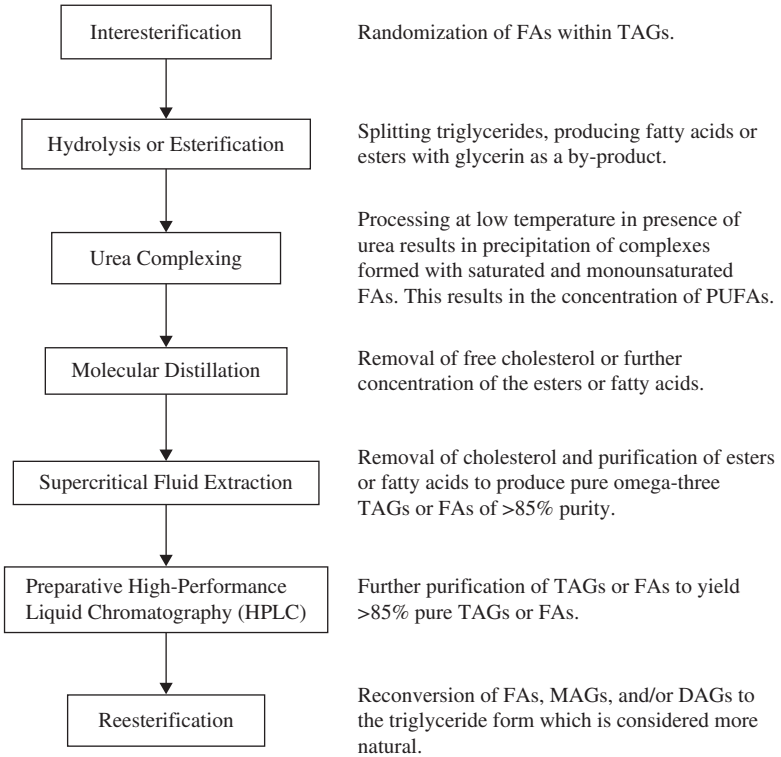

FIGURE 21.2 Additional processing steps for the production and purification of marine oil omega-3 fractions from refined fish oil.

to fats from terrestrial animals, more than 95% of fish oils produced are used for human consumption, being mainly utilized as supplements. Fig. 21.2 outlines the processing steps required for the purification of marine oil omega-3 fractions for the production of nutraceuticals and pharmaceutical products [19]. The process involves oil extraction, refining, purification, and enrichment [20].

Traditionally, krill oil is extracted using either the wet reduction process onboard the sea vessel, or via a solvent extraction of the dried krill at a factory on land. A novel onboard process has been developed for the extraction of krill oil. The technology which was patented in 2009 does not involve solvents, and produces an oil that is said to be of superior value compared to that obtained from the wet rendering process, being richer in EPA and DHA, phospholipids (which are the main class of lipids in this oil), and the antioxidant, astaxanthin. Furthermore, the claimed absence of toxicants, such as residual solvents, dioxins, and PAHs, is of note [21].

Various methodologies are currently employed for producing highly purified omega-3 fatty acids. PU oils obtained from fish, krill, and microalgae possess a myriad of beneficial effects, probably the most noteworthy being the prevention and management of cardiovascular disease. Various physical, chemical, and enzymic methods are utilized and include urea adduction, chromatography, rapid solidification [22], low-temperature fractional crystallization, supercritical fluid extraction, and distillation [23]. Of the developed methods, urea crystallization is the most simple and efficient. Crystallization is essential for purification and separation with urea being able to form crystals with saturated and MU fatty acids. Urea readily forms solid-phase complexes with SFAs thereby allowing for the separation of PUFAs and branched from saturated FA [24]. Urea complexation allows for the handling of large quantities of material, allows the use of simple machinery and low-cost solvents, and is associated with milder processing conditions. Urea complexation also protects PUFAs from autoxidation reactions [25].

21.4 NUTRACEUTICAL APPLICATIONS

Nutraceuticals promote health, prevent disease, and are considered as semimedicinal. They are chemically diverse and include isoprenoid derivatives, phenolics, carbohydrate derivatives, amino acid derivatives, capsaicinoids, and fatty acids and their derivatives. Various nutraceuticals are available commercially. Nutraceuticals beneficial to the heart, also referred to as cardioprotective nutraceuticals, include omega-3 fatty acids, vitamins, minerals, antioxidants, and

dietary fibers. Their mode of action is that of serving as biochemical metabolites, directly intervening in intermediary lipid metabolism, or regulating proteins of the vascular system [26]. The nutraceutical applications of fish (marine) and bird (terrestrial) oils are presented below.

Cardioprotective nutraceuticals include omega-3 fatty acids, vitamins, minerals, antioxidants, and dietary fibers.

21.4.1 Fish Oils

Fish oils account for the majority of lipidaceous substances that are applied for health-beneficial purposes. They are usually encapsulated and utilized as dietary supplements. Dietary supplementation with fish oils has become quite popular in the last few decades, in association with its effect on cardiovascular health, fetal development, cancer, and diabetes. Omega-3 fish oil is considered as a nutraceutical, exhibiting anti-inflammatory activity, protection from age-related macular degeneration (a common eye ailment), and hypocholesterolemic activity. Its application for the treatment of diabetic dyslipidemia was reported by several researchers in the late 1980s [27]. In Japan, the observation was made that there was a reduced occurrence of CVD in communities consuming fish, which catalyzed investigative research on the relationship between fish and its nutritional components on CVD [28,29]. An inverse association has been shown in most of the studies conducted between fish consumption and the risk of CHD. The consumption of fish and elevated blood levels of omega-3 fatty acids are also associated with reduced risk of sudden death. When a comparison was done between fish eaters versus non fish eaters, average concentrations of HDL were found to be modestly higher in fish eaters and LDL concentrations lower, resulting in an improved HDL:LDL ratio. Interestingly, in one study, it was observed that the Inuits who are regular eaters of fish, had a low incidence of cardiovascular disease in spite of there being a high prevalence of obesity and smoking [30,31]. It is apparent that habitual fish consumption offers protection against CVD, the benefits are, however, influenced by the type of fish consumed. Fatty fish (e.g., tuna, mackerel, trout, salmon) provide more DHA and EPA, conferring greater cardioprotection than white fish [32]. Fish oil supplements are generally derived from tuna, sardine, mackerel, herring, anchovy, menhaden, cod, or salmon, as they contain high levels of EPA and DHA [20].

Currently the quantity of omega-3 fatty acids consumed in the average Western diet is approximately 0.15 g/day. Intakes of 1.5 g of EPA plus DHA per week, or approximately 0.2 g/day, for healthy individuals are recommended by The UK Department of Health. This would be equivalent to two servings of fatty fish per week. The recommendation is that the ratio of omega-6 to omega-3 in the diet be 4 to 1.

Habitual fish consumption (especially fatty fish including salmon and mackerel) offers protection against CVD. Approximately 0.2 g EPA plus DHA per day, which is equivalent to two servings of fatty fish per week, is recommended for healthy individuals.

21.4.2 Bird Oils

In the USA, the emu, ostrich, and rhea, all from the Ratite family, are raised and produced for their oil, meat, and leather. The basic composition of their oils is quite similar, consisting mostly of oleic, palmitic, stearic, and linolenic acids. The oils are mainly used in the cosmetic industry, being incorporated into products such as moisturizing creams and lotions, lip balms, soaps, and sports ointments. The emu (*Dromaius novaehallandiae*), a flightless bird native to Australia, will be discussed here.

Emu oil has a characteristic bright yellow color. The medicinal properties of the emu oil appear to be related to its high content of unsaturated fats (especially oleic and LAs) as well as the presence of carotenoids, flavones, and other antioxidants. All three families of omega fatty acids (9, 6, and 3) are present in levels greater than that of chicken and beef [33,34]. As shown in Table 21.3, oleic acid is present in the largest quantities ($>45\%$ of the total), with saturated fats comprising close to a third of the total percentage [35]. Emu oil may be utilized as a supplement and is available commercially in Australia and the USA.

Liquid fat extracts from the emu have been utilized by natives of Australia for various ailments. Administration has been topical and oral. The oil possesses potent anti-inflammatory activity [36] and has been utilized in the treatment of various inflammatory conditions such as mucositis, inflammatory bowel syndrome, and auricular inflammation. It also has significant hypocholesterolemic and antiatherosclerotic activities [37], which have been attributed to its high content

of mono- and PU fats [38]. The antidiabetic and anti-inflammatory properties of oleic acid are well documented [39,40]. The oil is also of interest to dermatologists and cosmetic scientists [41] due to its applicability in promoting enhanced skin permeation, skin proliferation, stimulated melanogenesis in skin and hair growth, moisturization, reduction of skin wrinkles, and rejuvenation of age- and photo-damaged skin. It may also be utilized in the treatment of hypopigmentation, baldness, and chemotherapy-induced alopecia [42].

Emu oil possesses potent antiinflammatory, hypocholesterolemic, and antiatherosclerotic activities, the latter two activities being attributable to its high content of mono- and PU fats. Due to its applicability, including promoting enhanced skin permeation and proliferation, stimulated melanogenesis in skin and hair growth, as well as moisturizing properties, emu oil is of interest to dermatologist and cosmetic scientists.

21.5 PHARMACEUTICAL APPLICATIONS

Animal lipids including phospholipids, stearic acid, cholesterol, and bile acids have been applied in a number of pharmaceutical applications, where they are used for their emulsifying and lubrication properties, to name a few [42–44]. Caproic (C6:0), caprylic (C8:0), and capric (C10:0) acids have been used in the synthesis of TAGs termed medium-chain triglycerides (MCTs). These were originally manufactured for use as dietary fats for treatment of individuals who lacked the ability to digest and absorb long-chain TAGs, such as those experiencing pancreatitis, cystic fibrosis, biliary cirrhosis, and Crohn's disease. MCTs are still being used in this way [6]. They have also been incorporated in the meals of newborn infants, being rapidly metabolized to provide energy for growth and development. Axona is a prescription dietary supplement composed of MCTs, which has been approved by the US Food and Drug Administration as a *medical food* for the treatment of Alzheimer's disease. This is partly due to the rapid production of ketone bodies which supply the brain with an additional source of energy. Several animal lipids have also been used as drug carriers. Phospholipids have found extensive use, and will be discussed in detail below.

21.5.1 Phospholipids

The application of phospholipids in pharmaceuticals is linked mainly to their powerful emulsifying properties. In recent years phospholipid carriers have gained considerable interest in the arena of drug delivery, the use of PC revolutionizing drug delivery technology. Several drug delivery systems are based on PC, including liposome, ethosome, phytosome, and transferosome. Dioleoyl–phosphatidyl–ethanolamine and distearoyl–phosphatidyl–choline are examples of synthetic phospholipids [45], with egg and soy being the main food sources. It is necessary that pharmaceutical agents be able to traverse cell membranes in order to facilitate their absorption, distribution, and elimination from the body. Due to their amphipathic nature, phospholipid systems are compatible with cell membranes. Lipid emulsion systems have appealing properties as drug carriers, due to their biodegradability, biocompatibility, stability, and ease of production. Applications of phospholipid-based carrier systems are shown in Table 21.5, and advantages [46] when compared to other modes of drug delivery are presented below:

1. Enhanced permeability of drug through the dermis
2. Delivery of large and diverse drugs, e.g., peptides and proteins
3. Safe composition
4. Approved for cosmetic and pharmaceutical applications
5. Low-risk profile
6. Toxicological properties of phospholipids have been well documented
7. High market attraction

Problems that may be encountered in the use of emulsions for drug administration include the uncertainty of how they navigate the circulatory system to the desired site of action. Lipid emulsions have been stabilized with phospholipids. Different phosphotidylcholine–surfactant mixtures are also being evaluated for their ability to produce small and stable emulsions, improving emulsification efficiency [47]. Phospholipid systems have the potential of serving as carriers for site-specific drug delivery and providing a sustained release system. The main applications of PC are those of intravenous treatment, prevention of fat embolisms in polytraumatized patients, treatment of metabolic disorders, and as

TABLE 21.5 Applications of Phospholipid-Based Carrier Systems

Phospholipid Carrier Systems	Drug	Application
Liposomes	Insulin	Oral, ocular, pulmonary, and transdermal delivery Decrease glucose level
Ethosome	Acyclovir	Treatment of herpetic infection Improved drug delivery
Phytosome	Botanical extracts	Enhanced bioavailability
Transferosomes	Corticosteroids	Treatment of skin diseases
Long circulating liposomes	Serum proteins	Increased circulation time
Nanocochelates	Nanocochleates	Deliver proteins, peptides, and DNA for vaccine and gene therapy applications

Adapted from Verma, P, Ram, A, Jha, AK, Mishra, A, Thakur, A. Phosphatidylcholine: a revolution in drug delivery technology. Int J Pharm Sci Res 2010;1:1–12 [41].

a liver protectants [48]. PC is of importance as a drug carrier due to significant advantages associated with it. Phospholipid-based carrier systems [45] include:

Liposomes: used as therapeutic tools in tumor targeting, topical applications, genetic vaccination
Ethosomes: provide enhanced delivery through the skin
Phytosomes: contain active ingredient surrounded by the phospholipid producing better absorption of herbal products
Transferosomes: transdermal drug carriers
Long circulating liposomes: modification of liposomes with lipids to increase circulation time
Nanocochelates: consist of at least 75% soy-based phospholipid and a multivalent cation

Solubility is a primary issue in drug development. Mixed micelle (MM) systems have been developed to improve the solubility and bioavailability of drugs. MM are thermodynamically stable, nanosized vehicles with enhanced vascular permeability. Classical MM systems include PC and bile salts. Alternative MM systems with enhanced solubilization properties for hydrophobic drugs are being developed. Novel MM systems composed of PC and other surfactants can provide enhanced solubilization. In a study conducted by Rupp et al. [49], enhanced solubilization was achieved for water-insoluble drugs in a MM system comprised of sucrose laurate and hydrogenated PC.

The application of phospholipids in pharmaceuticals is linked mainly to their powerful emulsifying properties. The application of PC has revolutionized drug delivery technology. Drug delivery systems based on PC include liposomes, ethosomes, phytosomes, and transferosomes.

PC is being considered for treating “brain-centered” conditions, such as memory loss, Alzheimer’s disease, anxiety, manic-depressive disorders, and a movement disorder called tardive dyskinesia, due to the body’s use of PC to make the brain chemical, acetylcholine. It has been recommended that patients consume omega-3 fatty acids, especially DHA and EPA. Clinical evidence supports the benefit of DHA for individuals with mild memory complaints [50]. PC has also been used for treating other conditions such as hepatitis, eczema, gallbladder disease, circulation problems, high cholesterol, and premenstrual syndrome.

PC and the bile salt, sodium deoxycholate (DOC), are the active ingredients in products applied in injection lipolysis, a procedure used in cosmetic medicine to reduce localized fat accumulation. This is facilitated by the intralesional injections of substances that induce destruction of adipocytes. DOC is an ionic detergent capable of dissolving phospholipids thereby reducing adipose tissue [51]. In the cosmetic industry, several countries initially imported a prescription intravenous drug product from Germany known as Lipostabil (contains both compounds), which was used subcutaneously for cosmetic purposes. The manufacturer of this product however does not promote it for this use due to lack of scientific evidence. Lipostabil is unapproved for drug use in the United States. Other cosmetic products utilized for lipolysis include Lipodissolve, Lipolight, Lipolyse, and Lipotherapy.

21.6 FATS AND HEALTH

Compared with other food groups, the relationship that exists between fats and health is probably one of the most controversial. With the progression of time, scientific findings have revealed new biological activity of fats, corroborated previous knowledge on the subject, led to the elucidation of mechanisms underlying existing observations, and in other cases, totally contradicted previously held beliefs. This section will summarize the currently held views on the effect of selected types of fats on health. Although fats have not enjoyed the best reputation as far as their association with health is concerned, a growing body of evidence has been changing this with the progression of time. This is greatly attributable to studies of the effects of different kinds of fats on various health markers. For example, high levels of omega-3 fatty acids are known to have a protective effect against a number of degenerative diseases, including cardiovascular disease and diabetes. On the other hand, degenerative diseases, which are oftentimes negatively impacted by high levels of fats in general, contribute to the premature death of the majority of individuals globally: in both developed and developing nations. Almost 80% of deaths due to noncommunicable diseases (NCD) occur in low- and middle-income countries [52,53]. Cardiovascular disease and cancer are two of the leading causes of death globally. Diabetes and chronic obstructive pulmonary disease are also among the top 10 leading causes of death globally. In the United States, 51% of adults who are 65 and over have diabetes [54]. It is estimated that NCD will account for approximately 52 million deaths by the year 2030 [54–57]. During the last century, CVD has grown from a relatively minor disease globally to a leading cause of morbidity and mortality. This disease was responsible for 50% of all deaths in the year 2000 in Europe [58]. More than 58 million Americans have at least one form of cardiovascular disease—stroke, hypertension, or CHD. One in nine women, and one in six men aged 45–64 have some form of heart disease. Myocardial infarction (MI), more commonly called heart attack, is a major cause of death among Americans [59], albeit cancer is the leading cause of death for people under 65 years of age. From this data it is clear that NCD are major public health problems globally, regardless of socioeconomic status.

What will become apparent from reading this section is that it would be quite misleading to view fats as a single entity rather than looking at specific fat classes and their effects on different physiological systems. Due to their overwhelming abundance within this group of compounds (i.e., lipids), the focus of this section will be on fatty acids, important components of TAGs.

21.6.1 Saturated Fats

Although much association has been made about the negative effect of high dietary SFAs on cardiovascular health, the scientific evidence of this is lacking [60]. Many of the shorter-chain fatty acids found in milk fat and coconut oil have positive effects on health. Medium-chain fatty acids possess several unique properties resulting from their shorter chain lengths. They provide 10% less calories compared to long-chain fatty acids (i.e., 8.3 vs 9 cal/gram), are more rapidly absorbed and burned as fuel, are stored in fat deposits to a much lesser extent than long-chain fatty acids, and enhance thermogenesis. Additionally, they are oxidized by the liver to ketone bodies, which serve as an alternative energy source. It has been demonstrated that including dietary fats which are high in medium-chain fatty acids may be advantageous in individuals experiencing mild Alzheimer's disease (particularly those who are $APOE4^{-}$, a status that can only be genetically determined), as the increase in energy due to the ketone bodies may improve neuronal metabolism and survival, resulting in improved cognitive functioning. Medium-chain fatty acids have been found to provide fuel to the brain via the generation of ketones bodies, an advantage for type 1 diabetics, as this facilitates the preservation of brain function under hypoglycemic conditions without raising blood glucose levels [61–64].

Myristic acid is abundant in dairy fats as well as coconut and palm oils. Lauric acid is the major SFA in coconut oil. Fifty percent of the cholesterol-elevating effect of myristic acid is actually due to its effect on HDL-C. Hence, the relative cardiovascular health effects of these three fatty acids may well be comparable. However, contrary to unsaturated fatty acids, these three fatty acids raise LDL-C. The medium-chain SFAs in coconut oil and butterfat (milk) increase total serum cholesterol, but their positive effects on HDL-C are protective in many ways.

The shorter-chain SFAs in milk (C4–C12) are rapidly metabolized for energy in infants [65]. In fact, fat accounts for about half of the calories in milk (whether human milk or infant formula), and are a major energy source to adequately support growth and other metabolic requirements [60]. Additionally, antitumor, antimicrobial, antiviral, and immune response functions are among the health-promoting effects of milk that have been reported [66]. Lauric acid is effective in preventing tooth decay and plaque buildup [67]. Some studies also indicate that dairy products are not associated with a higher risk of CVD [68]. SFAs are not susceptible to lipid peroxidation. They therefore, unlike PUFAs (especially LA), do not contribute to the deposition of oxidized LDL particles in artery-lining macrophages. The cooking

methods used for foods high in saturated fats may actually be contributing significantly to the related negative health effects via the production of substances from the degradative reactions of PUFAs and other food constituents in those foods that are associated with disease [65]. All that being said, based on clinical studies, the likelihood of adverse coronary events is reduced upon dietary substitution of saturated fats by PU oils, and in particular, n-3 PUFAs [69–71]. It is important to note here that not all PUFA are equal. A meta-analysis of randomized controlled trials revealed that, whereas diets in which total fatty acids and SFAs were substituted for mixed n-3/n-6 PUFAs resulted in reduced risk of fatal and nonfatal MI and CHD death, the opposite was true when these FAs were replaced by n-6 PUFAs. The American Heart Association advises that vegetable oils that are rich in n-6 PUFAs be used to substitute for SFAs in the diet, both on an individual and population level, in order to reduce CHD risk. However, it has been posited that, based on the foregoing evidence, it may be best that this recommendation be reconsidered due to the likelihood of harm rather than the intended benefit [71].

Oftentimes there are several other factors related to overall health that correlate with the unsaturated-to-SFA ratio, including exercise, nonsmoking habits, and the maintenance of healthier diets. Other factors such as body mass index, male/female, geography, and level of physical activity must also be considered. However, various societies on CHD prevention consider a healthy diet to be comprised of a low intake of saturated fat, that is, less than 10% of energy, 2 g/day of ALA, and 200 mg/day of very long-chain omega-3 PUFAs (EPA plus DHA) per day [72].

Many of the shorter-chain fatty acids found in milk fat and coconut oil have positive effects on health, including antitumor, antimicrobial, antiviral, and immune response functions. The medium-chain SFAs in coconut oil and butterfat (milk) increase total serum cholesterol, but their positive effects on HDL-C are protective in many ways.

Medium-chain fatty acids possess several unique properties resulting from their shorter chain lengths including: the provision of lower calories compared to long-chain fatty acids; are more rapidly absorbed and burned as fuel; are stored in fat deposits to a much lesser extent than long-chain fatty acids; enhance thermogenesis; and are oxidized by the liver to ketone bodies, which serve as an alternative energy source.

21.6.2 Long-Chain PU Fatty Acids

Although long-chain fatty acids were previously described as having 12 to 18 carbon atoms earlier in this chapter, the term *long-chain polyunsaturated fatty acids (LCPUFAs)* is generally used in the literature to encompass fatty acids ranging from 18 to 24 in chain length, and this is the case here. PU fatty acids are generally considered to be good for health, playing vital roles in the prevention of cardiovascular complications, osteoarthritis, diabetes, hypertension and autoimmune diseases. Normal human development and growth requires a balanced concentration of omega-3 and omega-6 PUFA, as increased concentrations can alter physiological functions in the body. The term omega-3 fatty acids refers to a group of three fatty acids, namely, EPA 20:5n-3, DHA 22:6n-3, and alpha-linolenic acid (ALA) 18:3n-3. They have a final carbon–carbon double bond in the n-3 position (third bond from the methyl end) in common. Omega-6 fatty acids include LA 18:2n-6 and arachidonic acid (AA) 20:4n-6. Both of these families of PUFAs are required for the development of the brain and retina, as well as proper functioning of the cardiovascular system. An increased interest in the potential beneficial health effects, particularly of omega-3 fatty acids, started in the 1970s. This was catalyzed mainly by epidemiological research on Greenland Eskimos that revealed their extremely low incidence of CVD, even though their diets were traditionally rich in SFAs and cholesterol. Interestingly, the life expectancy of the Eskimos at that time was just about 40 years, which is not really long enough to develop CVD, and later studies revealed that non-Eskimos within the population had similar low incidence of CVD [73]. Notwithstanding this, the observation was explained by the significant consumption of long-chain omega-3 PUFAs from marine sources (including whale and seal) and resulted in global scale research on omega-3 fats, particularly EPA and DHA. Omega-3 PUFAs are important for the prevention of cardiovascular disease and the control of LDL cholesterol. In the race for the most health-beneficial fatty acids, omega-3 fatty acids (especially EPA and DHA) come out as the clear winners. Omega-3 fats protect against blood clots, irregular heartbeats, and high blood pressure. They are associated with proper mental function, growth, and development, as well as other beneficial properties such as antiinflammatory, hypolipidemic, antithrombotic, and vasodilatory properties.

Whereas the human body possesses the ability to synthesize saturated and MU fatty acids, it cannot synthesize the omega-3 and omega-6 PUFAs. ALA and LA, the parent fatty acids of these families, are considered essential fatty acids as neither is synthesized by humans, nor can the fatty acids of these families be interconverted. ALA and LA are converted to longer chain, more highly unsaturated fatty acids (i.e., to EPA then to DHA [n-3]; and to AA [n-6], respectively). DHA is an important component of cell membranes, especially in the brain and the retina, and therefore plays an important role in fetal brain development and vision development. AA, on the other hand, is both a membrane component as well as a precursor to prostaglandins and leukotrienes, which are powerful signaling molecules [60].

Even though the body can convert ALA to EPA and DHA, there is still a requirement for the latter FAs in the diet, this being so for two reasons. Firstly, whenever there is a high level of LA in the plasma, there is preferential biosynthesis of n-6 FAs over n-3 FAs. Secondly, it has been found that the conversion of ALA to EPA, as well as EPA to DHA, is inefficient [74,75]. ALA is found in plant oils, with flaxseed oil (also called linseed oil), berry oils, and algal oil being good sources. EPA and DHA are both commonly found in marine oils (fish oils, egg oil, squid oils, krill oil). Studies comparing supplementation using linseed oil (ALA) versus fish oil (EPA+DHA) have demonstrated that, whereas linseed oil produces a moderate increase in platelet EPA, fish oil produces a large rise in both platelet EPA and DHA [76]. A more recent study found corroborative results with dietary supplementation with n-3 PUFAs derived from fish resulting in increased levels of plasma adiponectin, suppression of inflammation, and prevention of cardiac dysfunction under pressure overload conditions. These results were not obtained by diets in which n-3 PUFAs (ALA) were obtained from a vegetable diet (linseed oil) [77]. According to Koletzko et al. [60], the consumption of ALA is significantly less effective in promoting optimal DHA status than consumption of preformed DHA and is insufficient for the maintenance of adequate levels of DHA in the fetal brain. It is clear that while n-3 PUFAs are generally good for health, the long-chain PUFA found in fish oils are superior in their effect. It must be noted that some studies have not found a relationship between the intake of fish and CHD. Such inconsistencies could be attributable to several factors including differences in methods, study populations, fish or dosages [78].

The role of DHA in cognitive and vision development is well established. Between the second half of pregnancy to the first year of life, brain growth is accelerated, and continues for the next several years. Significant cognitive, visual, and motor development takes place during this period. DHA is accumulated in the brain in utero and continues after birth, there being marked deposition in the second half of gestation, the level of deposition in the brain reaching about 4 g between the ages of 2 and 4 years. DHA is preferentially transferred via the placenta from mother to fetus, is an important structural component of retina lipids, and is the only n-3 LCPUFA that accumulates to any significant extent in the growing brain and eye. These provide strong indications of the physiological importance of DHA [60]. AA is also accumulated in the brain during pre- and postnatal development. An average daily intake of at least 200 mg DHA is recommended for pregnant and lactating women, which may be achieved by consuming one to two portions of marine fish per week, including fatty fish [20]. The consumption of oils rich in n-3 LCPUFA during pregnancy reduces the risk for premature birth. Breast milk contains a good supply of DHA, AA, and other nutrients required for the baby, and is therefore recommended for healthy term infants. When breastfeeding is not possible, infant formula having DHA levels between 0.2 and 0.5 weight percent of total fat is recommended when breastfeeding is not possible. Consumption of fish and fish oils during pregnancy is associated with slightly increased gestation period, marginally higher birth weight, and a lower risk of preterm delivery. Additionally, there are indications that a reduced occurrence of postpartum depression results from higher levels of DHA in maternal milk and increased seafood consumption.

PU fatty acids are generally considered to be good for health, playing vital roles in the prevention of cardiovascular complications, osteoarthritis, diabetes, hypertension, and autoimmune diseases. However, not all PUFAs are equal.

Omega-3 fatty acids include EPA 20:5n-3, DHA 22:6n-3, and ALA 18:3n-3. Omega-6 fatty acids include LA 18:2n-6 and AA 20:4n-6. Both of these families of PUFAs are required for the development of the brain and retina, as well as proper functioning of the cardiovascular system. Normal human development and growth requires a balanced concentration of omega-3 and omega-6 PUFAs.

Dietary supplementation with n-3 PUFAs derived from fish results in increased levels of plasma adiponectin, suppression of inflammation, and prevention of cardiac dysfunction under pressure overload conditions.

21.7 CONCLUSION

Fats—the small and intriguing biomolecules—will continue to be the subject of wide and varied research for a long time to come. Their important place in the nutraceutical and pharmaceutical arenas is well established. So what is the conclusion of the matter as far as the relationship between fats and health is concerned? It is this: fats are a *requirement* for health, and inappropriately balanced consumption of the various types of fats may, on the other hand, be detrimental to health. The topic remains a controversy. Much has been reported about the health benefits of PUFAs. However, clear distinctions should be made in discussing PUFAs, as n-3 and n-6 PUFAs have different effects on health, though both are necessary for the promotion of health. The crucial thing is the maintenance of diets comprised of an appropriate balance of fats in appropriate quantities, and for those foods to be prepared using methods that minimize fat degradation [52]. One must bear in mind also that "one size does *not* fit all," when it comes to the composition and amounts of fats in the diet are considered, as variables such as age, metabolic rate, health status, and physical activity result in different requirements for different sets of people.

21.8 PRACTICE QUESTIONS

1. Discuss the classification of lipids.
2. How does the structure of triacylglycerols impact on their physical properties?
3. Elaborate on the different types of omega fatty acids: their systematic and numerical nomenclature and their impact on health.
4. Comment on the fatty acid compositions of terrestrial fats and marine oils.
5. Outline the steps involved in the extraction and purification of fish oils.
6. Discuss the industrial preparation of omega-3 fatty acids, explaining the use of urea complexation in the process.
7. Elaborate on the applications of animal fats/oils in nutraceutical applications.
8. Elaborate on the structure and functional properties of phospholipids, and discuss their use as drug carriers.
9. In regard to their effects on health, comment on the statement: "Not all polyunsaturated fatty acids are equal."
10. Discuss the relationship between saturated fatty acids and health.
11. What are your thoughts on the controversial topic of "fats and heath"?

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Chapter 22

Waxes

W.F. Tinto[1], T.O. Elufioye[2] and J. Roach[1]
[1]University of the West Indies, Cave Hill Campus, St. Michael, Barbados, [2]University of Ibadan, Ibadan, Oyo State, Nigeria

Chapter Outline

Learning Objectives

- To define waxes
- To understand the origin and various applications of waxes
- To have a comprehensive understanding of select waxes
- The biological activities of waxes

22.1 INTRODUCTION

There are various definitions of wax, a term originally derived from the Anglo-Saxon word "weax" meaning beeswax. To the biochemist, waxes are the layer of fatty component on the surface of plant leaves, insect body, and animal skins, while technologists use the term to refer to any products that contain fatty materials obtained from plants, insects, marine, or mineral origin that are of commercial value. Waxes can also be described as hydrophobic organic substances of medium chain length. Regardless of their definition, there is no dispute that waxes have a wide range of applications. In this chapter, classic wax examples are discussed based on their origin: plant, animal, and mineral [1].

22.2 CHARACTERISTICS OF WAXES

Generally, the texture of waxes varies from soft and tacky to hard and plastic or breakable at 20°C. Most waxes have relatively low viscosity. They are insoluble in water and solubility in organic solvents is largely temperature dependent [2].

22.3 COMPOSITION OF WAXES

Waxes are made up of long-chain aliphatic substances. Generally, they contain very long-chain fatty acids, primary and secondary alcohols, hydrocarbons, sterol esters, aliphatic aldehydes, ketones, β-diketones, triacylglycerols, triterpenes,

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and sterols [2]. Genetic and environmental factors, however, influence the quality and composition of waxes [3]. Sources of wax also influence the nature of their constituents such as the chain length, degree of unsaturation, and branching. However, the aliphatic skeleton is usually unsaturated and monoenoic except for some waxes of marine origin and from some higher animals [4].

22.4 CLASSIFICATION OF WAXES

Waxes can be classified as natural or synthetic. The natural waxes can be further classified as renewable or nonrenewable. The nonrenewable natural waxes are the mineral waxes that are obtained from lignite or brown coal and may be crude or refined, e.g., montan and petrolatum.

Renewable natural waxes can be chemically modified by methods such as hydrogenation and re-esterification or chemically unmodified, e.g., animal and plant waxes [2].

22.5 SOURCES OF WAXES

Waxes are obtained from various sources and these include:

Plant: This may be different plant parts such as leaves of the carnauba (*Copernicia pruniera*) and candelilla (*Euphrobia antisyphilitica*), flower (sunflower wax, *Helianthus annuus*), fruits (berry wax, *Myrica cordifolia*), Hull (rice bran wax, *Oryza sativa*), or the seed in the case of jojoba (*Simmondsia chinensis*).

A**nimals:** Such as insects (bees, *Apis mellifera*), whale (shellac, *Physeter macrocephalus*), sheep (*Ovis aries*).

Minerals: e.g., Montan wax from brown coal or peat deposits.

Waxes can come from renewable or nonrenewable sources.
Waxes can be obtained from plants, animals, minerals, or they can be synthetic.

22.6 BIOSYNTHESIS OF PLANT WAXES

All the aliphatic components of plant waxes are synthesized in the epidermal cells from saturated very long-chain fatty acids (commonly $C_{20}-C_{34}$). 16:0 and 18:0 fatty acids are first synthesized in the stroma of plastids by the soluble enzymes forming the fatty acid synthase complex. This is followed by multiple elongation steps and is catalyzed by membrane-associated multienzyme complexes, known as fatty acid elongases. Each two-carbon extension of the chain involves four reactions:

- condensation between a CoA-esterified fatty acyl substrate and malonyl-CoA
- β-keto reduction reaction
- dehydration reaction
- an enoyl reduction to produce saturated very long-chain fatty acids with 24−36 carbon atoms.

Many different forms of the elongases have been identified, and these must interact in some manner to produce the chain length specificity observed.

There are two main pathways for biosynthesis of wax components: an acyl reduction pathway, which yields primary alcohols and wax esters, as seen in Fig. 22.1, and a decarbonylation pathway that results in synthesis of aldehydes, alkanes, secondary alcohols, and ketones, seen in Fig. 22.2.

In the acyl reduction pathway, acyl-CoA esters produced by chain elongation are reduced in a two-step process via a transient aldehyde intermediate, catalyzed by the enzyme, an acyl-CoA reductase.

The fatty alcohol produced can then be esterified via an acyl-CoA alcohol transacylase to form a wax ester. Similar mechanisms have been observed in studies with insects, algae, and birds (uropygial glands). It seems probable that wax diols are produced by insertion of a hydroxyl group into the alkyl chain of an acyl-CoA precursor.

In the decarbonylation pathway for the synthesis of wax constituents, the first step is the reduction of acyl-CoA ester to an aldehyde by means of an acyl-CoA reductase. Removal of the carbonyl group by an aldehyde decarbonylase yields an alkane, with one fewer carbon atom than the fatty acid precursor.

Further metabolism of the hydrocarbon is then possible, for example, by insertion of a hydroxyl group into the chain via a hydroxylase or mixed-function oxidase to form a secondary alcohol.

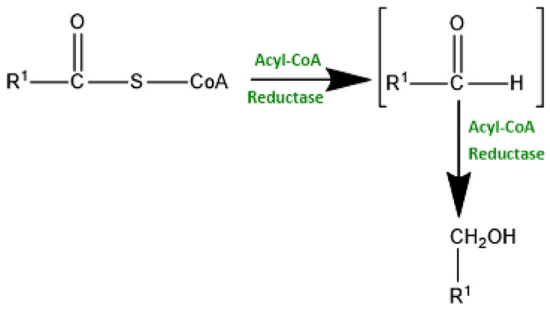

FIGURE 22.1 Biosynthesis of primary alcohols and wax esters in plants.

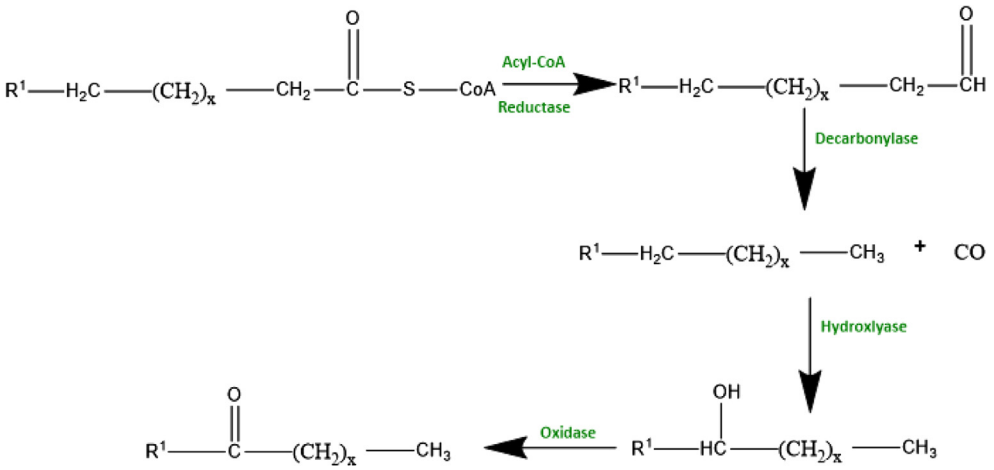

FIGURE 22.2 Biosynthetic pathway for the synthesis of alkanes, secondary alcohols, aldehydes, and ketones in plants.

The position of the substitution depends on the species, and the specificities of the enzymes involved. Secondary alkanols can in turn be esterified to form a wax ester. Alternatively, the hydroxyl group can be oxidized with formation of a long-chain ketone. An associated pathway leads to the formation of β-diketones and 2-alkanols. These processes have been studied most in plants, but similar biochemical reactions appear to occur in insects and birds.

The final step in the production of wax esters from long-chain alcohols and fatty acids involves the action of an acyl-CoA:alcohol transacylase. This is an enzyme that is also required for triacylglycerol biosynthesis, i.e., an acyl-CoA:diacylglycerol acyltransferase (DGAT) or more specifically an isoform of the enzyme known as DGAT1 [5].

22.7 APPLICATIONS OF WAXES

Waxes have a wide range of applications due to certain properties they possess. They are mostly useful for their texturing, oil gelling, and viscosity increasing properties.

Examples of other applications are:

- oil binding in shoe polish and lipsticks
- water repellence in dragees and industrial coatings
- release performance in bakery and plastics
- scratch resistance in car polish and inks
- plasticizing in hot-melts and chewing gum
- lubrication in pencils and metal working
- dispersing in mascara and toners
- retard release in agriculture and pharma matrices
- binding properties in ceramic and cosmetic powders [6].

22.8 SYNTHETIC WAXES AND ESTERS

They are made of ethylene glycol diesters or triesters of long-chain fatty acids (C_{18}−C_{36}). Their melting points range between 60°C and 75°C and can be used to confer rigidity to sticks and to modify the product's crystallinity. While having the structure of waxes, esters of alcohols and fatty acids either with a straight or branched chain, but shorter than for waxes, are manufactured for cosmetic applications. Depending on the chain length and structural arrangement of the two starting materials, esters are tailored to provide different physical properties and types of emolience. Straight chain esters, such as cetyl palmitate and cetostearyl stearate, which are solid at room temperature, are used to increase the viscosity of emulsions. Liquid branched-chain esters, such as isopropyl myristate or cetostearyl ethylhexanoate, provide products with good spreading properties. Furthermore, the choice of the ester influences both the solubility and spreadability of sunscreen agents and their ability to penetrate the skin [7,8].

22.9 PLANT WAXES

22.9.1 Carnauba Wax

22.9.1.1 Source

Carnauba wax is produced by the Brazilian palm *Copernicia cerifera* Martius, common name carnauba wax palm. It is the most commercially important plant wax. The extraction and exportation of carnauba wax is a major industry in Brazil. Wax is found on both the upper and lower surfaces of the palm leaves. For harvest, the leaves are cut from the palm and left to dry then the wax is beaten off the dried leaves [4,9].

22.9.1.2 Production of Carnauba Wax

The wax is obtained from the palm leaves in a systematic process of collection, drying, beating, refining, and finally purification by filtration, centrifugation, and bleaching.

The palm produces the wax in the cuticles of the palm fronds. The leaves are collected from the trees grown in the wild or cultivated by cutting the leaves, drying in the sun, and then threshing/beating. Carnauba wax can also be gotten from organic harvesting. Organic carnauba is produced just by melting and filtration of crude untreated carnauba wax of highest quality. It has a faint pleasant smell, shows good oil binding properties, and is light colored [10].

22.9.1.3 Composition

An early study of the alcohols of carnauba wax showed for the first time that octacosanol (C_{28}), triacontanol (C_{30}), and dotriacontanol (C_{32}) were present. The report demonstrated that hexacosane-1,26-diol can be separated by distillation at 0.5 mm [9]. A series of articles by Murray and Schonenfeld reported on the alcohols, *n*-acids, diols, and hydroxyl acids of carnauba wax. The alcohols were separated by acetylation of the nonsaponifiable fraction followed by fractionation in a spinning band column. The straight chain even carbon number alcohols C_{24}−C_{34} made up the majority of alcohols present [11]. The normal acids had been found to make up 38% of the acids present and these were separated by amplified distillation of their methyl esters as C_{18} (3%), C_{20} (11.5%), C_{22} (9%), C_{24} (30%), C_{26} (12%), C_{28} (16.5%), and C_{30} (7%) [12]. Four α-ω-diols were isolated from the unsaponifiable fraction of carnauba wax and were identified as *n*-docosane-1,22-diol, *n*-tetracosane-1,24-diol, *n*-hexacosane-1,26-diol, and *n*-octacosane-1,28-diol [13]. Seven ω-hydroxy acids were identified as the even carbon number C_{18}−C_{30} hydroxy acids [14].

Three polymerizable diesters containing cinnamic acid, its *para*-hydroxy, and its *para*-methoxy derivatives have been isolated from carnauba wax. These diesters are thought to be responsible for certain properties of carnauba wax [15]. A quantitative analysis of hydrolyzed carnauba wax was carried out by gas chromatography, while a chromatographic isolation of the original constituents of the natural waxes was accomplished [16,17]. The structures and molecular dynamics of carnauba wax was performed by X-ray powder diffraction, differential scanning calorimetry, and NMR techniques and the results obtained were compared to those of beeswax [18].

Carnauba wax is classified into several commercial grades based on purity and oil content. They range in color from prime yellow (Type 1, pure) to shades of gray or black (Type 4). Type 1 wax is produced by melting the collected wax over water then filtering [19]. Unhydrolyzed type 1 carnauba wax composition by weight as reported by Vandenburg and Wilder is: hydrocarbon (0.3-1%), aliphatic esters (38−40%), *p*-hydroxycinnamic aliphatic diesters (20−23%),

ω-hydroxy aliphatic esters (12−14%), *p*-methoxycinnamic aliphatic diesters (5−7%), monohydric alcohols (10−12%), triterpene diols (0.4%), free acids, and other unknown constituents (5−7%). Similar results were obtained in a separate study [20].

Carnauba wax is one of the hardest plant waxes. It is soluble in nonpolar solvents and insoluble in polar solvents. Type 1−4 grades have melting points from 82.5°C to 83.0°C, acid numbers from 2 to 4 mg KOH/g, and saponification numbers of 88 mg KOH/g [19].

22.9.1.4 Uses of Carnauba Wax

Carnauba wax has various applications and uses and these include food, cosmetics, automobile and furniture wax, molds for semiconductors devices, and as coating for dental floss.

Carnauba wax has very good emulsification properties and excellent oil-binding capacity for ester oils and mineral oils. It also raises the melting point of gels, thus making it the preferred additives in lipsticks, lip balms, and mascara. It provides glossy and slippery surfaces [21].

Carnauba wax can form solvent resistant superhydrophobic films from selfemulsifying mixtures with alcohol emulsions. These films are resistant to solvent etching by chloroform, toluene, acetone, and alcohols [6]. Carnauba wax is used as a hardener for other waxes and to raise the melting points of wax mixtures. It is also a component of furniture, leather, and shoe polishes [4]. In the cosmetic and food industries, carnauba wax is added to formulations of lipsticks and balms and chewing gum [19].

22.9.2 Candelilla Wax

22.9.2.1 Source

The main source of candelilla wax is the Mexican plant *E. antisyphilitica* Zuccarini. The plant grows into clusters of nearly leafless, thin stems, which are covered in wax [22]. The desert conditions of northern Mexico and south-western Texas promote abundant wax production [23]. Candelilla stems are boiled in a solution of about 0.2% sulfuric acid. The wax appears as foam on the surface of the solution. It is skimmed off and refined by boiling again in sulfuric acid solution. The wax is allowed to solidify, after which the residual water is removed along with the bottom of the wax cake, which contains debris. The clean wax cake is heated to eliminate excess moisture, leaving light brown candelilla wax [4,19,23].

22.9.2.2 Preparation of Candelilla Wax

The wax is obtained by boiling the dried areal parts (the leaves and stem) with water or dilute sulfuric acid. It is then skimmed off the surface by decanting. The dark brown crude wax is further processed to produce pale yellow wax [19].

22.9.2.3 Composition of Candelilla Wax

The composition of unhydrolyzed candelilla wax varies with the season when the plant was harvested, age of plant, region, and climate [23,24]. The average candelilla wax constituents by weight are hydrocarbons (42%), wax, resin, and sitosteroyl esters (39%), lactones (6%), free wax and resin acids (8%), and free wax and resin alcohols (5%) [25−27].

Candelilla wax is hard and brittle. It is insoluble in water, but soluble in many organic solvents. The chemical and physical properties vary with composition. Generally melting points range from 68.5°C to 72.5°C, relative density at 15°C is 0.950−0.990, acid number is 12−22 mg KOH/g, and saponification number is 43−65 mg KOH/g [19].

22.9.2.4 Uses of Candelilla Wax

Microemulsions of candelilla wax are used as coatings for fruit. It is mixed with other waxes to harden them without raising their melting point. It is used in cosmetics and as a food additive as well as in shoe and furniture polishes [4,23,28].

22.9.3 Jojoba Oil

22.9.3.1 Source

Jojoba wax is obtained from the plant *S. chinensis*, a shrub belonging to the family Simmondsiaceae. The plant is commonly called jojoba pignut, deer nut, goat nut, wild hazel. The plant is found in the semiarid regions of Mexico and in the United States. It is of very high economic value in the Sonoran desert.

The jojoba industry started in 1971 and jojoba has been commercially harvested since 1982. *S. chinensis* is a drought-resistant shrub and the leaves are thick and leathery. Female flowers are axillary and usually solitary while male flowers are smaller and usually grouped in dense clusters. Fruits are dehiscent capsules containing one seed but may contain up to three seeds. The seeds are large, light brown to black in color.

Jojoba wax is pale with a melting point ranging from 15°C to 70°C. It is odorless, colorless, and could be liquid to hard mass. Its properties, mainly texture and crystallinity, can be modified by rapid cooling thus affecting its cosmetic properties but they are very resistant to oxidation because methylene interrupted double bonds are absent.

Jojoba oil is obtained from the plant *S. chinensis*, a drought-resistant shrub found mainly in the Sonoran desert where it has very high economic value. The fruit capsules of the jojoba bush contain one to three seeds. The seeds are large, light brown to black in color [28]. Jojoba oil is extracted from the seeds by cold pressing. A crystalline, hard wax can be prepared by hydrogenation of the oil [29].

22.9.3.2 Composition of Jojoba Wax

Jojoba oil consists of about 97% wax esters with free alcohols, acids, and sterols composing the other 3%. It ranges in color from colorless to yellow with melting point 6.8−7.0°C, acid number 2 mg KOH/g, and saponification number 92 mg KOH/g [19]. One study found that the reserve wax of jojoba contained C_{20} unsaturated fatty acids of types 20:1 and 22:1 but these were not present in the leaves [30]. Seven individual jojoba plants were investigated in Aguanga, California, where the average wax content was 48%. It was found that variation in the wax content of these plants was due to environmental factors [3]. Jojoba seeds from Spain were analyzed by thin-layer chromatography (TLC) and high-performance liquid chromatography and found to contain four major waxes of which 11-eicosenoic acid was the major component [31].

22.9.3.3 Uses of Jojoba Wax

Jojoba oil is used for medicinal purposes and as a substitute for coffee by Native Americans. It is also used as a substitute for sperm whale oil, which has been prohibited. However, jojoba oil's main application is in the cosmetics industry [32].

A comparison of the wax from jojoba was compared to that obtained from other plants, by gas chromatography and they were all found to be unique [33]. A biosynthetic study was done on the waxes of developing jojoba seeds using ^{14}C-decanoic and ^{14}C-lauric acids and these were found to be elongated and desaturated. On the other hand, although ^{14}C-myristic and ^{14}C-longer chain fatty acids were incorporated, they were insignificantly modified. Labeled acetate contributed to chain elongation, whereas labeled glucose were uniformly distributed throughout the fatty acid acyl chain [5].

22.9.4 Sunflower Wax

22.9.4.1 Source of Sunflower Wax

Sunflower wax is obtained from *H. annuus* (sunflower). It can be found in different parts of the plant including the seed, seed hulls, and corncorbs [34,35].

22.9.4.2 Preparation of Sunflower Wax

The sunflower wax is obtained through the winterization of sunflower oil. Eight groups of lipids were obtained from the sunflower seeds during ripening and postharvest treatment and these include sterols; hydrocarbons; free fatty acids; mono-, di-, and triglycerides; and wax substances. A direct extraction procedure using a twin screw extruder was examined in a feasibility study [36−38].

22.9.4.3 Composition of Sunflower Wax

Sunflower wax is a hard, crystalline, high melting point vegetable wax. It consists of long-chain saturated $C_{42}-C_{60}$ esters derived from fatty alcohols and fatty acids. The major esters in some varieties were $C_{40}-C_{44}$ of which C_{42} predominated [37–39]. *n*-Triacontanol (C_{30}) is a plant growth regulator and was found in both free and bound forms [39].

22.9.4.4 Uses of Sunflower Wax

Sunflower wax is useful in cosmetics: Lipsticks, Mascaras, Decorative Cosmetics, Lip Balms, and Emulsions. This wax functions as a consistency modifier in sticks and emulsions. It thickens formulations by providing a rigid structural network of wax crystals, improving oil binding, emolliency, film formation, and lubricating capacity.

It can be used as an alternative for rice bran wax, carnauba wax, and candelilla wax. Sunflower wax functions to regulate consistency in sticks contributing to hardness, texture, strength, and mold release. It also regulates consistency in emulsions and has very strong oil gelling properties when used in concentrations as low as 4%. It can be used as a replacement for jojoba beads in decorative cosmetics. Sunflower oil is also used in food as a frying oil [40].

22.9.5 Rice Bran Wax

22.9.5.1 Source of Rice Bran Wax

Rice bran wax is a hard, crystalline, high melting vegetable wax obtained from husks of rice *O. sativa* [41].

22.9.5.2 Preparations of Rice Bran Wax

Rice bran wax is obtained through the cold press dewaxing of rice oil and this yields a yellow, hard natural wax with a high melt point, which is often compared to carnauba wax.

However, there are functional differences between the two. Rice bran wax is a superior binder of oils and has been useful in combining with and stabilizing oils in both anhydrous and emulsion systems.

Rice bran wax is usually refined through batch chromatography technology and is not solvent extracted. This method of refinement retains low concentrations of policosanols, phospholipids, phytosterols, and squalene. The resulting rice bran wax is of superior quality [41].

22.9.5.3 Composition of Rice Bran Wax

Rice bran wax consists of high molecular weight monoesters. These are very long-chain saturated $C_{46}-C_{62}$ esters from $C_{20}-C_{36}$ fatty alcohols and $C_{20}-C_{26}$ fatty acids. The major components of rice bran wax are aliphatic acids (wax acids) and higher alcohol esters. The aliphatic acids consist of palmitic acid (C_{16}), behenic acid (C_{22}), lignoceric acid (C_{24}), and other higher wax acids. The higher alcohol esters consist mainly of ceryl alcohol (C_{26}) and melissyl alcohol (C_{30}). Rice bran wax also contains constituents such as free fatty acids (palmitic acid), squalene, and phospholipids. Rice bran wax is compatible with most vegetable and mineral waxes, as well as vegetable oils, mineral oils, and petrolatum [42,43].

22.9.5.4 Uses of Rice Bran Wax

Rice bran wax has been historically used in a wide variety of cosmetics, replacing carnauba wax in some applications. It is used in paper coatings, textiles, explosives, fruit and vegetable coatings, confectionery, pharmaceuticals, candles, molded novelties, electric insulation, textile and leather sizing, waterproofing, carbon paper, typewriter ribbons, printing inks, lubricants, crayons, adhesives, chewing gum, and cosmetics (Creams, Glamour Products, Lotions, Sun Care, Mascara, Lip Balms). Rice bran wax can be used as a thickener and has emollience properties. Rice bran wax also works well as a binding, coating, or gelling agent. It has exceptional oil gelling properties in relatively small concentrations. It is an interesting wax to use in emulsions, creating new textures. It is seen as particularly effective in reducing syneresis in lipstick and other oil-based systems [44].

22.10 ANIMAL WAXES

22.10.1 Beeswax

22.10.1.1 Source of Beeswax

Beeswax is a naturally occurring wax produced in the bee's hives by honeybees *A. mellifera*. Glands under the abdomen of the bees secrete this wax and it is used to build the honey comb. There are eight glands in the bee abdominal segment (4−7) of female worker bees that produce the wax. The wax is recovered as a by-product when honey is harvested and refined [45].

22.10.1.2 Production of Beeswax

Wax glands on the underside of the abdomens of the young bees secrete small wax platelets, after feeding with royal jelly and taking part in the construction of the hive. These are scraped off by the bee, chewed, and masticated into pliable pieces with the addition of saliva and a variety of enzymes.

The quality of wax depends greatly on the method of production. Basically, two methods are used for wax extraction—melting and chemical extraction—with melting being more frequently used because chemical extraction by solvent is only feasible where small-scale wax production is required, such as in the laboratory. Another disadvantage of chemical extraction is that organic wax contaminants can also be extracted along with the wax. Thus, the quality of the wax can be compromised.

Melting of wax can be done using boiling water, steam, or by electrical or solar power. Beekeepers can however produce raw beeswax by directly heating in the sun 2−3 times daily. This is a simple and cheap method of producing quality wax [6,46].

22.10.1.3 Wax Collection and Processing

Wax is usually removed from the capping during honey extraction. This produces high-quality, light-colored wax. Different qualities of wax can be produced by separating new white honeycombs from darker ones. Since whole combs are harvested and crushed or pressed, the proportion of wax per kilogram of honey (10−15%) will be much higher than with frame hive beekeeping, where the yield is only 1−2%.

Beeswax mainly refers to wax produced by the honeybee *A. mellifera*. However, beeswax can also be obtained from other honeybee species, including *A. dorsata*, *A. florea*, and *A. indica*. The wax is secreted by abdominal glands and used to construct honey combs, which are homes to bees and their larvae as well as storage for honey and pollen [47].

To produce high-quality wax, melting honey combs is the preferred method. This can be achieved by boiling the combs in water in stainless steel containers before separating the pure yellow wax from the comb residue. Cooled and dried wax should be stored in containers made of glass, plastic, or stainless steel to avoid color changes due to contamination by metals [48].

22.10.1.4 Characteristics of Beeswax

Virgin beeswax, immediately after being secreted, elaborated and formed into comb, is white. It becomes darker with use inside the hive as pollen, silk, and larval debris are inadvertently incorporated. Natural beeswax when cold is brittle. The melting point of beeswax is not constant since the composition varies slightly with its origin. It ranges from 61 to 66 °C. Its relative density at 15°C is 0.958−0.970 g/cm^3 and its electrical resistance ranges from 5×10^{12} to 20×10^{12} Ωm. Its thermal conductivity coefficient is 2.5×10^{-3} Jcm/s °C cm^2. The saponification value of beeswax is 85−100. Beeswax is inert with high plasticity. It is insoluble in water and resistant to many acids, but soluble in most organic solvents and, after warming, in alcohol and fatty oils [49,50].

22.10.1.5 Composition of Beeswax

The composition of beeswax varies depending on place of production (Europe, Asia, or Africa), species of honeybee, and age of wax [51]. Generally, unhydrolyzed beeswax contains hydrocarbons (15%), esters (71%), free acids (8%), and other compounds (6%) [47]. There have been many studies on beeswax over the years and an early study showed that English beeswax contains 14.45% cerotic acid and 88.9% myricin [50]. A later study demonstrated that beeswax mainly contained a complex mixture of *n*-alkanes, alcohols, and acids [52]. Two studies from Indian beeswax showed that it contained mainly hydrocarbons, unsaturated, saturated, and hydroxy acids [53,54]. The relationship between the

properties and chemical composition of Japanese beeswax demonstrated that for good plasticity, the presence of higher alcohols and hydroxyl acids is important [55]. The composition of the beeswax from Spain was determined by high-temperature gas chromatography after treatment with diazomethane followed by acetylation and the components identified as hydrocarbons ($C_{21}-C_{41}$), free alcohols, acids, esters, and hydroxyl acids [56]. The alkyl esters of beeswax after separation from the unhydrolyzed wax by preparative TLC were analyzed by gas chromatography and shown to contain carbons ranging from C_{36} to C_{54} [57]. The hydrocarbon composition of beeswax collected from light- and dark-colored combs, from Poland, showed that the main difference was that the darker combs had a higher content of total *n*-alkanes, including even-numbered and odd-numbered alkanes [58].

Virgin beeswax is white. It becomes darker with use inside the hive as pollen, silk and larval debris are incorporated. Natural beeswax when cold is brittle. Beeswax is inert with high plasticity. It is insoluble in water and resistant to many acids, but soluble in most organic solvents. The melting point of beeswax ranges from 62 to 65°C. Its relative density at 15°C is 0.958−0.970, acid number is 17−24 mg KOH/g, and saponification number 85−100 mg KOH/g [6].

22.10.1.6 Physiological Effects of Beeswax

It has no direct effect on humans or larger animals. If mixed with medicinal drugs or poisonous baits, it preserves the active materials longer and releases them slowly. It protects against external damage such as corrosion and abrasion as well as against moisture loss. It is a good electric insulator and, when saponified with borax, allows the mixture of very stable and smooth emulsions for cosmetics. It has little antiinflammatory and antioxidant activities [6].

22.10.1.7 Uses of Beeswax

Beeswax is used for the making of wax foundations, and commercially beeswax has many applications, including candle making, metal castings, and modeling, in cosmetics, food processing, industrial technology, textiles, varnishes, and polishes [48]. The detection of adulteration on commercial Spanish beeswax showed that these were mainly paraffins, cow tallow, stearic acid, and carnauba wax [51].

22.10.2 Shellac Wax

22.10.2.1 Source of Shellac Wax

Shellac is hard, brown in color, and has excellent shine production properties.

This wax is mainly produced in India and Thailand. It is an exudation of the parasitic insect *Laccifer lacca* (Kerr) [59].

22.10.2.2 Production of Shellac Wax

It is obtained from the bark of the trees where the female insects live. The insect secretes it to form a tunnel-like tube as it traverses the branches of tree. It is basically a by-product. The insects excrete the wax as it sucks sap of the tree. The least colored shellac is produced when the insects feed on *Schleichera trijuga* [60].

22.10.2.3 Composition of Shellac Wax

Shellac wax is made up of long-chain esters of monovalent alcohols and acids. It contains more than 30% of free wax alcohol with chain length of $C_{28}-C_{32}$. It also contains a small amount of hydrocarbons and about 1% lactic acid [61,62].

Raw shellac contains 60−80% pure shellac, 4−6% shellac wax, and impurities such as wood, dead insects, moisture. Purified shellac consists of 85−90% pure shellac, 5−8% shellac wax, and 2−5% impurities.

22.10.2.4 Uses of Shellac Wax

Shellac wax is highly useful in cosmetics, furniture polish and vanish, aluminum foil coating, paper coating, cosmetics, printing ink and paints, pharmaceutical tablet, agricultural fertilizers, and confectionery [63].

22.10.3 Spermaceti

22.10.3.1 Source of Spermaceti

Spermaceti is obtained from the cavity in the head of the sperm whale *P. macrocephalus.*

The frontal organ, used as a sonar by the animal, contains about 3 tons of spermaceti for a 15-m long animal [64].

22.10.3.2 Production of Spermaceti

It is extracted by cooling the oil from the adipose tissues of the sperm whale. The adipose tissue contains about 10−12% spermaceti wax [65].

22.10.3.3 Composition of Spermaceti

It contains fatty esters (65−95%) but also triglycerides (5−30%), free alcohols (1−5%), and acids (0−3%). Fatty esters are formed essentially of cetyl palmitate (C_{32}) and cetyl myristate (C_{30}) [66].

Spermaceti was used in medicine in England (15th century) and later in cosmetics, pharmacy, and also in candles. However, after the recent international regulation concerning whale captures, it is no longer produced and sold. It is now replaced by synthetic spermaceti made of pure cetyl palmitate or mixtures based on jojoba [67].

22.10.4 Wool Wax

22.10.4.1 Source of Wool Wax

Wool wax is a naturally occurring substance secreted by the sebaceous glands in sheep skin. Thus the source is renewable. It coats and softens the wool fibers, protecting both sheep skin and fleece against exposure [68].

22.10.4.2 Preparations of Wool Wax

Wool wax or lanolin is obtained from sheep wool by scouring. Crude lanolin constitutes about 5−25% of the weight of freshly shorn wool. The wool from one sheep will produce about 250−300 mL of recoverable wool grease.

Heavy impurities such as sand and dirt are first removed by gravity. Lanolin is extracted by washing the wool in hot water with a special wool scouring detergent to remove dirt, wool grease (crude lanolin), sweat salts, and anything else stuck to the wool. The wax is then obtained using either centrifugal separation or solvent extraction. The harvested wool wax is a dark, highly viscous, and greasy paste with a distinct sheep-like odor. In this crude form, it can be used in several technical applications. However, it has to be refined for it to be useful, especially in cosmetics.

Some characteristics of wool wax that make it so valuable may complicate the process of refining. For example, the powerful surfactant activity makes the purification process difficult. To solve this, the emulsifying power of the wax has to be held to a low and controlled level during the refinement process, without affecting quality of the product [69,70].

22.10.4.3 Composition of Wool Wax

It consists of long-chain waxy esters, lanolin alcohols, lanolin acids, and lanolin hydrocarbons [71,72].

22.10.4.4 Uses of Wool Wax

Wool wax is used extensively in both the personal care (e.g., high value cosmetics, facial cosmetics, lip products) and the health care sectors. It has commercial industrial application as rust-proof coatings, lubricant grease, wood polish, as well as leather treatments. Lanolin is often used as a raw material for producing cholecalciferol (vitamin D_3) using irradiation. It is also used in lip balm [68,73].

22.11 MARINE WAXES

Many marine animals from invertebrates to whales contain some amount of waxes in the form of hydrocarbons and wax esters. Also, glycerol ethers and sterols could be classified as components of wax in some species.

Wax esters from C_{32} to C_{44} and very long midchain ketones from C_{31} to C_{43}, along with sterol ethers, were identified by glass capillary gas chromatography/mass spectrometry from a diatomaceous ooze.

Waxes are found in a variety of tissues from fish roe, to liver and muscle tissues. The wax esters consist of the normal range of saturated, monoenoic, and polyunsaturated fatty acids typical of fish, esterified to mainly saturated and monoenoic alcohols Squalene and other terpenoid hydrocarbons are usually major components and can be accompanied by saturated straight-chain and methyl-branched, monoenoic, and polyenoic components. Waxes function as an energy source, insulator, buoyancy enhancer, and even echo locator in fish [74,75].

22.12 MINERAL WAXES

22.12.1 Montan Wax

22.12.1.1 Sources

Montan wax is a fossilized vegetable wax. The main source is lignite deposits in eastern Germany. It occurs as a component of bitumen. Solvent extraction of lignite using hydrocarbons such as toluene, followed by distillation to remove the solvent, gives the best yield of crude montan wax when compared to using polar solvents such as ethanol [76].

22.12.1.2 Composition

Crude montan wax composition depends on the plant material fossilized as well as the conditions used for solvent extraction. Quantities of wax, resin, and asphalt are usually present [77]. The main components of crude montax wax include wax acids (35%), wax alcohols (20%), resin acids (15%), hydroxycarboxylic acids (10%), and sterols (10%) [78,79].

Montan wax is obtained by the fractionation of a mineral soil by column chromatography to give mainly *n*-alkanoic and hydroxy acids [78]. Montan wax was recovered in 14.6% yield from Neyveli lignite tar and was characterized by modern spectroscopic methods, including NMR spectroscopy and mass spectrometry, and was shown to be mainly long-chain paraffins of $\sim C_{30}$ together with smaller quantities of long-chain fatty acids and esters and some unsaturated compounds [80]. The chemical constituents of montan resin from Yunnan Essan was determined using modern spectroscopic methods including high field NMR spectroscopy and, of the complex mixture obtained, 14 compounds were found in montan wax for the first time [77].

22.12.1.3 Uses

Crude montan wax is hard and brittle. It is soluble in organic solvents. Acid number and saponification number are 20–40 and 70–120 mg KOH/g, respectively. Crude montan wax needs to be refined and derivatized for industrial applications. Montan wax derivatives are used in polishes and plastic lubricants [81].

22.13 BIOACTIVITY OF WAXES

There have been limited reports on the isolation of bioactive waxes from natural sources [82]. There have been reported antibacterial and antifungal activity of wax extracts from several *Citrus* spp. peels [83], while there has been a report of antioxidant activity of *Eucalyptus globulus* leaf waxes [84]. In light of this, the possibility that the observed activity might be due to other components should not be excluded.

22.14 PRACTICE QUESTIONS

- Describe the general characteristics, composition, and uses of waxes.
- Discuss the sources of waxes, giving specific examples.
- Give a full account of any two of the following:
  - Jojoba wax
  - Beeswax
  - Wool wax

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Chapter 23

Form and Function of the Animal Cell

R. Young and S. Francis
University of the West Indies, Kingston, Jamaica

Chapter Outline

Objectives

At the end of this chapter, students should be able to:

- Describe the composition of the animal cell
- Describe key features of the organelles
- Explain key functions of the organelles
- Understand key physiological functions of cellular components and the organelles in ensuring cell regulation

23.1 INTRODUCTION

Plants and animals have coexisted on the earth for millennia. Plants directly use sunlight, to convert CO_2 and minerals into biomass, dominating the biosphere, whilst herbivores, from microbes to man, harvest a significant fraction of plants' annual productivity for energy and structural materials. Carnivores in turn exploit the rich energy and material resource provided by herbivores, helping the survival stratagems evolved by plants, to keep the threat of overgrazing in check. This relatively simple scenario quickly develops into an "evolutionary arms race," generating a complex, dynamic web of interactions in which plants not only produce defensive mechanisms to discourage animals from excessive grazing, but also provide food and shelter in exchange for pollination and dispersal services, and use their waste products as nutrients and their degradative activities as a method of recycling. Animals, in turn, generate mechanisms for circumventing plants' defenses—developing methods of detecting and avoiding or detoxifying noxious chemicals produced by plants. Animals in their interactions with each other, devise similar strategies and counterstrategies—resulting in an intricately tuned set of coevolved biological processes showing not only integration within organisms, but also balanced complementarity and antagonisms between the chemistry of organisms. The management of the metabolic pathways executing the required chemistry is embedded in the genome of the organisms, necessarily incurring a cost, which must be carefully husbanded. The concentration of defensive chemicals, e.g., may be greater in more vulnerable juvenile plants than in mature plants which may employ more structural defenses to discourage browsing [1]. Intense grazing, moreover, may induce "juvenile reversion" to the production of high levels of defensive chemicals in mature plants [1]. The understanding of the properties, variability, and potential medicinal uses of this finely tuned, interdependent chemistry of living things, evolved with a multiplicity of defensive, offensive, and cooperative purposes,

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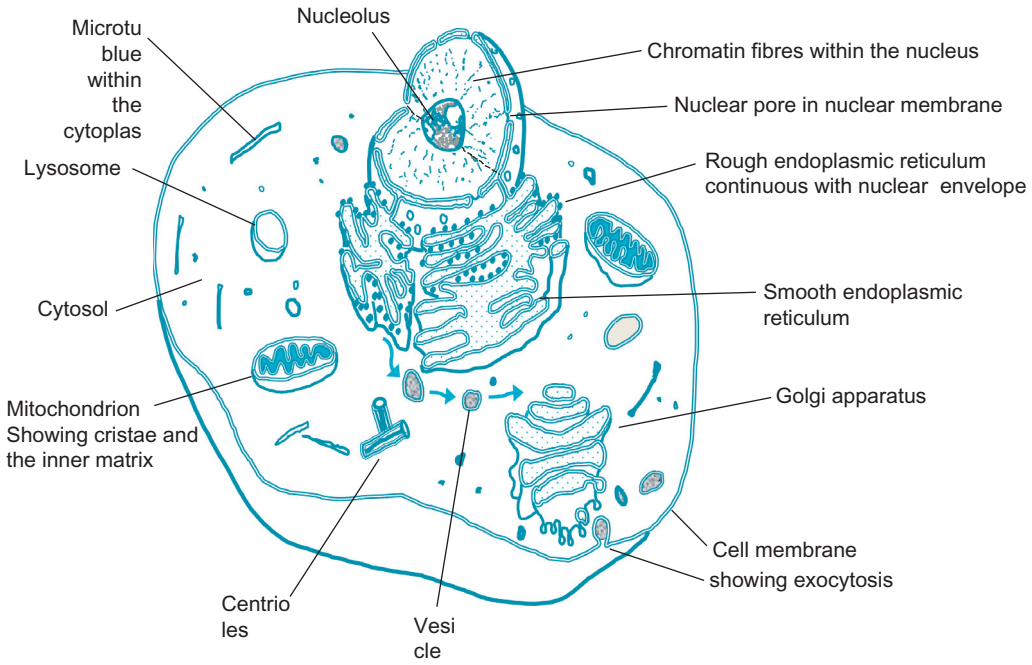


FIGURE 23.1 *The basic eukaryotic animal cell* is a small (10−20 μm diameter; widely variable) assemblage of dynamically interacting macromolecules, organic and inorganic ions in an aqueous suspension, the cytoplasm. The cell is bound by a limiting plasma membrane and divided into compartments by internal membrane systems. The membrane systems are formed and supported by a meshwork of interlacing filaments and microtubules, the cytoskeleton, which also function in motility in the cell. A prominent, usually ovoid, nucleus, enclosed by an extension of the ER, the nuclear membrane, contains the nucleoplasm, made up of the chromosomes and other nucleic acids, and proteins. Also suspended in the cytoplasm are membrane-bound organelles (mitochondria, lysosomes, vesicles, etc.) [2].

and embedded in the varying structures of the cells of the organisms, forms the basis of pharmacognosy. With this orientation, we focus here on the biology of the animal cell.

In this chapter we will focus only on those areas pertinent to pharmacognosy, in which we believe that our readers might benefit from detailed perspectives which are not widely treated. Accordingly, we will deal only peripherally with cell division and motility, but will look more closely at the cell membrane, organelles, particularly the mitochondria, and cell signaling, highlighting the complex interactions coevolved to cope with an environment rich in synergistic and antagonistic chemical and physical cues of both intra- and extraorganismal, as well as intra- and extracellular origin.

The basic form of the animal cell is well known (Fig. 23.1) comprising the:

- cytoplasm
- cell membranes
- mitochondria
- lysosomes
- peroxisomes
- nucleus
- endoplasmic reticulum
- Golgi apparatus

which will be described in the order listed.

For detailed and broader understanding of each component of the cell, see our recommended reading list at the end of the chapter.

23.2 THE CYTOPLASM

Enclosed within the bounding plasma membrane, the typical animal cell contains a highly organized, dynamically changing, hydrophilic framework of fibrillar and globular protein anions (Pr^-) along with amino acids, phosphates, carbohydrates, and other macromolecules and inorganic ions in an aqueous medium [3].

The fibrillar proteins, primarily of three types, form a mesh-like cytoskeleton [3]. The 8-nm microfilaments of polymerized actin subunits, and the 25-nm microtubules assembled from α- and β-tubulins, play an essential role in cell motility and intracellular translocation. In this, they are assisted by associated proteins such as myosin, kinesin, and dynein [4]. These two types of fibrils readily assemble and disassemble in response to conditions in the cell, with their so-called dynamic instability being an important functionally [5]. By binding to tubulin subunits and inducing depolymerization of the microtubules, colchicine, from the autumn crocus *Colchicum autumnale*, blocks mitotic spindle formation and impairs the motility of neutrophils [6]. This forms the basis for the use of colchicine as an antimitotic/anticancer agent and in blocking inflammation in the treatment of gout, and affirms the importance of microtubules in intracellular (chromosomal) translocation and cell (neutrophil) motility. Interestingly, whilst the vinca alkaloids vincristine and vinblastine function similarly to colchicine, the well-known agent taxol has the opposite effect of stabilizing microtubules and inhibiting depolymerization [7].

The third type of fibril, the 10-nm intermediate filaments, is of varied composition, and may be made up from keratins (mainly in epithelial cells), vimentin (mesenchymal cells), desmin (muscle cells), glial fibrillary acidic protein (GFAP; glial cells), or form neurofilaments in axons [8]. Intermediate filaments of lamin form a distinctive meshwork, the nuclear lamina, associated with the inner nuclear membrane, nuclear pore proteins, and the enclosed chromosomes. Intermediate filaments are more stable than the microfilaments and microtubules and are important in maintaining the structure of internal membrane systems as well as of the overall cells and their attachments to each other and to the substratum [4].

The free, aqueous phase elements comprise the cytosol, containing soluble proteins, carbohydrates, and ions. The fibrillary proteins, although not strictly soluble in their polymerized form, are typically regarded as a part of the cytosol, as are suspended inclusions such as lipid droplets and glycogen granules. The cytoplasm then comprises this heterogeneous gel/sol complex, along with suspended vesicles and cellular organelles, such as lysosomes, mitochondria, cilia, and the like, with water still comprising 70% of the total volume [3].

Proteins within the cytoplasm are enzymatic (catalyzing the metabolic processes of the cell), or architectural (contributing to the cell's structure), or both. The viscosity of the cytosol is dependent on the dissolved carbohydrates and ions and the concentration, type, and organization of the cellular proteins [3] and organelles, and varies depending on location within the cell, cell type, and functional status.

Given the high concentration of materials enclosed within the semipermeable bounding plasma membrane, the osmotic entry of water is a constant threat. A considerable amount of energy, therefore, must be spent in maintaining osmotic balance to avoid rupture of the delicate cell membrane and leaching of the critical constituents from the cell, whilst still maintaining intimate interaction with the external milieu. We will now examine the various components of the cell membrane and how it enables communication with its external environment whilst maintaining osmotic balance.

23.3 THE CELL MEMBRANES

The living cell is made up of a system of fragile membranes comprised basically of a 5-nm thick, phospholipid bilayer, the cell membrane, with fibrillar and globular proteins attached to the outer or inner surfaces (Fig. 23.2). These peripheral proteins may be "anchored"—linked covalently to membrane structures—or merely "associated" via weaker bonds (e.g., hydrogen bonding). Integral proteins are embedded in and run through the membrane to protrude on both sides. The high-energy, low-entropy intracellular contents are separated from the external environment/extracellular fluid by the plasma membrane, which, simultaneously, provides the link between the two. The phospholipids which make up the

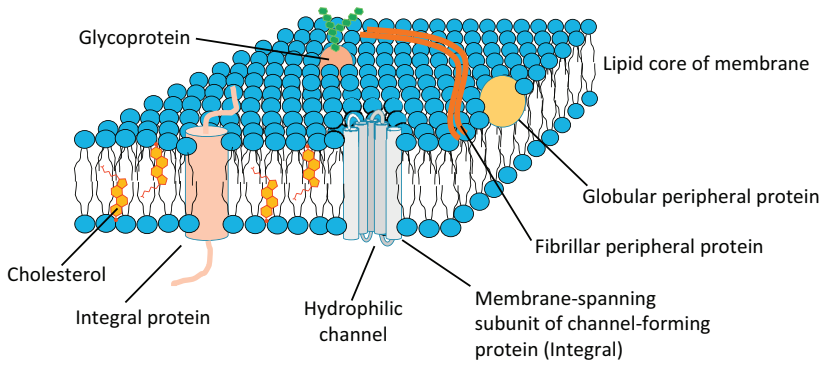
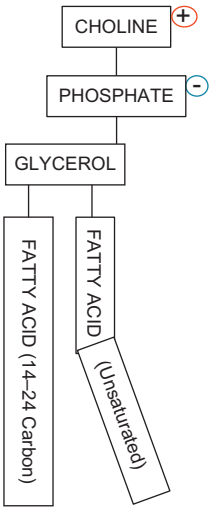


FIGURE 23.2 *Structure of the cell membrane and its associated proteins.* These proteins move freely laterally in the membrane, but do not readily move across it—hydrophilic regions which interface with the extracellular fluid or the cytosol do not readily cross the lipid core. Cholesterol helps to maintain fluidity of the animal cell membrane.



Phosphatidylcholine—Schematic Representation (left) and Typical Image of a phospholipid (right). In **Sphingomyelin**, a serine as opposed to a glycerol phospholipid, the glycerol is replaced by serine. **Glycolipids** are serine phospholipids in which the phosphate is conjugated with a carbohydrate such as glucose.

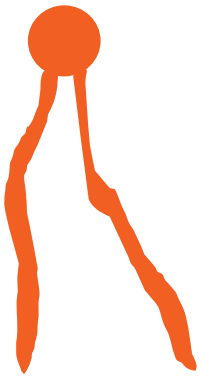


FIGURE 23.3 *The basic structure of a phospholipid* which, along with cholesterol, forms the core of the animal cell's membranes.

cell membrane are basically glycerol molecules esterified with two long, aliphatic fatty acid chains and one phosphate (phosphatidic acid). The fatty acid chains form the lipid, hydrophobic "tails" of the molecule, which partition into the core of the membrane. The phosphates which may in turn be conjugated to molecules such as choline (forming phosphatidylcholine—Fig. 23.3), serine or ethanolamine (aminophospholipids (APLs)), or inositol (making phosphatidylinositol) comprise the hydrophilic "heads" which interface with the hydrophilic intra- and extracellular media [9]. These phospholipids, along with sphingomyelin and glycolipids (see Fig. 23.3), comprise about 50% of the membrane lipids. The outer leaflet of the plasma membrane (see below), abutting with the extracellular fluid, is rich in glycolipids and glycoproteins with attached oligosaccharides (e.g., sialic acid), giving the surface a fuzzy coat called the glycocalyx. These carbohydrate molecules have characteristic "fingerprints," and assist in cell−cell, cell−substrate, and in immunological interactions. In animal cell membranes, cholesterol is present in roughly equimolar quantities with the phospholipids, with its hydrophobic steroidal rings partitioned into the lipid core and its hydrophilic −OH group adjacent to the hydrophilic head groups, and contributes significantly to maintaining membrane fluidity [10].

In aqueous media, the amphipathic phospholipids self-assemble into a phospholipid bilayer comprised of two leaflets made up of planar arrays with the hydrophilic phosphate ends facing the adjacent aqueous media and the hydrophobic fatty acyl tails facing inward toward each other. The cylindrical shape of the molecules leads to formation of a relatively flat bilayer, the borders of which tend to fuse together spontaneously producing an enclosed space, with primarily, choline phospholipids (sphingomyelin and phosphatidylcholine) in the outer and APLs (phosphatidylserine and phosphatidylethanolamine) in the inner leaflet. The curvature of the membrane is determined by the precise phospholipid composition, membranes richer in phosphatidic acid being more curved [10].

The phospholipids are synthesized in the endoplasmic reticulum (ER) and transferred to the cytosolic leaflet of the cell membrane. Whilst lateral mobility is great, passive transfer across leaflets is extremely slow, and is accelerated by transporters, in particular a protein known as a "floppase," which transports phospholipids nonspecifically across from the inner to the outer leaflet. A protein that closely resembles the nonspecific floppase of red blood cells tends to be "abundantly expressed in drug-resistant tumor cells" [11]. Floppase works in tandem with an adenosine triphosphate (ATP)-dependent APL-translocase which specifically transports APLs back from the outer to the inner leaflet [12]. This differential distribution is functionally important, and in red blood cells and platelets may, when disrupted, initiate clotting. A third transporter, a "scramblase" when activated at high intracellular Ca^{2+} concentrations, can randomize distribution of the phospholipids across the leaflets, a change, which, if persistent, could initiate apoptosis—a process of programmed cell destruction. The three transporters work together to maintain a dynamically balanced distribution of phospholipids in the plasma membrane [11,12].

Materials moving into or out of the cell must traverse the barrier of the plasma membrane—either directly through the lipid core or via hydrophilic channels formed in integral proteins running through the membrane.

According to Fick's Law [11], the rate (J) at which a substance diffuses across a barrier depends upon the concentration difference across the barrier (ΔC), the thickness (Δx), and surface area (A) of the barrier, and the "diffusion coefficient" (D). That is, $J = D_{*}A(\Delta C/\Delta x)$, where $\Delta C/\Delta x$ is the concentration gradient across the membrane.

The diffusion coefficient (D) depends upon the nature of the barrier and the molecular size of the diffusing material.

The Meyer–Overton Rule [11] asserts that nonpolar substances with a large oil/water partition coefficient[1] will move through biological membranes more readily than those with a low coefficient. This is because such substances in the aqueous extracellular fluid will preferentially partition into the hydrophobic core of the cell membrane, amplifying the concentration gradient across the membrane. In general, lipophilic substances of low molecular weight move readily across cell membranes.

Hydrophilic substances, conversely, will not readily enter the hydrophobic core and so will cross the membrane with difficulty. Such substances must traverse biological cell membranes through hydrophilic pores, formed by integral, multiloop membrane proteins, as discussed above. Typically, the subunits (polypeptide chains) forming these channels loop backward and forward through the cell membrane, and aggregate to form a hydrophilic channel traversing the cell membrane (Fig. 23.2). The side chains and loops of the proteins are arranged to create a negatively or positively charged environment in the channel. The hydrophilic pores through the membrane allow enhanced movement of charged particles, with varying degrees of specificity, either passively down their concentration gradient (facilitated transport), or actively against their concentration gradients (active transport). Specificity of the channels is conferred depending upon charge, size, affinity for the transported species, and shape—allowing energetically appropriate stripping of the hydration shells surrounding the charged particles [11].

In the case of active transport, the required energy may be provided either directly by ATP, as for the phospholipid transporters (above), or by via exchanges with other charged particles [11] which move down their concentration gradient, thereby providing the energy for upgradient movement of the transported particle (e.g., Na^+/H^+ counter-transport) (Fig. 23.4).

Where the transport energy is derived directly from ATP (e.g., the Ca^{2+}-ATPase pump), phosphorylation of the carrier molecule by ATP (releasing ADP) may induce a configurational and affinity change from state I to state II (Fig. 23.5), with dephosphorylation leading to reversion.

Water, a polar molecule, traverses the membrane readily through specific channels in tetrameric integral proteins termed "aquaporins" which exclude the passage of ions [11].

These channels allowing the movement of water or ions across the cell membrane may exist in varying numbers and in varying states—open or closed, active or deactivated. Their state may be altered depending upon stereospecific binding with particular ligands (ligand gated channels) or conditions in the intracellular or extracellular environment. It is probably best to think of these channels as populations which exist in a dynamic equilibrium between open and closed states, with the point of equilibrium being dependent upon the concentration of ligands or the membrane potential.

Na^+ channels in sweet-sensitive taste cells, e.g., are typically closed, but are opened when sucrose binds to the outside of the channel, allowing the entry of Na^+ into the cell and exciting it, sending action potentials to targeted sites in the brain, and eliciting the sweet sensation. Cells, then, can respond in a complex and dynamic fashion to their environment, regulating the inward or outward movements of water, ions, and other substances. Thus, cell membranes are selectively permeable [13].

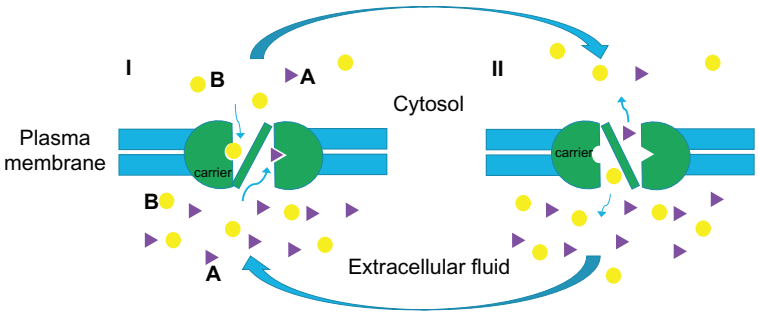


FIGURE 23.4 *Model of counter-transport*. The carrier oscillates between states I and II. In state I particle A (triangle) at a high concentration in the extracellular fluid (ECF) binds stereospecifically to a site exposed to the ECF. This stimulates cooperative binding of particle B (circle) to its high-affinity site exposed to the cytosol, and a shift to configuration II. The bound particle A, now exposed to the cytosol where its concentration is low, dissociates from its binding site, causing release of particle B from its site—now exposed to the ECF, and reversion to state I. The movement of A down its concentration gradient provides the energy for moving B out of the cell, against its concentration gradient.

1. A substance is shaken with equal amounts of olive oil and water. After settling, the oil–water partition coefficient will be given by (Concentration of substance in oil layer)/(Concentration of substance in water layer)

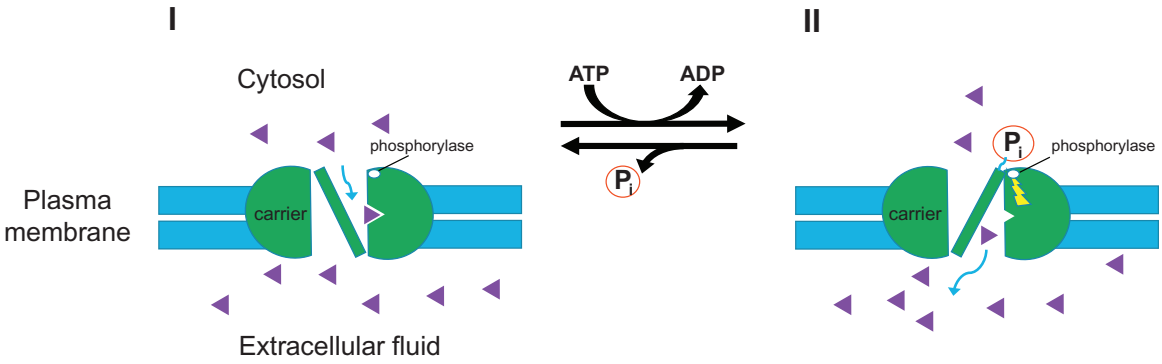


FIGURE 23.5 *Model of ATPase uniport*. The carrier oscillates between states I and II. In state I the transported particle (triangle), at a low concentration in the cytosol, binds stereospecifically to a high affinity site exposed to the cytosol. This facilitates phosphorylation of the carrier by ATP, a shift to configuration II and release of the bound particle (fall in affinity), now exposed to the extracellular fluid (ECF). The release activates a phosphorylase site dephosphorylating the carrier and causing reversion to state I. The breakdown of ATP thus provides the energy for moving the particle out of the cell, against its chemical or electrochemical gradient.

As indicated above, the cytosol in animal cells contains a high concentration of large polyions which are trapped and unable to move freely across the cell membrane, which is semipermeable to inorganic ions and freely permeable to water. This imbalance will result in the movement of water into the cell which will tend to swell and rupture unless the immobile, osmotically active particles in the cytosol are balanced by a corresponding impermeant particle in the extracellular compartment. The cell achieves this by making the membranes quite impermeable to Na^+ ions and actively pumping these ions out of the cytosol into the extracellular space, in exchange for K^+, using an ATP-dependent $3Na^+/2K^+$ exchange pump. The extracellular space therefore has a high concentration of Na^+ whilst the cytosol has a low concentration of Na^+ and a high concentration of K^+, along with impermeant polyanions. K^+, however, unlike Na^+, can move relatively freely through open K^+ channels in the cell membrane, and so tends to flow out, down its concentration gradient, leaving a negative charge, referred to as the membrane potential, on the inner, cytosolic side of the membrane, relative to the extracellular face. By regulating the opening and closing of Na^+ and K^+ channels in the membrane, the cell then has a means of varying the membrane potential and has used this as a powerful signaling mechanism in many cells particularly in muscle and nerve cells [14].

Although the cytoplasm is not an ideal solution [15], the distribution and movements of ions and the voltage changes across the cell membrane associated with changes in ion permeability, are well described by the Goldman-Hodgkin-Katz equation which assumes approximate ideality, and which was developed in order to elucidate the origins of the resting membrane potential in all cells and action potentials in neurons and related cells [15].

The Ca^{2+} concentration in the cytosol (10^{-7} M) is even more stringently regulated than the Na^+ levels (12×10^{-3} M) using powerful ATPase Ca^{2+} pumps and Ca^{2+}/Na^+ exchangers in both the plasma membrane and in the endoplasmic reticular membranes. The differential between the intra- and extracellular concentration is enormous compared with that for Na^+ (2×10^4 vs. 12-fold) although the actual concentration is low by comparison with Na^+ even in the extracellular compartment (2×10^{-3} M vs. 140×10^{-3} M for Na^+). This is important since many of the components of the cytosol may form insoluble compounds with Ca^{2+}. The Ca^{2+} concentration in the cytosol then can be varied by controlling the opening and closing of Ca^{2+} channels in the cell membranes, and has become a potent signaling element in cellular functioning, linked with many of the critical responses of cellular metabolism to environmental factors [16].

ATP is a necessary molecule for membrane regulation and in general cell function. The organelle primarily responsible for ATP manufacture is the mitochondrion, a critical evolutionary inclusion in eukaryotic cells.

23.4 MITOCHONDRIA

The mitochondrion (plural mitochondria) is a unique double-membrane bound organelle within the eukaryotic cell thought to be an independent bacterium that became incorporated into the eukaryotic cell forming a symbiotic relationship with the cell that revolutionized cellular respiration, driving the evolution of eukaryotic cells into very efficient aerobes.

The basic structure of the mitochondrion comprises:

1. an outer mitochondrial membrane, separated by
2. an intermembrane space, from

3. an inner membrane with expanded surface area, folded into cristae, projecting into
4. an internal matrix.

The membranes of the mitochondria are similar to the general cell membranes: a phospholipid bilayer, with attached integral and peripheral proteins [17]. The nature and quantity of the proteins vary between membranes and among mitochondria, depending on the function of the particular cell, and the duties it requires from the mitochondria. The mitochondria have their own circular, bacterial-style genomic DNA, ribosomes, and RNA—the tools to manufacture their own proteins; some proteins found in their membranes and within their membrane-bound spaces, however, are encoded by nuclear DNA, synthesized in the ER and transported to the mitochondria [17]. These genes were originally within the mitochondrial chromosome, but have become translocated to the nuclear chromosomes incrementally over time. Whilst mitochondrial replication is partly encoded by nuclear genes, the method of replication remains distinctly bacterial—by binary fission.

The outer membrane contains large barrel-shaped integral proteins called porins, allowing free passage of water, ions, and small molecules into the intermembrane (IM) space. Thus, the composition of amino acids, peptides, sugars, and small ions in the IM space is similar to that of the cytoplasm. Larger molecules transit across the outer membrane via specific translocator proteins [3], so that their concentrations in the IM space are different from in the cytoplasm.

The inner membrane of the mitochondria engages in many complex metabolic functions and is more selective in the transit of molecules than the outer membrane, allowing free passage to only oxygen, carbon dioxide, and water. The expanded folds of the inner membrane, the cristae (singular crista), greatly enhance the capacity to carry out membrane-linked enzymatic activity such as oxidation−reduction, transport, and synthesis of ATP. They also serve to minimize the matrix-to-membrane diffusion distance. Other proteins within the inner membrane strictly regulate the transport of molecules across the membrane, control lipid and protein synthesis, and fatty acid β-oxidation, or are involved in the growth, the movement, and the division of the mitochondria [18]. The matrix is the site of the Krebs cycle reactions.

The generation and maintenance of an H^+ gradient across the inner membrane is a major function of mitochondrial metabolism (Fig. 23.6).

Briefly, carbohydrates, fats, and proteins are metabolized in the cytosol to generate pyruvate or fatty acids. These compounds are shuttled across the mitochondrial membranes into the matrix, where they acetylate Coenzyme-A to

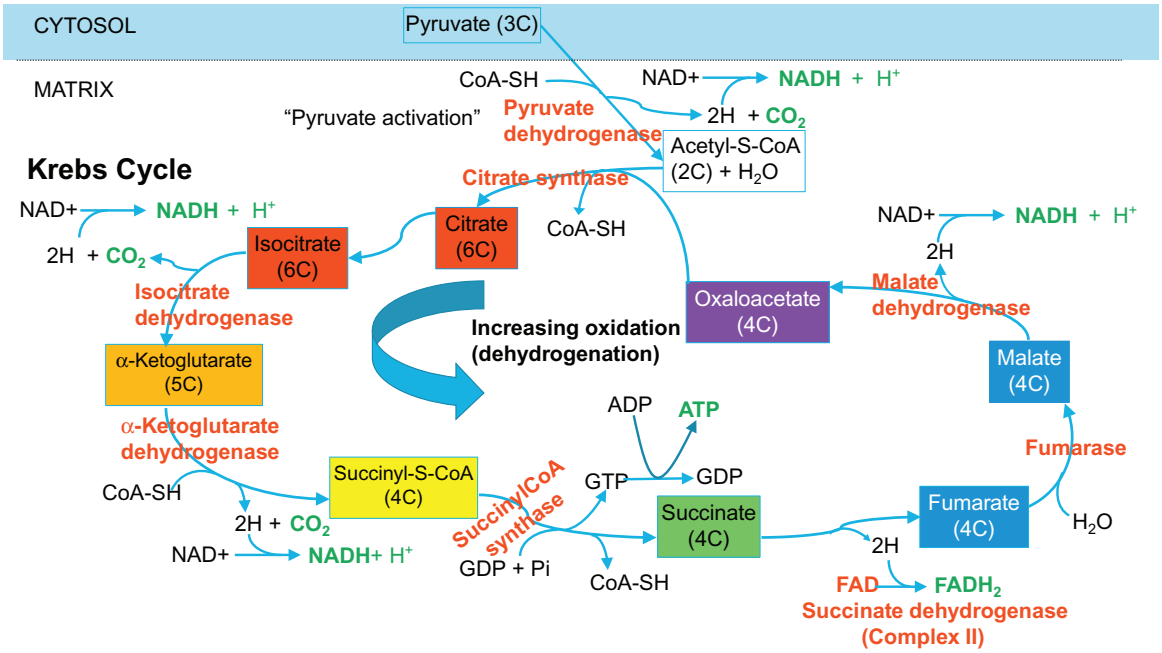


FIGURE 23.6 *Pyruvate activation (not a part of the cycle) and the Krebs cycle*. Dotted lines indicate that minor steps have been omitted. Note that succinate dehydrogenase/FAD is a part of Complex II of the electron transport chain (ETC). Each cycle "burns" two carbon atoms and produces one $FADH_2$, three NADH, and one ATP. Pyruvate activation "burns" one carbon and produces one NADH. Substrates are oxidized in a stepwise fashion to oxaloacetate which is then "recharged" by combination with acetyl-CoA to form citrate. $FADH_2$ generated in Complex II is restored (reoxidized) to FAD by passing electrons to Coenzyme Q (see Fig. 23.7).

form acetyl-CoA. Short-chain fatty acids (<10 carbons) can readily move into the mitochondrial matrix, but longer-chain molecules first have to be activated by combining with Coenzyme-A, to form acyl-CoA. A transferase enzyme (CT-I—carnitine acyltransferase I) in the outer mitochondrial membrane swops the Co-A for L-carnitine in the IM space, to form acyl-carnitine, which is then shuttled across the inner membrane and into the matrix, by a translocase enzyme, and reconverted to acyl-CoA by another enzyme, CT-II, bound to the inner membrane. The carnitine then diffuses back from the matrix into the IM space. This comprises the "carnitine cycle" and its importance underlies the reason why L-carnitine promotes the burning of fats as an energy source. The fatty acyl-CoA then undergoes "β-oxidation" whereby 2-carbon fragments are sequentially cleaved off to form acetyl-CoA molecules, which can enter the Krebs cycle.

These are by no means the sole metabolites entering the Krebs cycle. Many products of amino acid metabolism (e.g., α-ketoglutarate, succinate, fumarate) enter the matrix and are acted upon by suitable enzymes of the Krebs cycle. The Krebs cycle enzymes transform more reduced to more oxidized metabolites, in the process, reducing the electron-carrying coenzymes, nicotinamide adenine dinucleotide (NAD^+) or flavin adenine dinucleotide (FAD) to NADH and $FADH_2$, respectively. These reduced coenzymes, in turn, by donating their high-energy electrons, reduce a series of proteins comprising the electron transport chain (ETC) bound to the inner membrane of the mitochondria. Energy generated by the process, as electrons pass from one energy level to next, allows the pumping of hydrogen ions across the inner membrane, from the matrix into the IM space. The last electron carrier within the electron transport chain (cytochrome oxidase of Complex IV) transfers its electrons to oxygen to form, with $4H^+$ from the matrix, two molecules of H_2O (Fig. 23.7).

NADH enters the electron transport chain at Complex I (NADH-Coenzyme-Q reductase) and moves $2H^+$ ions into the IM space, while passing a pair of electrons to the complex. $FADH_2$ is formed in Complex II (the Succinate dehydrogenase complex, of which FAD is a constituent part) and releases $2H^+$ ions to the matrix whilst releasing two electrons to reduce Complex II (no H^+ ions are transferred to the IM space). Both complexes (I and II) independently reduce Coenzyme-Q (a peripheral protein on the matrix face of the inner membrane) by passing electrons to it, but again, no H^+ ions are translocated. Further H^+ ions are transported when Complex III (another integral protein, Cytochrome *bc*1) is reduced by the reduced Co-Q complex. Complex III then reduces Cytochrome *c* (another peripheral protein) and the reduced Cytochrome *c* complexes cooperate to reduce Complex IV (cytochrome oxidase) which in turn reduces oxygen to produce, along with $4H^+$ ions from the matrix, $2H_2O$ molecules, whilst pumping more H^+ ions into the IM space. As hydrogen ions are pumped from the matrix across the inner membrane, a proton gradient, the chemiosmotic gradient, is established and maintained across the inner mitochondrial membrane.

Depending upon the demands of the tissue (i.e., the ADP/ATP ratio in the matrix), these protons are allowed to reenter the matrix, moving down their concentration gradient via a protein complex [19], the F_0 subunit, consisting of a proton channel and proton-wheel (think water-wheel—yes nature did invent the wheel) and an ATP synthase, the F_1 subunit, within which the proton-wheel is turned by the force of H^+ ions moving down the chemiosmotic gradient, into the matrix (Fig. 23.8). The energy harnessed from the hydrogen ion flow drives the phosphorylation of ADP to ATP, the reaction being increasingly resisted as ATP builds up in the matrix, and promoted as ATP level falls.

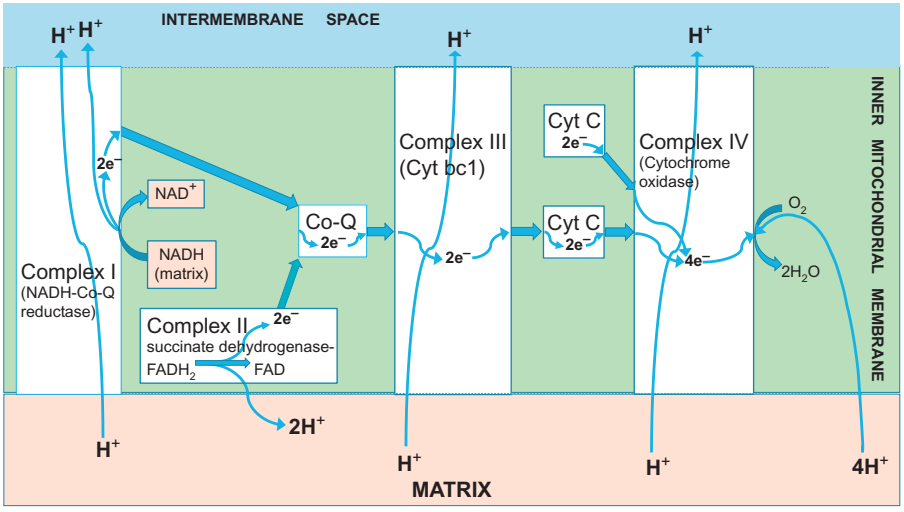


FIGURE 23.7 *The electron transport chain (ETC) of the mitochondria.* Coenzyme Q can be reduced independently by either Complex I or Complex II. The complexes involved in H^+ transfer are integral proteins whilst the others are peripheral proteins; all are freely mobile laterally in the membrane and interact as they collide.

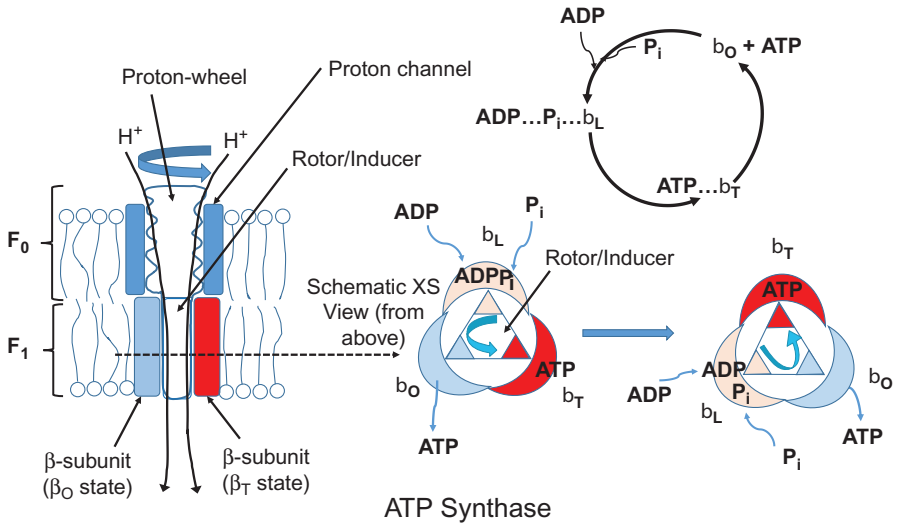


FIGURE 23.8 *ATP synthase* comprises two components—a *proton wheel*—a rotor driven by the H^+ flow through the channel (the F_0 subunit) and *ATP synthase* (the F_1 subunits) comprising three β-subunits, each of which can assume one of three forms (Loose L, Open O, or Tight T) depending upon the region of the rotor that is adjacent to it. The β_L configuration binds ADP and P_i at adjacent sites. The β_T configuration has a very high affinity for ATP, driving the combination of the bound ADP and P_i to form ATP. The β_O configuration has no tendency to bind ATP, releasing the bound ATP into the matrix. As the rotor turns, each β-F_1 subunit sequentially passes from L to T then to the O configuration, continuously binding ADP and P_i, producing, and releasing ATP into the matrix.

Heat is an important by-product of this process, playing a critical role in body temperature regulation in endotherms. Indeed, so-called uncoupling proteins (UCPs) may provide alternative pathways to the F_1 subunit (ATP synthase) allowing proton leaks across the inner membrane, uncoupled from ATP synthesis, but producing heat, contributing significantly to the basal metabolic rate. The UCPs may be regulated by thyroxine levels and are prominent in brown fat, found in some hibernating mammals and infants, and which is involved in "nonshivering thermogenesis" [20].

The number and size of mitochondria present within the cell and the abundance of proteins within their membranes vary in relation to cell function. A single-cell organism may have one mitochondrion, however, in multicellular organisms, cells with high-energy demands, e.g., muscle cells or liver cells and cells of the digestive tract, have numerous mitochondria.

The electron transport chain, enzymes involved in the Krebs cycle, and ATP synthase are well known for playing starring roles in cellular energy generation; however, many proteins within the mitochondria play other important roles in cellular physiology and pathology.

During cellular respiration, the electron transport chain of the mitochondria can generate highly reactive oxygen species (ROS) such as H_2O_2, O_2^-, and ·OH which can damage many macromolecules. High levels of antioxidants, which destroy these reactive species before they can effect damage, mitigate these side effects and positively enhance growth in developing organisms, increasing protein levels involved in growth regulation, cell proliferation, and differentiation [21]. Cellular oxidative stress, the imbalance of cellular ROS to antioxidants [17,21], can inappropriately induce cell-cycle regulators and genes involved in apoptosis (programmed cell death—suicide) and necrosis (unprogrammed cell death—murder) [22]. Generally, perturbation in cell function can lead to the overexpression of stress proteins, such as Jun N-terminal kinase,[2] inducing Cytochrome *c* release from mitochondria [23]. Cytochrome *c*, a protein of the electron transport chain, when released from the mitochondria, initiates the activation of proteins involved in apoptosis, thereby engaging mitochondria in the pathophysiological pathways of the cell.

Mitochondria play a broader role in cell signaling. Calcium ion regulation is necessary for activation of transport proteins and enzyme regulation [24] and many intracellular processes, with mitochondria acting in conjunction with the ER as a major Ca^{2+} source and sink. The sometimes close association between mitochondria and the ER is thought to be essential in both mitochondrial/ER Ca^{2+} signaling and the exchange of phospholipids between the ER and the mitochondria [25]. Cellular functions mediated by Ca^{2+} ions, e.g., muscular contraction, can be altered by abnormal mitochondrial uptake or release of Ca^{2+} which may significantly upset the dynamics of the changes in cytosolic [Ca^{2+}] essential for the activity [25].

Overall, then, mitochondria are responsible for the metabolism of pyruvate and fatty acids, producing energy in the form of ATP (or heat), to serve nearly all cellular as well as mitochondrial activities, including, e.g., movement, growth, division, protein synthesis, and translocation. Other functions include cell fate determination and cell signaling.

2. Jun N-terminal Kinases are mitogen-activated protein kinases activated by stress factors, that phosphorylate cJun and trigger gene transcription, leading to responses as diverse as cell proliferation, differentiation, growth or apoptosis via mitochondrial or other pathways (Dhanakesaran & Reddy, 2008).

Mutated or dysfunctional mitochondria within the neuron have been implicated in causing several neurological diseases, e.g., Parkinson's disease [26], primarily due to the high and unceasing energy demands of neurons.

Whilst the mitochondria digest macromolecules primarily for energy extraction, lysosomes, and peroxisomes digest particulates for their degradation, recycling, and egestion. Like the mitochondria, the peroxisome is also involved in β-oxidation of fatty acids and generate ROS as a by-product [27]. Typical features of lysosomes and peroxisomes will now be discussed.

23.5 LYSOSOMES

Lysosomes are membrane-bound vesicles, ranging in size from 0.1 to 1.2 μm, and containing inactive hydrolytic enzymes, mainly cathepsin [28]. The membrane is a phospholipid bilayer, with glycosylated proteins on the outer leaf, and in the inner leaflet, the unusual phospholipid bis(monoacylglycero)-phosphate, which is exclusive to lysosomes [29]. Lysosomes are present in almost all eukaryotic cells except red blood cells. Lysosomes are involved in digestion of phagocytosed microorganisms, endocytosed macromolecules, and unwanted cellular organelles. These entities, contained, respectively, in phagosomes, endosomes, and autophagosomes are degraded, after fusion with lysosomes and activation of the enzymes, to their most basic constituents, which are transported to the cytoplasm and can be recycled by the cell.

Increased intracellular [Ca^{2+}] consequent to cell-membrane damage, induces synaptotagmin-mediated fusion of lysosomes with the cell membrane, assisting in repair and wound healing [30]. With extreme damage, the cathepsins of lysosomes can enable the release of Cytochrome *c* from mitochondria to the cytoplasm, thereby activating proteins regulating apoptotic cell death [31]. Other studies have shown that lysosomal accumulation of cholesterol protects the cell from oxidative stress-induced apoptosis [2].

23.6 PEROXISOMES

Peroxisomes are self-replicating membrane-bound vesicles which use molecular O_2 to oxidize several organic substrates, generating H_2O_2. They contain flavin oxidases, mainly D-amino oxidase, and catalase which uses H_2O_2 to oxidize potentially harmful toxins (e.g., alcohol) and degrades excess H_2O_2 to H_2O. The enzymes, tagged with specific markers, are synthesized on free ribosomes in the cytosol and transported into the vesicles. A significant peroxisomal reaction is the β-oxidation of longer-chain fatty acids to acetyl-CoA. Acetyl-CoA and acyl-CoAs of < 8 carbons can be transported to the mitochondria and metabolized to generate ATP [32]. Peroxisomes play a role in the synthesis of myelin phospholipids [33], important in myelinating neurons peripherally and in the central nervous system, allowing secure and speedy transmission of action potentials. Peroxisomal disorders may impair myelination, leading to neurological illnesses. Peroxisomes are involved in cholesterol and bile acid synthesis and are abundant in the liver.

23.7 THE NUCLEUS

A defining organelle in the eukaryotic cell is the nucleus, which encloses a dense, strongly staining nucleolus (Fig. 23.1). The nucleus is bounded by double lipid membranes, perforated by openings, the nuclear pores, which allow only selected, appropriately tagged molecules to enter and exit the enclosed nucleoplasm. The nucleoplasm houses the genetic material—long, paired, double-helical strands of deoxyribonucleic acid (DNA)—the chromosomes, which are faithfully replicated in each nucleus of each cell. Segments of these chromosomes, the genes, code for the structure of the vast majority of proteins (except some mitochondrial proteins) needed by the organism, in the form of particular sequences of nucleotides along specific portions of the strings, demarcated by regulatory DNA sequences. The regulatory sequences, along with associated proteins govern the cell cycle and determine which genes become translated into proteins, when, and in which cells. Molecules entering the nucleus can affect DNA synthesis and transcription, which can alter the cell cycle and protein expression in the cell/organism. The nuclear membrane and the nuclear pores therefore play an important regulatory role in the cell.

According to the Central Dogma of Biology the DNA of a gene is never directly translated into a protein, but is first transcribed into a messenger ribonucleic acid (mRNA) molecule which is then transported from the nucleus and translated into a protein in the cytoplasm with the help of another type of RNA, the ribosomal RNA (rRNA). It is within the nucleolus that the several genes coding for rRNA are brought together for transcription, processing, and the assembly of the ribosomal subunits. After synthesis and assembly the ribosomal subunits exit the nucleus and, either free in the cytoplasm or associated with the rough ER, are responsible for protein synthesis. The separation of genetic transcription

in the nucleus from translation in the cytoplasm allows modification of transcribed mRNA prior to it leaving the nucleus [34]. This permits "proofreading" and quality control to correct interrupted or missing sequences that may have been copied from damaged DNA or miscopied from normal DNA, and for the removal of introns (see below). Not all genes, however, are translated into proteins. Indeed, in eukaryotes, the vast majority (over 90%) of transcribed RNA is not protein-forming [35]. Transfer RNA (tRNA) carries the appropriate amino acid to its assigned location on the mRNA when it associates with the ribosome, and regulatory (small nuclear) ribonucleic acids (snRNA) help, among other things, in the assembly of rRNA into ribosomes.

It is the nucleus then that conducts the synthesis of deoxyribonucleic acid (DNA); the transcription of ribosomal, messenger, transfer, and regulatory snRNA (rRNA. mRNA, tRNA, and snRNA) and their posttranscriptional modification; and the assembly of ribosomes, separating these processes from protein synthesis, which occurs outside the nucleus.

23.7.1 The Structure of DNA and RNA and the Genetic Code

DNA and RNA are polymers of nucleotides. The nucleotides of DNA consist of the pentose sugar deoxyribose, bound at C1 to one of the nitrogenous bases adenine (A), guanine (G), cytosine (C), or thymine (T). RNA contains the pentose sugar ribose instead of deoxyribose, and the nitrogenous bases A, G, C, and uracil (U) which replaces thymine. Based upon the differences in sugar molecules and nitrogenous bases, DNA and RNA vary in their structure and function. DNA is a dimeric, double helical, deoxyribonucleotide polymer (Fig. 23.9). The nucleotide subunits in each half of the double helix are joined together by covalent phosphodiester bonds between the −OH group at C3′ on the pentose ring, and the phosphate group, which is situated at C5′ of the pentose in the adjoining nucleotide. Hydrogen bonds between the nitrogenous purine (A, G) and pyrimidine (T, C) bases form rungs holding the two halves of the helix together, with bonding interactions only between adenine and thymine (two bonds), and between cytosine and guanine (three bonds). The two strands of the double helix therefore contain complementary base sequences carrying essentially the same genetic information, however, one strand runs in the reverse order of the other, and is referred to as the complementary, antiparallel strand.

The now well-known genetic code guiding the translation of the DNA information into a protein, is a triplet code, whereby successive sequences of three nucleotides (codons), reading from the 5′ to the 3′ end of the so-called sense strand[3] of the double helix, indicate which amino acids and in what sequence, are incorporated into the protein molecule. Given the four nucleotides which make up the DNA, this allows for more than sufficient triplets (4^3 or 64) to code

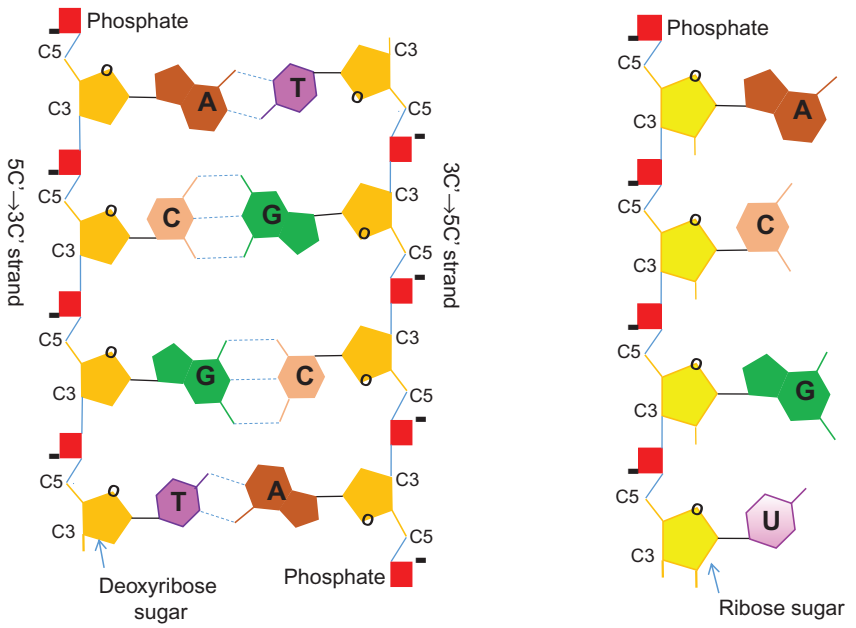


FIGURE 23.9 Basic structure of deoxyribonucleic acid, DNA (left) and ribonucleic acid, RNA (right). In DNA the nucleotides (deoxyribose sugar with phosphate on C5 and nitrogenous base [adenine A, thymine T, cytosine C or guanine G] on C1) polymerize via phosphodiesterase bonds (solid blue lines) to the −OH on C3. The separate chains, running in opposite directions (5′→3′ vs. 3′→5′), dimerize by hydrogen bonding (broken blue lines) between complementary bases (A−T and C−G). Note that the ribose sugar in the RNA backbone has an extra sidegroup (−OH) on C2 and the base thymine (T) in DNA is replaced by uracil (U). RNA is copied (transcribed) from the antisense DNA strand, after separation of the dimer, by pairing and joining up of complementary ribonucleotides.

3. The complementary strand (which runs in the opposite direction—3′ to 5′) is termed the "antisense strand."

for the 20 amino acids that make up the body's proteins. There is even some degeneracy, whereby several different triplet sequences (codons) may code for the same amino acid. As examples, the mRNA triplet UGG codes for the amino acid tryptophan, whilst the codons UAU and UAC code for tyrosine. The codons UAG, UGA, and UAA code for no amino acids and are called "stop" sequences. The start of the "reading frame" of a meaningful sequence will usually be marked by (among other factors) the AUG "start codon," which also codes for the amino acid methionine. It is the antisense DNA strand that actually serves as the template for transcription of the mRNA, which therefore has the same 5′→3′ base sequence as the "sense" strand, with U substituting for T.

RNA, unlike DNA, does not readily form a helical dimer, but is a single-stranded linear structure (Fig. 23.9) which may fold upon itself into loops due to intramolecular hydrogen bonding interactions between segments with complementary sequences of the nitrogenous bases cytosine/guanine and uracil/adenine. This gives rise, e.g., to the characteristic "clover leaf" configuration of tRNA.

23.7.2 DNA Replication

DNA is replicated by opening up of the double helix, and attachment of complementary deoxyribose nucleoside triphosphates (dNTP) (e.g., ATP, GTP), to each strand, successively at the 3′ end of the new, growing, complementary strands. Therefore the complementary DNA strands elongate in a 5′ to 3′ direction. DNA polymerase forms phosphodiester bonds between the 3′ −OH of the new strand being sequenced, and the 5′ C phosphate of an incoming dNTP complementary with the next available nitrogenous base on the template strand (A with T, and C with G), releasing pyrophosphate in the process. Each strand of the helix serves as a template for a growing complementary strand. DNA replication is thus semiconservative since one parent strand remains in each newly replicated DNA chain. To initiate replication, origin recognition complexes bind to specific replication points called origins.[4] Helicase unwinds the helix at each origin, separating the strands to produce a pair of replication forks (Fig. 23.10). Topoisomerase helps to relieve the stress developed in advance of the forks due to the unwinding of the helix. Primase forms a complementary 10-base RNA sequence on each separated single strand near the fork. This initial sequence, termed the RNA primer, is important since DNA polymerase can only add nucleotides to a preexisting nucleotide chain. DNA polymerase can then link the free 3′ −OH of the growing strand with an incoming complementary dNTP as described above, simultaneously

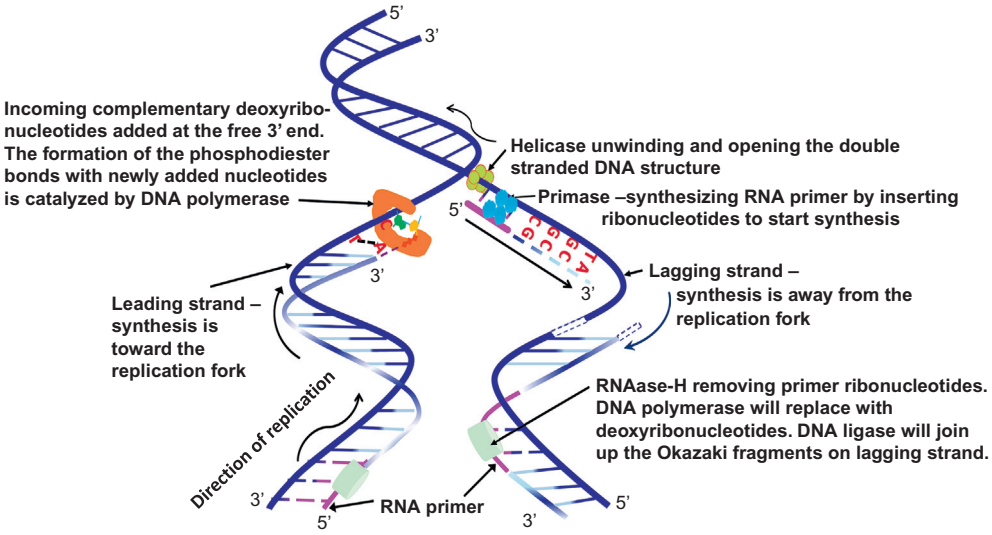


FIGURE 23.10 *DNA replication.* After the original double helix is separated and unwound, replication is initiated by primase which forms a complementary 10-base RNA sequence at the fork, on each separated strand. This initial sequence is called the RNA primer. Complementary deoxyribonucleotides are then added to each strand starting at the free 3′ end of the primer, elongating the new DNA strands in a 5′ to 3′ direction. For the leading strand synthesis of the new, growing strand continues as the fork advances. However, replication on the lagging strand will encounter a segment of double helical DNA or a preexisting primer, stopping further growth. When this happens, the fork will have advanced by about 200 nucleotides in eukaryotes (distance represented by broken lines in the strands) and a new RNA primer is inserted at this point on the lagging strand, with growth resuming from this point. Thus the lagging strand grows in segments, called Okazaki fragments, each about 200 nucleotides in length.

4. These origins are usually rich in A−T base pairs which open more readily because they each have only two as opposed to three hydrogen bonds.

"proofreading" to ensure that only correctly paired nucleotides are admitted. For the parent $3' \rightarrow 5'$ strand, leading into the fork (the leading strand) a single primer is sufficient for sustained growth of the newly synthesized strand at its $3'$ end toward the advancing fork. For the complementary strand (the lagging strand), however, growth at the $3'$ end of the primer (away from the fork) soon encounters a segment of double helical DNA (or a preexisting primer), stopping further growth. This induces insertion of a new primer on the lagging strand at the new location of the advancing fork, usually some 200 base pairs on from the previous primer (in eukaryotes), with growth of the new strand proceeding up to the preexisting primer.

In this way the lagging strand grows in segments, called Okazaki fragments, separated by intercalated primers. These RNA primers are eventually degraded by RNAase-H and replaced by DNA using a slow variant of DNA polymerase; the separate segments of newly formed DNA are joined enzymatically by DNA ligase (see Fig. 23.10).

23.7.3 Transcription and Translation of Genes

All cells in the body putatively have exactly the same complement of genes. The orchestration to determine which set of genetic units is actively synthesizing products in any cell at any given time, is therefore the essence of differentiation of tissues and of regulation to ensure the development of a functional organism, operating as a harmonious entity. To control this process, each genetic unit within the DNA comprises both a coding sequence which will be transcribed into mRNA then translated into a protein chain, and regulatory sequences located upstream (at the $3'$ end) and downstream (at the $5'$ end) of the coding sequence on the antisense strand (and even at remote sites in the DNA) which are not transcribed. The coding sequence comprising both used (expressed—exons) and nonused segments (introns) are transcribed into mRNA. Introns are excised as a part of the posttranscriptional processing before export of the final coding-mRNA from the nucleus. Translation of the mRNA into a protein takes place outside the nucleus, guided by rRNA in conjunction with tRNA, in the cytosol or on the rough ER (RER). These processes are further described in the chapter on proteins and will not be discussed here.

23.8 ENDOPLASMIC RETICULUM

The ER is a system of flattened, membrane-bound sacs and tubules which ramify through the cytoplasm of the eukaryotic cell. The membranes appear to be continuous with the nuclear membranes [36]. The lumen is a single continuous space, quite separate from the cytosol, and contains a variety of specialized synthetic and other proteins depending upon the functions of the particular cell. The lumen comprises $> 10\%$ of the total cell volume. In the typical cell the ER is differentiated regionally into two parts—the rough ER (RER) and the less extensive smooth ER.

The RER, most abundant close to the nucleus, has a granular, beaded appearance, because numerous ribosomes, dense, 23-nm particles, made up of about 40% RNA, plus associated proteins, are bound to the cytosolic surfaces of its membranes. Ribosomes are also found, to a lesser extent, bound to the nuclear membrane. Free ribosomes are also located in the cytosol and in the mitochondrial matrix. Both forms, free and bound, work cooperatively with mRNA and tRNA in the synthesis of proteins. The membrane-bound ribosomes of the RER synthesize proteins for transport to specific destinations within the cell, or for export from the cell. As they are being formed, these proteins enter the lumen or become associated with the membranes of the RER, and are delivered to their final destinations in vesicles via the smooth ER and/or Golgi complex. The free ribosomes synthesize cytosolic proteins. Ribosomes may constitute over 25% of the total mass of a eukaryotic cell [31].

The smooth ER is involved in the synthesis of lipids and steroid hormones, and in the detoxification of agents such as barbiturates and alcohol. The smooth ER therefore is abundant in cells of the liver and some glands, and can proliferate or shrink greatly, depending upon need—contributing, in this way, to the development of tolerance to some drugs or toxins. The sacroplasmic reticulum (SR) of muscle cells is a part of the smooth ER. The membranes of the SR are rich in the Ca^{2+}-binding protein calbindin, Ca^{2+} channels, and Ca^{2+}-ATPase pumps, and the lumen has high concentrations of calsequestrin [2] which loosely binds to large quantities of Ca^{2+} ions, which can readily be released into the cytosol to trigger the contractile mechanism, then rapidly resequestered to terminate the process [33].

The ER stakes a good claim for being the manufacturing center of the cell, and the most outstanding exponent of this claim is the ribosome. Ribosomal RNA is synthesized and assembled with its associated proteins[5] in the nucleolus (see above). The particle, with a Svedberg sedimentation coefficient (S) of about 80 (80 S) in most eukaryotes (smaller

5. The genes for these proteins, like all others, are transcribed into mRNA which is exported and translated into proteins which are then reimported into the nucleus.

in prokaryotes, mitochondria, and chloroplasts), comprises two subunits—a large subunit (60 S), associated with over 40 proteins, and a small subunit (about 40 S) made up of a 16 S rRNA chain plus some 21 proteins. The two subunits remain free and separate until the small subunit binds to a suitable mRNA chain to initiate the process of protein synthesis (see chapter on proteins for more details).

23.8.1 Protein Management

Synthesized proteins undergo posttranslational modification as required. Cytosolic proteins, whether fibrillary (tubulins, filaments, actins, etc.) or enzymatic, undergo minimal modification. They may be tagged with side-groups, which modify their activity and which, along with N-terminal amino-acid "presequences," help to determine their destination in the cell. Nuclear pores and mitochondrial or peroxisomal membrane receptors recognize specific sequences and allow entry of appropriately labeled molecules. RER proteins undergo extensive posttranslational cleavage, splicing, phosphorylation, acetylation, glycosylation, methylation, acylation, or other transformations as required. Proteins which are misfolded or incorrectly translated are tagged for destruction and are not processed further. This posttranslational processing takes place in the lumen of both the rough and smooth ER, in the Golgi apparatus and even in the destination organelle, whether they be vesicles, secretory granules, endosomes, lysosomes, or the cell membrane (for reviews see [34] and [32]).

23.9 GOLGI APPARATUS

The Golgi apparatus is a stack of three to eight flattened, membrane-bound sacs or cisterns, held together by microtubules, usually located close to the rough ER. The disc-like structures are oriented with a concave cis- face toward the nucleus and ER and the opposite, convex trans- face toward the cell membrane [33]. Vesicles carrying lipids and proteins processed in the ER in their lumens or membranes, bud off from the transitional zone—smooth areas of the RER [35]—known as the Endoplasmic reticular intermediate compartment (ERGIC), and join with the receptive cis-face of the Golgi complex [34]. There is evidence of bidirectional movement of material between the Golgi and ER at this interface, with a return traffic of wrongly directed or mislabeled materials and recycling membranes. After further processing and separation within the medial Golgi cisterns, the materials are segregated based upon the final destination and packaged into three types of vesicles at the trans-face of the Golgi complex. The processes involved are poorly understood. Vesicles rich in acid hydrolase activity may be lysosomal precursors. Glycoproteins to be packaged in these vesicles are recognized by the specific phosphorylation of mannose residues as they pass through the Golgi cisterns to the trans-face. The second type of vesicles involved in cell membrane renewal will fuse with and become incorporated into the plasma membrane, carrying new membrane with new, bound proteins, glycolipids, etc., whilst older membrane areas are reclaimed in clathrin-coated vesicles for fusion with lysosomes, degradation, and recycling. These vesicles, which are continuously (constitutively) released, may also carry materials contributing to the extracellular matrix (e.g., collagen). The third category of vesicles carries constituents (e.g., neurotransmitters, hormones) in solution for exocytotic (regulated) release upon presentation of suitable stimuli. For a review see [35].

23.10 CELL SIGNALING

It would not be an exaggeration to say that signaling is the essence of cellular existence—sending appropriate signals at the correct times and responding appropriately to signals received from neighboring cells or the general environment. A failure to respond or an excessive response to a given signal often lies at the center of both cellular and organismal dysfunction.

Cellular signal transduction is most often mediated by chemical messengers sent by other cells in the body of the animal, or internalized from the external environment. Significant chemicals in the extracellular fluid may bind with receptor proteins in the cell membrane, which have evolved a high specificity and affinity for the chemical. This primary cellular interaction can mediate varied physiological responses within the cell, by activating secondary messengers. These "second messengers" may trigger oxidation, reduction, acetylation, or phosphorylation reactions which amplify the original response, and accelerate or decelerate cellular metabolism, change cell motility, or activate or deactivate genes. Multicellular animals comprise a variety of differentiated cells. Gene regulation informs cell division and differentiation, e.g., the transformation of a totipotent embryonic cell to either a muscle cell or an epithelial cell, and initiates developmental changes, which may include the synthesis and release of secretions, or the expression of structural, pigment, or metabolic proteins to carry out general cellular activities.

Low molecular weight lipophilic signaling molecules may enter the cell directly through the cell membrane and combine with intracellular transducers. Steroidal hormones for example may directly enter the nucleus of brain cells and bind stereospecifically with nuclear receptors, which when so activated, can bind with hormone responsive elements of the DNA and trigger or suppress the expression of related genes. This can, e.g., play an important role in the development of sexually dimorphic behavior patterns under the influence of the sex hormones.

Larger macromolecules may be internalized by receptor-mediated endocytosis, which involves the binding of chemicals to receptor proteins in a patch of the cell membrane coated internally by a cage of clathrin molecules. The receptor-bound macromolecule or particle (e.g., insulin or low-density lipoprotein (LDL)) and the associated cell membrane is then pinched off to form an endosome. This may allow cells to take up large particles such as LDLs but also may present a route whereby large signaling molecules (e.g., endothelial growth factor, EGF) can be carried more rapidly to their target site in the nucleus. As seen below, receptor-mediated endocytosis may also be involved in regulation of the sensitivity of cells to signaling molecules. Pinocytosis is another form of endocytosis, not involving receptor–ligand binding, enabling the cell to take up large quantities of material from the extracellular fluid. Chemicals internalized in these ways can be carried more directly to intracellular target sites.

Two prominent families of signaling proteins are highly conserved in some features, and yet extremely diverse in others. These are the cell surface receptors for growth factors, cytokines, and hormones, known as the receptor tyrosine kinase (RTK) proteins (Fig. 23.11)[6] and the GTP-binding proteins (Fig. 23.12) perhaps best characterized by the adrenergic receptors (for a review see [2]).

Examples of the RTK signaling pathway include the insulin receptor and the vascular endothelial growth factor (VEGF) receptor systems. The latter significantly promotes vascularization of solid tumors, and blockade of the associated RTKs can induce degeneration of some tumors. The putative anticancer agent catechin gallate found in green tea inhibits, at μM concentrations, VEGF-induced tyrosine phosphorylation [2].

The G protein–coupled receptors (GPCRs) are involved in the transduction of sensory signals. It is estimated that at least one-third and perhaps even one-half of the drugs marketed at the start of the 21st century were targeted at GPCRs [22].

Perhaps the best studied example of a GPCR is the β-adrenergic receptor, in which the activated G-protein Gα subunit binds to and activates the enzyme adenylyl cyclase (Fig. 23.12), which converts ATP to cyclic adenosine monophosphate, a "second messenger" which initiates several other downstream activities, including gene activation. Activation is terminated by dephosphorylation of the GTP bound to the Gα subunit (which has intrinsic GTPase activity) and

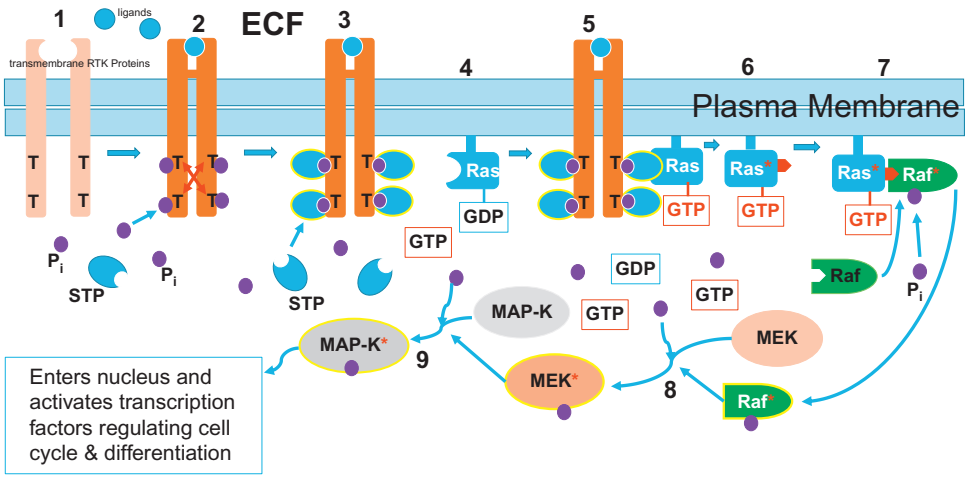


FIGURE 23.11 *RTK regulation of the MAP-Kinase cascade*. The RTK protein is a transmembrane monomer with an extracellular (ECF) receptor N-terminal and an intracellular tail displaying tyrosine (T) residues [1]. Combination with a suitable ligand (e.g., NGF) stimulates dimerization and activates the tyrosine kinase activity, causing cross-phosphorylation [3], and attracting various signal transducing proteins (STP) to bind to the phosphorylated sites [4]. The Ras G-protein [5] activated by a bound STP swaps GTP for GDP [6,7] and can then stimulate phosphorylation of Raf [8], which activates MEK [9], then, in turn, MAP-K [10]. This cascade leads to response amplification and the activation of several pathways leading to altered gene expression. Activation is terminated by dissociation of the ligand from the receptor, dephosphorylation of the phosphorylated entities, and breakdown of the GTP on Ras (a G-protein) to GDP.

6. Raf, MEK, and MAP-K are mitogen-activated kinases.

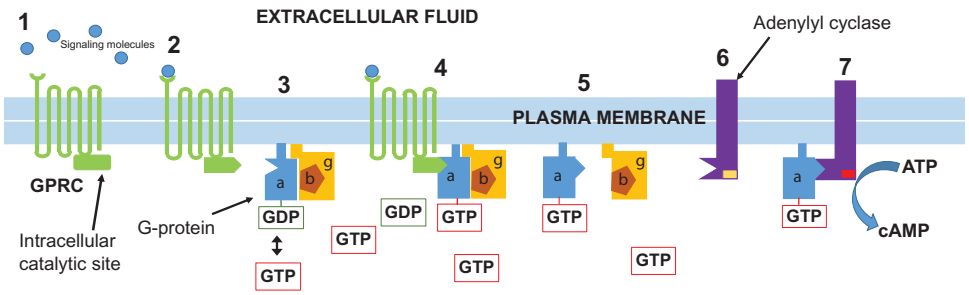


FIGURE 23.12 The serpentine G-protein coupled receptor (GPCR) has seven transmembrane domains [1] with a variable extracellular N-terminal receptor, which binds stereospecifically with a particular nonsteroidal hydrophilic signaling molecule. Ligand binding [3] activates an intracellular domain near the C-terminal, which and can then combine with the heterotrimeric G-protein in which the α-subunit is bound to GDP [4]. Linking of the activated GPCR to the G-protein allows GTP to displace GDP [5] causing dissociation of the α-subunit from the βγ-subunits and from the activated GPCR [6] which is then free to activate other G-proteins so long as it has a bound ligand [3]. Either of the G-protein subunits may then activate various intracellular pathways depending upon the particular cell involved. Here, the activated α-subunit links with adenylyl cyclase [7] inducing enzymatic conversion of ATP to cAMP [8] which serves as a second messenger to various downstream pathways. An intrinsic α-subunit phosphodiesterase activity eventually degrades GTP to GDP allowing reversion to the inactive state, and recombination with the βγ-subunits [4].

reassociation of the subunit with the Gβγ subunits. Signal termination is, however, much more complex than this alone, and involves receptor desensitization by phosphorylation of sites on the cytoplasmic loops of the GPCR, binding of these phosphorylated sites to tissue-specific cytoplasmic proteins termed "arrestins," internalization of the receptor into endocytotic vesicles via clathrin-coated pits, resensitization of the receptor, and recycling to the surface membrane. These processes play a critical role in regulation of sensitivity of the cell to signaling molecules over time, but the details are still being elucidated (for a review see [33]).

23.11 TEST YOUR KNOWLEDGE

1. Briefly describe the cell membrane.
2. Where are phospholipids synthesized?
3. What is shuttled across the membrane of the mitochondrion to generate acetyl-coenzyme A?
4. What are telomeres?
5. Why is the animal cell unable to resist osmotic swelling?

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Chapter 24

Proteins

J.E. Murray[1], N. Laurieri[2] and R. Delgoda[1]
[1]University of the West Indies, Mona campus, Jamaica, West Indies, [2]University of Oxford, Oxford, United Kingdom

Chapter Outline

Learning Objectives

- Explain the structure and biosynthesis of proteins
- Explain the general function of proteins and how it relates to their structure
- Discuss how man has utilized proteins for his benefits, especially in the pharmaceutical industry.

The name protein that I propose. . ..I wanted to derive from Greek word proteios*, because it appears to be the primitive or principal substance of animal nutrition.*

Jacob Berzelius, 1838

24.1 INTRODUCTION

Proteins are essential biological macromolecules, capable of performing vital functions in the body as enzymes, hormones, and regulatory, transport, and structural molecules involved in key immune, circulatory, and homeostatic processes. They are the most predominant macromolecules present in living cells, representing over 50% of the dry weight of a cell [1] and accounting for roughly 20% of the total body mass [2]. In 1838, Gerardus Mulder, a Dutch chemist, introduced the term "protein" to scientific literature in his publication "On the composition of some animal substances" (originally written in French), where he described the presence of a common complex substance in blood fibrin, serum, egg albumin, gelatin, and the gluten of wheat. The credit for the name however was granted to his colleague, Jons Jacob Berzelius (Swedish: jœns jkb bæselis), who made the suggestion to Mulder in a letter dated July 10, 1838, stating [3,4]:

The name protein that I propose for the organic oxide of fibrin and albumin, I wanted to derive from Greek word proteios*, because it appears to be the primitive or principal substance of animal nutrition.*

Proteios, from which "protein" was derived, means of first rank or position and accurately represents the essential role they play for living organisms.

Pharmacognosy. DOI: http://dx.doi.org/10.1016/B978-0-12-802104-0.00024-X

24.2 GENERAL PROPERTIES

Below are properties shared by all proteins:

1. They represent a significant portion of the living cell, and participate in almost all biochemical reactions.
2. They are usually built from a reservoir of 20 standard amino acids.
3. While the constituent amino acids contribute to the characteristics of the protein through the side chains, the characteristics of the end product (i.e., the protein) is not necessarily a simple sum of the characteristics of the individual amino acids.
4. The structure of the protein plays a huge role in its function. This structure is affected by the conditions of the surroundings and therefore the temperature, pH, and salt concentration of its environment will affect both the structure and function of the protein.
5. Proteins can interact with other macromolecules or other proteins to form complex macromolecules with capabilities which would not be possible by the individual units.

24.3 FROM AMINO ACID TO PROTEIN: PROTEIN BIOSYNTHESIS

The synthesis of a functional protein involves construction at several levels, much like the construction of a room (see Fig. 24.1). The building blocks for the protein are the monomeric amino acid units, which are linked together to form a linear chain called the primary structure or polypeptide chain. This can be visualized as cementing concrete blocks side by side. From the primary structure, the chain can fold to form distinct structures, namely secondary and tertiary structures. This is much like building blocks stacking together to form a wall, and the four walls coming together to build the room. The protein construction can take a further step where multiple polypeptide chains and/or cofactors conjugate to form the quaternary structure, like adding a roof to the room. Proteins work based on their shape and structural changes; therefore knowledge on protein structure is critical to understand protein function.

24.3.1 Primary Structure: Piecing Together the Polypeptide Chain

As just mentioned, the first step to the actual synthesis of any protein is the formation of its *primary structure*, which is the linear polymer comprising of the protein's monomeric units, the amino acids. While there are 23 *proteinogenic* (meaning, protein building) *amino acids*, 20 are referred to as *standard amino acids* (Table 24.1). The canonical

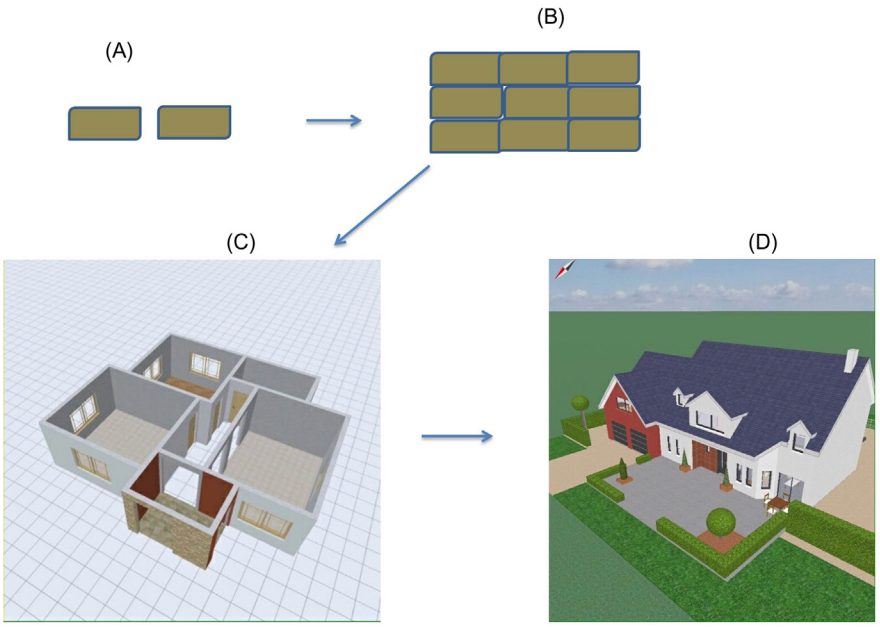
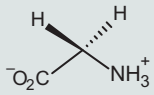
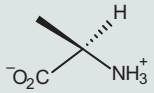
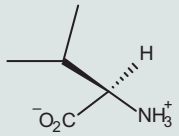
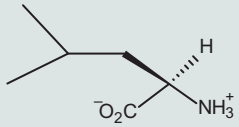
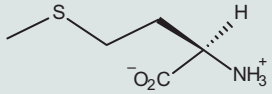
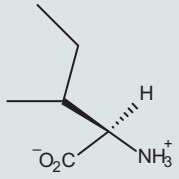
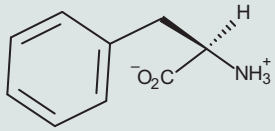
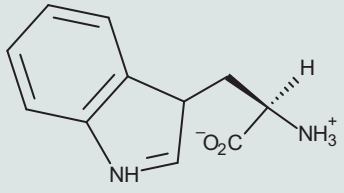
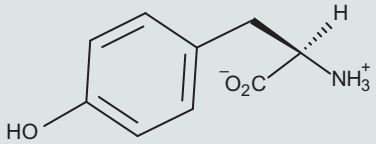
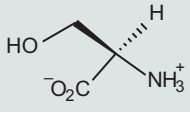


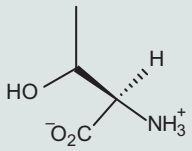
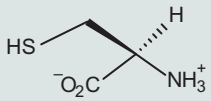
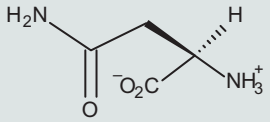
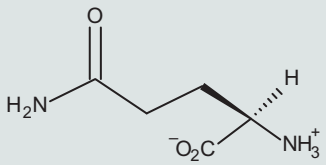
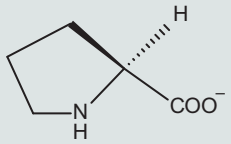
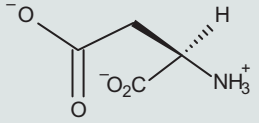
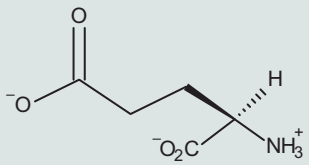
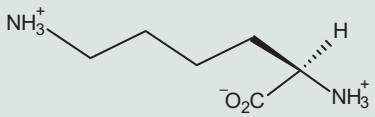
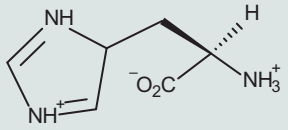
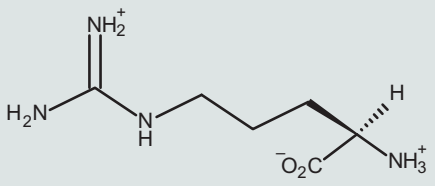
FIGURE 24.1 Structural elements of proteins. (A) The building blocks, the amino acid sequence, forms the primary structure; (B) the assembly of the building blocks into a uniform formation constitutes the secondary structure; (C) the assembly of the secondary structures into a further dimension forms the tertiary structure; (D) the assembly of the quaternary structure.

TABLE 24.1 Structures of the 23 Proteinogenic Amino Acids

Amino Acid Name (Three-Letter Abbreviation)	Structure
Nonpolar, Hydrophobic, Aliphatic	
Glycine (Gly)	
Alanine (Ala)	
Valine (Val)	
Leucine (Leu)	
Methionine (Met)	
Isoleucine (Ile)	
Nonpolar, Hydrophobic, Aromatic	
Phenylalanine (Phe)	
Tryptophan (Trp)	
Polar, Uncharged, Hydrophobic, Aromatic	
Tyrosine (Tyr)	
Polar, Uncharged, Hydrophilic	
Serine (Ser)	

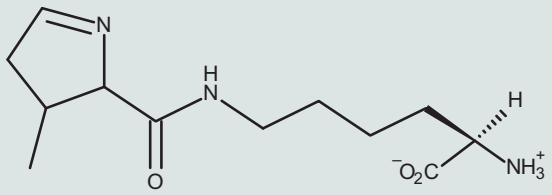
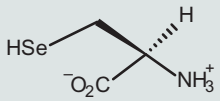
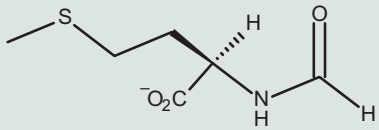
(*Continued*)

TABLE 24.1 (Continued)

Amino Acid Name (Three-Letter Abbreviation)	Structure
Threonine (Thr)	
Cysteine (Cys)	
Asparagine (Asn)	
Glutamine (Gln)	
Proline (Pro)	
Polar, Negatively Charged	
Aspartate (Asp)	
Glutamate (Glu)	
Polar, Positively Charged	
Lysine (Lys)	
Histidine (His)	
Arginine (Arg)	

(Continued)

TABLE 24.1 (Continued)

Amino Acid Name (Three-Letter Abbreviation)	Structure
Nonstandard Proteinogenic Amino Acids	
Pyrrolysine	
Selenocysteine	
N-formylmethionine	

The standard 20 amino acids have been grouped based on the polarity, aromaticity, and formal charge of the side chain groups.

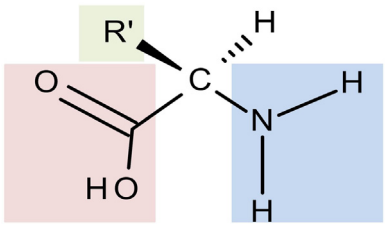


FIGURE 24.2 General structure of amino acids.

structure of amino acids consists of an alpha carbon (C_α) covalently bonded to three diverse substituents—a hydrogen, a carboxylic carbon, an amine nitrogen, and an amino acid−specific side chain (R) (Fig. 24.2).

The primary structure is produced from *condensation reactions* between amino acids, with each reaction resulting in the formation of a *peptide bond*, and this bond requires energy for its formation [5]. Mixing two amino acids together in an aqueous solution at room temperature, of which one contains an unprotonated amino group and the other contains a protonated carboxylic group, would therefore only form a salt because there is no input of energy. In the body, the carboxylic group of the amino acid that will take part in the peptide formation must first be activated before reacting with the amine group of a second amino acid. This activation comes from the conversion of the carboxylic group to an anhydride, which the Organic Chemist will recognize as a high energy structure with a good leaving group. In the biological system, *adenine trinucleotide phosphate* (ATP) is the typical energy store, containing a triphosphate anhydride group (Fig. 24.3). The energy contained in ATP can be transferred to the carboxylic acid group on an amino acid, in an activating adenylating reaction resulting in the formation of an intermediate aminoacyl-AMP anhydride. This activation precedes attack of the resulting anhydride by the 3′ or 2′ hydroxyl group of a *transfer ribonucleic acid* (tRNA) molecule. The tRNA is responsible for the translation of genetic code to amino acid sequence, which is discussed in more details below. The final product of the activation is known as *aminoacyl tRNA*, and this is what enters the ribosomal unit for attachment to the growing polypeptide chain. Each amino acid has its own specific tRNA molecule and an aminoacyl tRNA synthetase enzyme that catalyzes the activation of its substrate amino acid and subsequent transfer of the amino acid to its tRNA molecule. High fidelity between the amino acid and its tRNA is crucial to ensure insertion of the correct amino acid in a growing polypeptide chain (Fig. 24.3).

Since the peptide chain sequence is very specific, the attack of the aminoacyl tRNA by a second amino acid must be a precise and ordered process. Different RNA molecules and the ribosome set the scene for such a process to take place, and

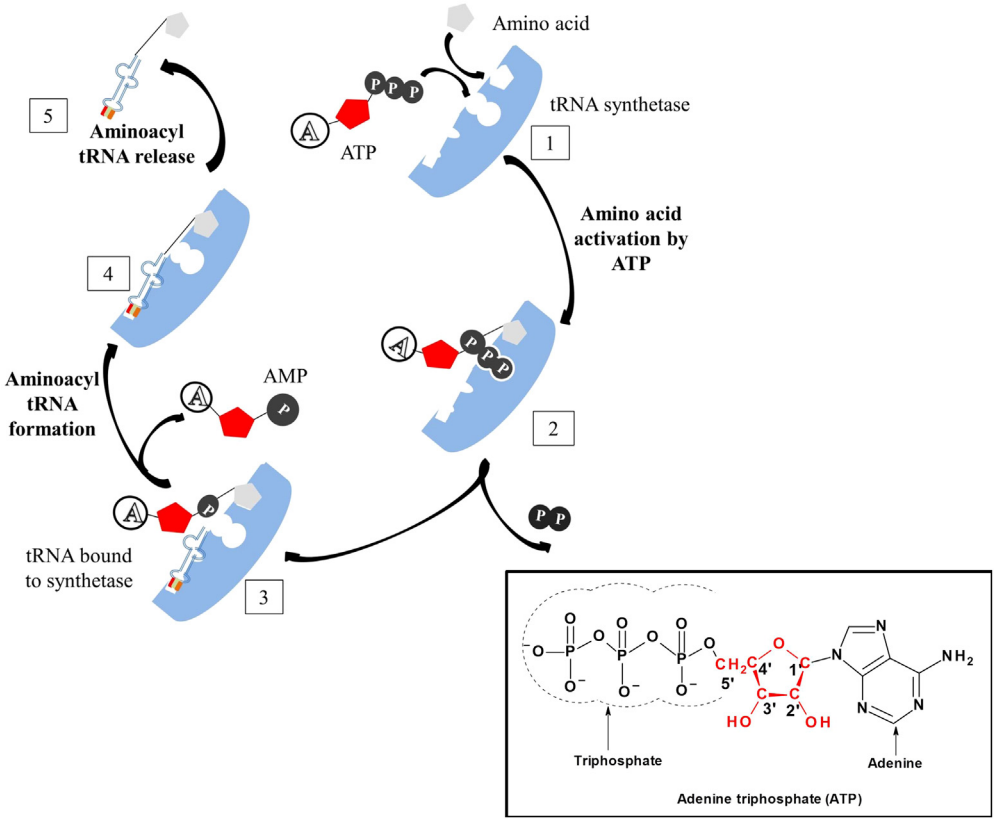


FIGURE 24.3 Synthesis of aminoacyl tRNA by tRNA synthetase. (1) The amino acid substrate and ATP bind to tRNA synthetase; (2) the synthetase catalyzes the activation of the amino acid by forming an amino acyl-AMP anhydride, resulting in the loss of diphosphate; (3) tRNA binds to tRNA synthetase; (4) the synthetase catalyzes the formation of the aminoacyl tRNA; (5) aminoacyl tRNA is released.

how they do so is discussed further in the section below. The condensation reaction between the amino acids may be repeated up to a 1000 times and the product is always a linear *polypeptide chain*, and takes place in the cytoplasm. This chain has directionality, like DNA, with the first amino acid located at the N terminus and the last at the C terminus. But before we continue, we should take note of the events that take place before amino acids are put side by side and joined together, as these events need to be taken into account in order to complete the story on protein synthesis.

24.3.1.1 Transcription

Before the primary structure of a protein can be synthesized, information dictating its sequence must first be found. This information is found in the genetic material, DNA, inside the nucleus of a eukaryotic cell. The DNA helix is first unwound to expose the region of interest and a copy of this region in the form of *messenger RNA* (mRNA) is produced. The mRNA may have coding and noncoding regions, and the coding region is called the reading frame. As expected, there are fundamental differences in transcription between prokaryotic and eukaryotic mRNA, with the eukaryotic mRNA processing being more extensive and highly regulated. In prokaryotic cells, transcription and translation occurs in the same location. In fact, translation can take place while the nascent prokaryotic mRNA is still being transcribed. In eukaryotic cells, however, transcription and translation are compartmentalized, with transcription taking place in the nucleus and translation taking place in the cytoplasm. In prokaryotic cells, the primary transcript is oftentimes what is translated into a protein. In eukaryotic cells, however, the primary mRNA (called *premRNA*) undergoes posttranscriptional modifications that will produce the final *mature mRNA*. There are three main modifications that take place before mRNA exits the nucleus and into the cytoplasm: splicing, poly(A) tail addition (*polyadenylation*), and 5′ capping. The latter two are suggested to affect mRNA stability, facilitate its transport to the cytoplasm and improve translation efficiency. Splicing is where noncoding sections of the preRNA (*introns*) are excised by a spliceosome and left in the nucleus, and the remaining (*exons*) are stitched together by RNA ligase and exit into the cytoplasm for translation to protein production. Like the saying goes, "there is more than one ways to skin a cat," and this applies well to splicing as one premRNA can result in different mature mRNAs, and hence different proteins, due to a phenomenon called

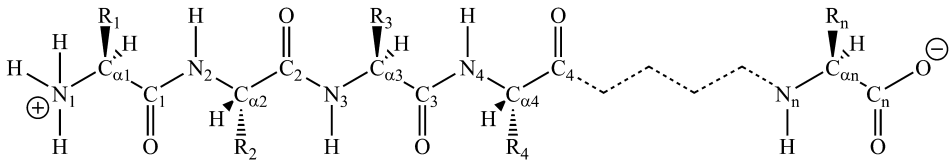


FIGURE 24.4 Stereochemistry of protein primary structure.

alternative splicing. This can take place in three different ways: (1) exon skipping, where an exon situated between two introns is excised; (2) alternative splice sites at one end of an exon; and (3) intron retention, where the intron is not excised and hence is found in the mature mRNA [6]. Alternative splicing debunks the old notion that for one gene you get one protein, and demonstrates the efficient design of human DNA. It also explains why humans can produce about 90,000 different types of proteins with only 25,000 protein coding genes, which represents a mere 1−2% of the entire genome [7,8]. The mature mRNA is then transported to the cytoplasm for protein production after posttranscriptional modification. At the 5′ end of the mRNA is the start codon where translation is initiated for both prokaryotic and eukaryotic cells.

24.3.1.2 Translation

Once in the cytoplasm (for eukaryotic cells), mRNA is translated from one language (nucleotide sequences) to another (amino acid sequences) in a 5′−3′ direction, with the aid of ribosomes. The ribosome consists of large and small subunits that come together on the mRNA template, providing a platform for the ordering of the peptide sequence. Basically, ribosomes travel along mRNA and allows for the reading of its information in groups of three nucleotide letters called *codons*. There is also the adaptor molecule tRNA, in the form of aminoacyl tRNA (see Section 24.3.1), that mediates this translation from genetic code to amino acid sequence. It is able to carry out this mediation because of the amino acid covalently attached to one end of its structure and a triplet genetic code present at the other end which binds to mRNA through base pairing. The ribosome then catalyzes the formation of a peptide bond between amino acids.

The large subunit of the ribosome contains three sites in which the tRNA occupies: the A site, which is occupied by aminoacyl tRNA which carries the next amino acid to be added to the polypeptide chain, the P site which is occupied by the peptidyl tRNA that is attached to the growing chain, and the E site where tRNA is released. The peptide bond is formed between the aminoacyl tRNA present in the A site and the preceding amino acid present in the growing chain attached to the tRNA molecule in the P site. For initiation, the initiator tRNA enters the ribosomal large subunit at the P site and is assisted by initiation factors, bringing with it the first amino acid. For prokaryotes, the first amino acid is *N*-formylmethionine while for eukaryotes it is methionine.

Once incorporated in a polypeptide chain, the repeating amino acid units are referred to as amino acid *residues*, since strictly speaking they are no longer amino acids; to form each peptide bond, the amino acids lose water and so it is the residue of the amino acid structure that is present in the polypeptide chain. The polypeptide chain consists of a repeating sequence of the amide N, the alpha C, and the carbonyl C and can be represented as N_i, $C_{\alpha i}$, and C_i', respectively, where i is the number of the residue in the chain starting from the amide end (Fig. 24.4).

24.3.2 Secondary Structure

Once the primary structure has been built, it is very likely that some of the amino acid residues will have hydrophobic nonpolar side chains, e.g., glycine and leucine, while others have hydrophilic polar and charged side chains, e.g., serine and glutamine, distributed along the polypeptide chain (see Table 24.1). In the aqueous environment of the cell, the hydrophilic side chains happily interact with water through hydrogen bonding but the hydrophobic side groups fold into each other to minimize contact with water, subsequently forming a compact structure. Within the hydrophobic core, the nonpolar groups can interact with each other through Van der Waals forces while the hydrophilic side groups are mostly left on the surface of the protein to interact with water. However, this folding-in of the hydrophobic side groups inevitably brings the peptide backbone for these side groups, which contain the amide (NH) and carbonyl (C═O) groups, into the hydrophobic core. The peptide backbone is therefore left with no choice but to form hydrogen bonds amongst themselves, and this interaction gives rise to well-defined structural elements called the *secondary structures*. The secondary structure refers to the localized form or shape of a segment of the polypeptide chain due to interactions between the peptide backbone [9]. The most common secondary structure is the α *helix*, followed by the β *pleated sheet*. There is

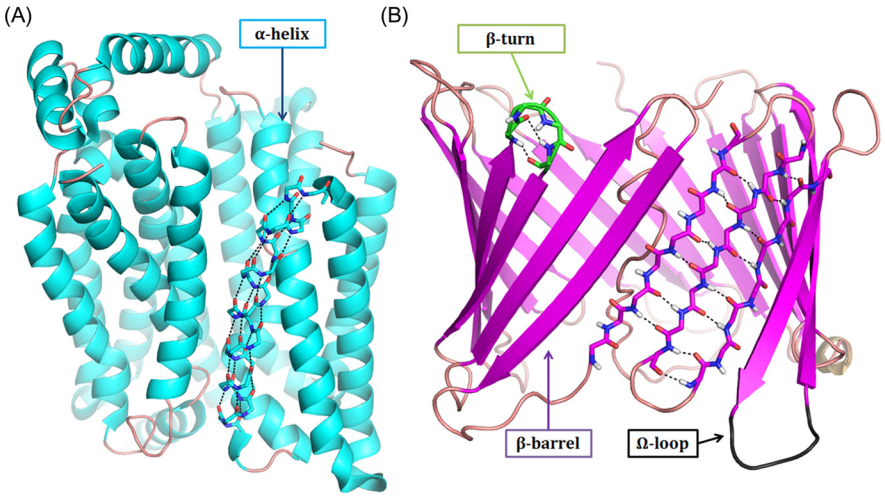


FIGURE 24.5 A selection of protein secondary structures. The proteins are represented by their peptide backbones, drawn in ribbon form, with β sheets shown as flat arrows pointing from the N-terminus to the C-terminus. α-Helices are colored in *light blue*, β strands in *fuchsia*, one β-turn in *green*, and one Ω-loop in *black*. Residue main chains of α-helices, β strands, and β-turn are partially represented in stick representation, with the alpha carbon colored in *light blue* (α helices), *fuchsia* (β strands), or *green* (β turn), hydrogen in *white*, nitrogen in *blue*, and oxygen in *red*; key intramolecular hydrogen bonds are labeled with *dashed line*. (A) X-ray structure of human glucose transporter GLUT1 [PDBid:4PYP] [11]. (B) NMR-solution structure of integral human membrane protein VDAC-1 in detergent micelles [PDBid:2K4T] [12].

also the β turn and Ω loop [10] (Figs. 24.5A,B). On their own, secondary structures would unravel in solution, as the backbone groups would prefer to hydrogen bond with water molecules instead of among themselves.

24.3.2.1 α Helix

The α helix (Fig. 24.5A) arises from intrastrand interaction, where the carbonyl group of each peptide residue in the helix hydrogen bonds with the amide group present on the peptide located four residues along the sequence. Linus Pauling was the first to propose the existence of an α helix structure in 1951, in attempting to predict possible structures that would exist in the protein that were sterically allowed and also would allow for hydrogen bonding between the atoms present in the peptide backbone [13]. Soon after, the first protein structure was elucidated using X-ray crystallography, and proved Pauling's hypothesis [14]. The helix, which is typically right-handed, consists of 3.6 residues per turn, with a rise of 1.5 Å per residue which results in a pitch of 5.4 Å. While any of the amino acid residues can be found in an α helix, different residues have varying tendencies of doing so. This tendency is called the *helix propensity*, with proline and glycine having the least propensity of appearing in a helix [15].

24.3.2.2 β Sheet

The β pleated sheet (Fig. 24.5B) is the second most abundant secondary structure, and arises when segments of a polypeptide chain, called *β strands*, overlap, and form hydrogen bonds between the strands. So, like the α helix, the β pleated sheet owes its stability to its hydrogen bonds as well as the Van der Waals forces that exist because of the close proximity of the residues in the structure. The β strands, almost fully extended, in the β pleated sheet structure may run parallel or antiparallel, using the conventional N-terminus to C-terminus directionality.

24.3.2.3 Reverse Turns

This feature is another common secondary structure, where the polypeptide chain folds onto itself, at almost 180 degree, and changes chain direction. The most prevalent reverse turn is the *β turn* (Fig. 24.5B), which comprises of four residues, where residue i and $i + 3$ interact through hydrogen bonding while $i + 1$ and $i + 2$ take part in the actual turning of the polypeptide chain. The carbonyl group and amide nitrogen on residues i and $i + 3$ can then hydrogen bond with each other, stabilizing the structure. If flanked by two β strands, the structure is called a *β hairpin*. Since the folding of the polypeptide chain is essential to the creation of the compact three-dimensional (3D) structure of a globular protein, reverse turns are a common feature in these types of proteins.

24.3.3 Tertiary Structure

The resulting 3D form that is adapted when the protein folds onto itself, stabilized by weak interactions between polar and nonpolar groups, is called the *tertiary structure* [9]. At this level, the secondary structure elements pack together to form the solid tertiary object. While tight turns do frequently connect the secondary structure elements together, such as

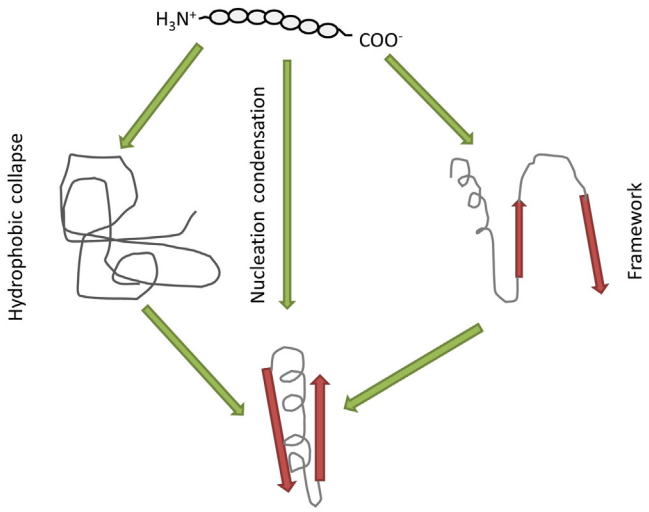


FIGURE 24.6 Hypothesized models on the early stages of protein folding. The framework model depicts early formation of defined secondary structures, followed by collapse of the chain, whereas the hydrophobic model depicts that the chain collapses first and then forms secondary structures. The nucleation condensation model is a marriage of the two, depicting that both events (the formation of the secondary structures and the hydrophobic collapse) occur concomitantly.

the α helices and the β strands, more often it is a long stretch of amino acids that is void of a regular structure that is found between the secondary structure elements. These stretches are usually found on the surface of the protein, protruding into the solvent, and are therefore convenient recognition, interaction, and binding sites, and hence are important to protein function. Since they are not as involved in the stabilization of the overall fold in comparison to the packed secondary structure interior of the protein, their corresponding DNA regions are more likely to undergo mutation and hence are also sites of evolution for protein function.

Minimizing the interaction between nonpolar side chains and water, by creating a hydrophobic core from which water molecules are expelled, and maximizing the weak interactions between like-groups is what drives the packing of the tertiary form. This is deemed the *hydrophobic effect*. There are different ways that the secondary structures can be arranged, e.g., helices can interact with other helices and/or with β sheets, and β sheets can interact with other β sheets, and the packing arrangement can be used to generally classify the tertiary structure. As just mentioned, interactions between the water molecules and the residues on the protein surface are also important in establishing the tertiary structure and so the solid form of the protein is always surrounded by bound water molecules. Water molecules can also be found inside cavities in the protein interior, if these open spaces are lined with polar side chains to interact with the water.

Despite having a vast number of possible conformations, proteins fold into their native state in milliseconds or even microseconds. How proteins fold into their native states so quickly is one question many have attempted to answer. Three hypothesized models on the events that take place at the earliest stages of protein folding that attempts to answer this question are presented here (Fig. 24.6). The "*framework*" *model* depicts that the tertiary structure is produced from well-defined local secondary structures that then collapse, arrange, and interact to form the tertiary element consisting of a hydrophobic core through the formation of an intermediate state called the *molten globule*. The "*hydrophobic collapse*" *model* states that the first step in protein folding is the collapse of the hydrophobic side chains, after which the well-defined secondary structures are formed. The "*nucleation condensation*" *model* is a combination of the two aforementioned models; it depicts that the coming together of the hydrophobic groups occurs concomitantly with the formation of the secondary structures [16,17].

Whatever happens in the early stages, what is for sure is that the spontaneous folding of a polypeptide chain to form its 3D tertiary structure is governed by favorable and unfavorable forces within the structure as well as between the structure and its environment [9]. A stable structure results in the loss of *free energy* (ΔG), which is a sum of *enthalpy* (ΔH) and *entropy* (ΔS):

$$\Delta G = \Delta H - T\Delta S \tag{24.1}$$

If a reaction is thermodynamically favorable, the ΔG should be a negative value, indicating the release of energy when the reaction takes place. When a polypeptide collapses, the hydrophobic side chains are brought together and form weak interactions, such as Van der Waals forces, that would not be possible in the unfolded state, which decreases

the enthalpy of the system and hence contributes to stabilizing the folded state. Hydrogen bonding between polar groups also contributes to the enthalpy of the system. Although these noncovalent interactions are individually weak, the strength comes from their numbers as hundreds of them are formed. In considering the effect on entropy when protein folding occurs, however, a folded structure has more order than the unfolded state, which means the entropy decreases. This decrease in entropy goes against the stabilization of the folded structure, so stabilizing forces should be able to counter this effect. In addition to the enthalpy mentioned before, the other driving force for the stabilization of the protein folded structure is the entropy of the solvent, water. In the folded state, water is expelled from the hydrophobic core and has a higher entropy than when the water molecules interact with the nonpolar groups individually, hence stabilizing the folded protein state.

Since weak interactions are the main bonds holding a protein 3D structure together, the protein is actually a fluid entity with the ability to make conformational shifts when necessary. This comes in handy when the protein is bound to a substrate that undergoes several structural changes to form the final product. The flexibility of a protein is therefore essential to its function. Other than hydrogen bonds and Van der Waals forces, other types of bonds exist within the protein infrastructure [9,18]:

Salt bridges—this is the ionic attraction between residues with ionized side chains of opposite charge.
Covalent bonds—the most common type of covalent bond is the disulfide bridge, which is formed from the oxidation between the sulfhydryl groups of two cysteines.
Metal ion coordination—this bond is typically formed between the protein side chain and a metal ion, stabilizing the protein structure.
Cofactor binding—the cofactor may be a separable organic or organometallic molecule or it may be covalent cross-linking between residue side chains that are not disulfide bridges. These are found in the active site and are crucial to protein function.

24.3.4 Quaternary Structure

Many proteins are actually made up of more than one polypeptide existing in their tertiary arrangement. These protein complexes are described as oligomeric and this level of structure is called the *quaternary structure*. Within this structure, each tertiary structure is called a subunit. The quaternary structures may be classified as homo-oligomers or hetero-oligomers based on whether their subunits are identical or a mixture of different types of tertiary structures. The simplest quaternary structure is a homodimer, which is a protein made up of two identical subunits (Fig. 24.7) [10]. The quaternary structure has the advantage of being able to repair more efficiently compared to the monomeric proteins [19]. If there is a defect in one of the subunits, that subunit can simply be replaced with another. The tertiary subunits in these complexes interact with each other via complementary regions on their surfaces. The flexibility of the individual subunits has also been recently suggested to assist in the building of the quaternary complex [20].

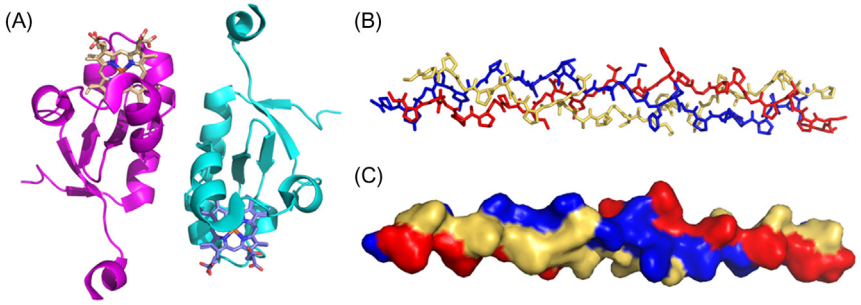


FIGURE 24.7 Three-dimensional structures of distinctive proteins. (A) Quaternary structure of the homodimer human NADH cytochrome b5 oxidoreductase (Ncb5or) is shown in cartoon representation with domain A in *magenta* and domain B in *cyan*. Each domain has a functional heme moiety as noncovalent ligand, which is represented in *pink sticks* within domain A and in *blue sticks* within domain B [PDBid:3LF5] [21]. (B,C) Example of the triple-helix chemical structure of collagen-like proteins. The von Willebrand Factor A3 domain binding region of type III collagen is shown in stick (B) and surface (C) representation with each domain differently colored [PDBid:4GYX] [22].

24.4 EFFECT OF HEAT, pH, AND CHEMICAL AGENTS ON PROTEIN FOLDING

Despite the hundreds of weak interactions holding a folded protein together, the difference in free energy between the folded and unfolded state is actually small. This means that the folded structure of a protein, called the *native state*, is only slightly stable under physiological conditions. This is because of the significant loss of entropy when the protein folds. When the weak bonds are broken due to an increase in temperature, a change in pH or the presence of chemicals denaturants such as urea, the protein structure unfolds exposing the hydrophobic groups that formed the interior to the aqueous environment. If the unfolding is to the extent where protein function is lost, the protein is said to be *denatured*. Usually with the return of ideal conditions for folding, denaturation is reversed, although this is not always the case (Fig. 24.8). With the change in the environment, the equilibrium that exists between the native and the denatured state shifts to favor unfolding.

24.5 PROTEIN CLASSIFICATION

Proteins can be classified based on numerous properties, structural features being an important one. We have just completed the section on the building of the protein structure, so classification based on structure will now be discussed. The two main categories for protein structure are *fibrous* and *globular*.

24.5.1 Fibrous Proteins

Fibrous proteins consist of elongated polypeptide chains that run parallel to one another and are stabilized by cross-linkages. In humans, their main role is to provide structure and support and aid in biomechanics. They are not found in differentiated plants [23]. The most commonly found fibrous protein (and protein overall) is collagen, which accounts for about 30% or more of the total protein in the body. The basic structure of all collagens is a triple helix (Fig. 24.7) however collagen can be grouped into at least 16 types based on the 3D structures formed at points where the helix is interrupted [24]. The basic triple helix comes about from a high abundance of glycine, proline, and hydroxyproline in its primary structure. Each of these amino acids contribute to the formation and stability of the triple helix; the glycine fits into the crowded center of the helix, where hydrogen bonding between its peptidyl group and another peptidyl group on an adjacent polypeptide chain can take place and keep the structure together; the proline and hydroxyproline allow the actual twisting of the chains resulting in the characteristic helical formation [24]. Collagens are major components of the bone (type I collagen), cartilage (type II collagen), blood vessels, tendons, and other body components. Like most fibrous proteins, collagen is insoluble in an aqueous environment.

24.5.2 Globular Proteins

Most proteins belong to this group, and take on the compact tertiary and quaternary structures that have been discussed in Sections 24.3.3 and 24.3.4. If large enough, the protein may possess two or more *domains*, although there are many cases of stand-alone domains. Domains are individual compact regions within the protein structure that are often folded in such a way that they may be stable in isolation. This stability is due to the formation of the hydrophobic core, just like in its protein whole. Domains are often made up of a continuous segment of amino acid sequence, but there are

FIGURE 24.8 Protein denaturation in eggs. Heat irreversibly changes the structures in eggs.

exceptions where the amino acid sequence for one domain is interrupted by the sequence that forms another domain. They also often retain part of the biochemical function of the protein from which they are derived. It seems there are a finite number of possible domain folds, as they are used repeatedly in the construction of proteins, but in different arrangements. This variation in arrangement gives rise to the diversity of protein function.

24.5.2.1 Enzymes

Enzymes found all over nature (Fig. 24.9) are a special and very important group of globular proteins, as they carry out crucial catalytic functions in the body. These proteins bind to their substrates in special regions within their compact structure called *active sites*, which is a pocket found on the enzyme surface, and catalyze reactions by increasing the rate of the reactions. This often enables these reactions to take place millions of times faster than if they were left alone to take place under physiological conditions. There are thousands of enzymes in the body, and almost all chemical processes that take place involve the action of enzymes. Essentially, they allow reactions to take place fast enough to sustain life at physiologically safe temperature and pressure. The enzymes themselves, though their conformation may change due to substrate binding, are not permanently altered by the reactions they catalyze.

The active site is a small area in the entire protein structure; however, this is where the catalytic power of enzymes lies. With the composition of the active site being very precise, the substrate(s) that can bind to the enzyme is also very specific, limiting the number of reactions that each enzyme can catalyze. The level of specificity can be categorized into five groups: *absolute specificity*, *group specificity*, *bond specificity*, *stereochemical specificity*, and *reaction specificity* (Table 24.2). Few enzymes exhibit absolute specificity, where they catalyze only one reaction with only one substrate.

Minor changes in the amino acids found in the active site greatly affect substrate specificity and consequently what reaction the enzyme catalyzes. An example can be seen when considering a group of pancreatic serine proteases:

FIGURE 24.9 Fruits containing proteolytic enzymes. Papain and bromelain are expressed in papaya and pineapple, respectively.

TABLE 24.2 Definitions for the Different Levels of Enzyme Specificities

Level of Specificity	Definition	Examples
Absolute	Enzyme can only catalyze one reaction with one substrate	Urea hydrolysis by urease: $H_2N(CO)NH_2 + H_2O \rightarrow CO_2 + 2NH_3$
Group	Enzyme catalyzes reactions involving substrates with specific functional groups	Trypsin hydrolysis of peptide bonds for amino acids with positively charged side chains only
Bond	Enzyme catalyzes reactions on specific chemical bonds	Serine proteases only act on peptide bonds
Stereochemical	Enzyme catalyzes reactions for one stereoisomer of its substrate	Action of lactic acid dehydrogenase on L-lactic acid but not on D-lactic acid
Reaction	Enzyme catalyzes a specific reaction for structurally related substrates	Serine proteases catalyze the hydrolysis of peptide bonds only

chymotrypsin, trypsin, and elastase. These proteases catalyze the hydrolysis of peptide bonds between aromatic and nonpolar amino acid residues on the carboxyl terminus and they utilize a *catalytic triad* Asp/His/Ser to carry out the hydrolysis. However, each protease hydrolyzes the peptide bonds linking specific types of amino acids due to the presence of other amino acid residues within the active site pocket. The active site of a trypsin-like enzyme has an additional Asp residue, which carries a negative charge and is neutralized when a positively charged amino acid enters the active site. This neutralization helps stabilize the enzyme substrate complex that is formed once the substrate enters the active site, and hence trypsin catalyzes the hydrolysis of the peptide bond between amino acids which have side chains carrying a positive charge. This Asp residue however is not present in chymotrypsin, which catalyzes the peptide bonds between amino acid residues with large hydrophobic nonpolar side chain groups. Elastase can only catalyze the hydrolysis of the peptidic−amide linking amino acid residues with small side chain groups because of the presence of two valine residues in its active site pocket. These two residues are located on either side of the pocket, blocking access to the depths of the pocket by residues with large side chains due to steric restrictions (Fig. 24.10).

Enzymes accelerate chemical reactions by lowering the *activation energy*, which is the energy barrier that needs to be overcome for a reaction to take place Fig. 24.11.

Enzymes help to lower this barrier through various *catalytic strategies*. Catalysis may simply take place due to providing a means for multiple substrates to come in close proximity of each other, and sometimes also in the correct orientation. This is called *catalysis by approximation and orientation*. Another important strategy is *transition state stabilization*. Here, weak interactions between the enzyme and the substrate are optimized in the transition state, which provides the energy needed to lower the activation energy barrier.

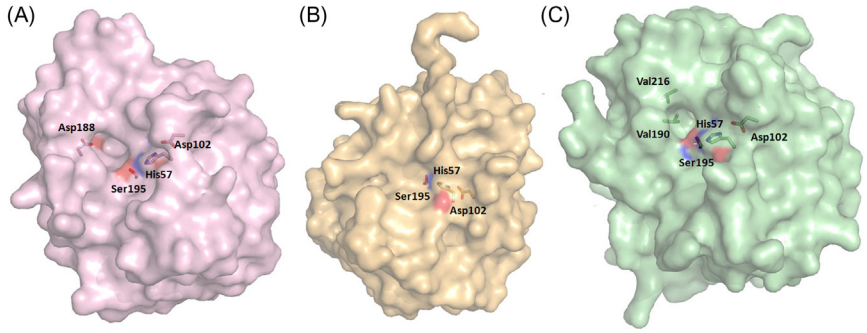


FIGURE 24.10 Effects of residue differences in serine protease family on substrate specificity. Three diverse serine proteases are shown in surface representation. Residues of the catalytic triad (Asp102-His57-Ser195) and key residues in substrate specificity are labeled and shown in stick representation. (A) X-ray structure of DESC1, a human trypsin-like type II transmembrane serine proteinase [PDBid:2OQ5] [25]. (B) Crystal structure of bovine α-chymotrypsin [PDBid:1OXG] [26]. (C) X-ray structure of human neutrophil elastase [PDBid:3Q76] [27].

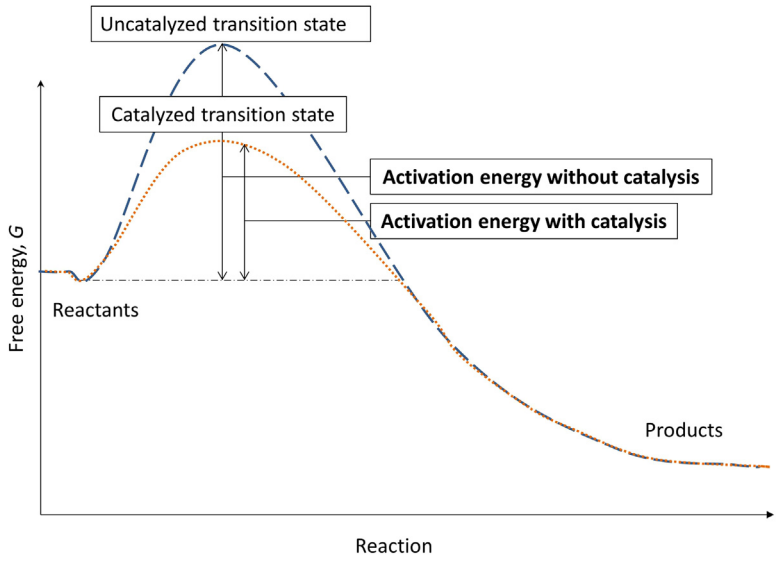


FIGURE 24.11 Influence of enzyme catalysis on activation energy. Enzymes increase the rate of reactions by decreasing the activation energy barrier.

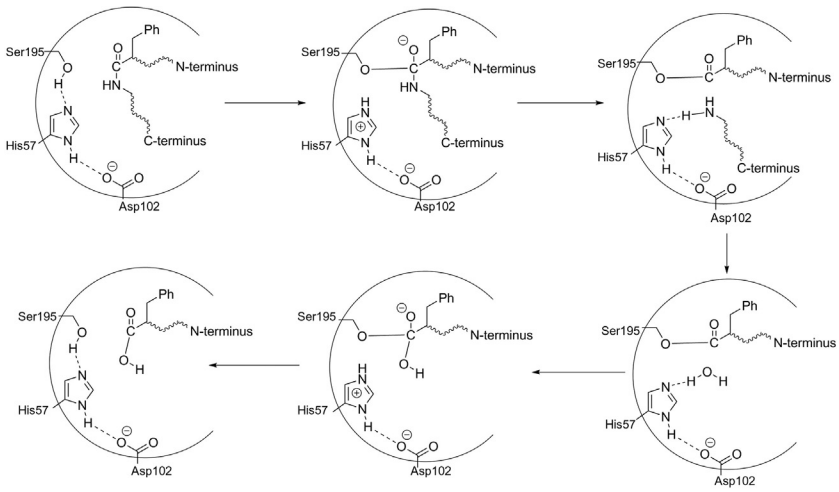


FIGURE 24.12 Catalytic mechanism of chymotrypsin. The polypeptide substrate binds noncovalently to the side chains of the hydrophobic active site, where H^+ is transferred from Ser195 to His57 and the resulting nucleophilic hydroxyl oxygen of Ser195 attacks the peptidic carbonyl carbon, thereby forming a tetrahedral intermediate with the enzyme. Subsequently, a proton is transferred from His57 to the peptidic nitrogen which results in a release of the C-terminal fragment, whereas the N-terminal peptide keeps bound through acyl bond to Ser195. A water molecule binds to His57 and attacks the carbonyl carbon linked to Ser195, thus forming a tetrahedral transition state which allows the release of the N-terminal fragment and the return of the enzyme conformation to its initial state.

Covalent catalysis is a commonly used mechanism which involves the formation of a transient covalent bond between the enzyme and the substrate that results in a more reactive complex compared to the substrate, hence reducing the energy requirements for the reaction to take place. It usually involves a powerful nucleophilic group present in the active site. *General acid–base catalysis* is another common mechanism employed, where a residue is able to remove or donate a proton as part of the catalytic process. A classic example for both covalent catalysis and general acid–base catalysis strategies is in the proteolytic action of the serine proteases, such as chymotrypsin (Fig. 24.12). In the catalytic triad, the hydroxyl group of the Ser195 nucleophilic residue is hydrogen bonded to the imidazole ring of the His57 residue, which polarizes the OH group and makes it a more reactive nucleophile. The carboxyl group of Asp102 in turn hydrogen bonds to imidazole ring of His57, stabilizing the orientation of His57 as well as the positive charge that forms on the His residue during the catalytic process. The oxygen in the OH group of Ser195 attacks the carbonyl carbon on a peptide residue substrate, forming a tetrahedral intermediate, and the His57 residue acts as a *general base catalyst* by accepting the hydrogen atom from Ser195. The tetrahedral intermediate carries a formal negative charge which is stabilized by interactions with NH groups on amino acids present in a site of the enzyme called the *oxyanion hole* [10]. Subsequently, the amino group is released following proton transfer to the amino nitrogen by His57, which acts as a *general acid catalyst* at this point. A water molecule then attacks the carbonyl group that is still covalently bonded to Ser195, forming the tetrahedral intermediate like before, while a proton is concomitantly transferred to His57. The intermediate breaks down to release the carboxylic acid. It is apparent that there is a network between the catalytic triad members that allows for the ready exchange of hydrogen atoms and hydrogen bonding which is crucial to the catalytic process and increases the nucleophilic nature of Ser195. This network is referred to as the *charge-transfer network* [28].

Metal ions are utilized by at least 30% of all enzymes for catalysis, and they can participate in various ways. They may directly bind to the substrate, increasing the amount of interactions between the enzyme and the substrate. Or, they may stabilize the negative charge formed during the catalytic process. This type of catalysis is also referred to as *electrostatic catalysis*. They may also mediate oxidation–reduction reactions through reversible changes in their oxidation states. Cytochrome P450 (CYP) enzymes are metalloenzymes that utilize an iron (Fe) heme-prosthetic center in their active site. In the catalytic cycle for the hydroxylation of various substrates by CYPs, it is generally accepted that catalysis involves changes in the iron oxidation state to facilitate molecular oxygen activation by accepting electrons from a redox partner protein, which are crucial steps to break the molecular oxygen bond before the oxygen atoms may be used for hydroxylation. The formal positive charge on the Fe group also stabilizes the negative charges formed by the oxy intermediates (Fig. 24.13). Metal ions may also help in the generation of a nucleophile, such as in carbonic anhydrases, where Zn helps in the deprotonation of bound water before it attacks carbon dioxide to form carbonic acid and bicarbonate ions for transportation by the red blood cells in the body.

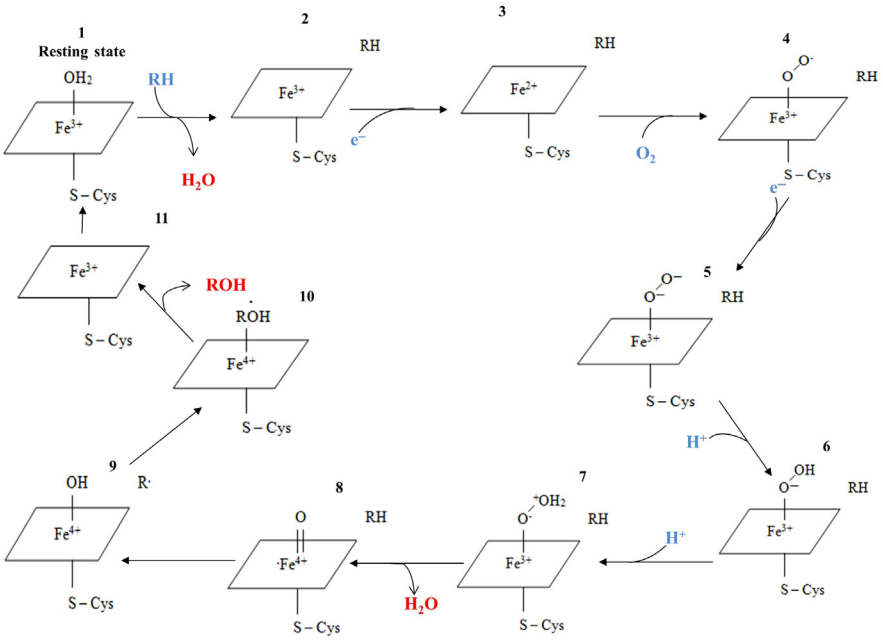


FIGURE 24.13 Metal ion catalysis: cytochrome P450 catalytic cycle [29]. (1) Ferric heme iron resting state, which exists predominantly in a low spin state usually with a sixth water ligand [30]; (2) substrate (RH) binding causes a displacement of the sixth axial water ligand, resulting in a penta-coordinated heme, a change in the ferric iron from low spin to high spin [31,32], and an increase in the heme redox potential; (3) electron transfer results in a change from ferric iron to ferrous iron; (4) oxygenated cytochrome P450 is formed as a result of oxygen binding to the ferrous iron through spin interactions; (5) ferric peroxy complex is formed as a result of a second electron transfer; (6) ferric hydroperoxy complex is formed after a proton enters the system; (7) a second protonation step takes place; (8) oxo metal complex formed due to loss of water; (9) hydrogen abstraction from the substrate to form bound hydroxyl group; (10) rapid radical recombination results in metal bound hydroxyl radical formation; (11) hydroxylated product is released.

24.6 PHARMACEUTICAL APPLICATIONS

24.6.1 Peptide Therapeutics

24.6.1.1 Nonribosomal Peptides

The peptides discussed thus far have been those built by the ribosomal machinery, but there is a diverse class of peptides that are synthesized independent of ribosomes known as nonribosomal peptides secondary metabolites, found mainly in bacteria and fungi. Monomeric units for these peptides consist of much more than the proteinogenic amino acids, as over 500 monomers may be incorporated in the peptide chains including nonproteinogenic amino acids, proteinogenic amino acids, heterocyclic compounds, fatty acids, and other carboxylic acids. Peptide chains are usually only 2−50 residues long. Unlike their ribosomal counterparts, NRPs usually contain complex cyclic elements, branching, and bonds other than peptide and disulfide bonds. Diversification of the primary structure in this class of peptides can take place at several levels of its biosynthesis, whereas diversification for ribosomal peptides mainly takes place at the posttranslational modification step. The extraordinary power possessed by this class of peptides has been exploited for years as pharmaceuticals, with penicillin being the first of its kind to be discovered (Fig. 24.14A) [33,34]. The pharmaceutical nonribosomal peptides have magnificently diverse application, including as antibacterial, antiviral, immunosuppressant, and anticancer drugs.

24.6.1.2 Nonribosomal Peptide Synthetases and Polyketide Synthase

Just as fascinating as their products are the enzymes that synthesize the nonribosomal peptides. Fascinating characteristics of the nonribosomal peptide synthetases (NRPSs) are shared with another class of enzymes called type I polyketide synthases (PKSs). Both classes of enzymes feature multifunctionality and multimodular characteristics, and consequently catalyze multiple biochemical reactions for the synthesis of their large and complex natural products. Like NRPSs, PKS products (polyketides) have found great and diverse use in the pharmaceutical arena. An example is the broad-spectrum antibiotic erythromycin (Fig. 24.14B). These large proteins are made up smaller catalytic units called

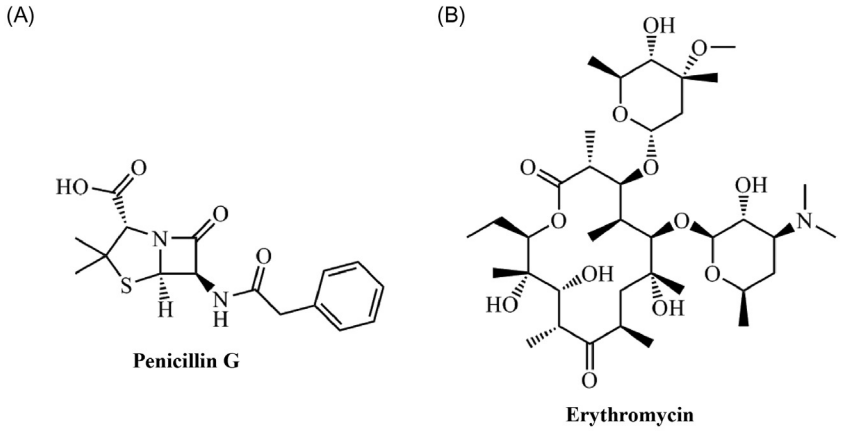


FIGURE 24.14 Chemical structures of penicillin G and erythromycin. (A) Penicillin G is a narrow spectrum penicillin antibiotic. (B) Erythromycin is a macrolide antibiotic useful for the treatment of a number of bacterial infections.

modules, and the modules further consist of distinct catalytic folds called domains. The order of the modular units determines the monomeric order in the growing chain, as well as the chemistry that takes place during assembly.

24.6.2 Protein Therapeutics

The protein therapeutics field has grown significantly since the introduction of the first human protein therapeutic, human insulin derived from recombinant DNA in 1982. Proteins have several advantages over small molecule drugs that currently dominate the pharmaceutical market. Proteins are capable of performing highly specific and complex functions, which is impossible for small molecule drugs. The high specificity of proteins also results in less drug toxicity through interference with normal body processes. Protein therapeutics is also less likely to elicit an immune response as many of them are naturally produced by the human body. They can be a suitable alternative to gene therapy for some genetic disorders, especially for those disorders where gene therapy is currently unavailable. There are limitations to protein therapeutics, such as high production costs, usually not available as oral drugs due to denaturation in the gut, and large proteins not being able to efficiently penetrate the tissue to reach their target sites due to size.

Most proteins are produced using recombinant DNA technology using various host systems such as bacteria, yeast, insect cells, and mammalian cells. The host choice is dependent on the cost of production as well as the biological activities that are required for its production. Engineering proteins not only allows for a ready source of the products, but enables modifications to be made that maximize clinical potential. For example, a protein with additional glycosylation sites has a longer half-life in vivo, thereby extending its effects on its target sites. Proteins may also be fused together by joining the genes of each protein to form *Fc fusion proteins*, which possess properties of its component parts. Proteins may also be attached to chemicals that improve their clinical potential, e.g., *PEGylated proteins*. In this case, proteins are joined to polyethylene glycol (PEG) which increases their half-life by reducing renal clearance. There are at least eight PEG-conjugated proteins approved for clinical use. *Antibody-based proteins*, such as IgG molecules, constitute the majority of protein therapeutics on the market, with 24 antibody-based protein therapeutics currently on the US market.

24.6.3 Proteins as Drug Targets

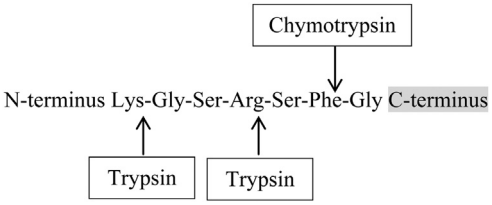
Proteins that are influential to a disease process are the most commonly used therapeutic drug targets, while the remainder of the drugs work on ribosomal, DNA, or pathogenic targets [35]. These proteins must exist in the disease state and contain sites within its structure where the drug can bind favorably. Attempts to estimate the number of approved drugs that act on these target proteins have consistently shown that receptor proteins and enzymes are the most common drug target proteins [36]. Chapter 33, Ethical Aspects of Working With Local Communities and Their Biological Resources in this book by Laurieri and Delgoda provides insights into novel targets which include many proteins. For receptor proteins, G protein-coupled receptors, the largest group of membrane receptors, are the most common targets.

24.7 CONCLUSION

Proteins have been known for years to be powerful molecular workhorses in the body, yet many questions still remain on their structure and function. As we continue to solve the mystery behind their extraordinary activities, this knowledge will improve applications to the health care industry.

24.8 SELF-ASSESSMENT QUESTIONS

1. A peptide chain (referred to as PC) has the following sequence: Lys-Gly-Ser-Arg-Ser-Phe-Gly
   a. Which amino acid is present at the N terminus?
   b. Which amino acid is present at the C terminus?
2. Given the sequence of PC above, indicate where the serine proteases, chymotrypsin, and trypsin are expected to cleave.



3. What would be expected in the presence of PC if there was a mutation at His57 of trypsin? Why?
4. Cytochrome P450 enzymes typically catalyze the hydroxylation of several substrates; however, to achieve this it must break the strong double bond of the oxygen molecule.
   a. How is this achieved?
   b. What catalytic strategy do the CYP enzymes utilize?
5. Currently, the pharmaceutical industry is dominated by small molecular drugs with singular targets. (a) What are the advantages and disadvantages of this drug design? (b) How can protein-based drugs overcome the disadvantages of small molecular drugs? (c) What are the disadvantages of protein-based drugs?

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Chapter 25

Pharmacokinetics

S.C. Turfus[1], R. Delgoda[1], D. Picking[1] and B.J. Gurley[2]
[1]University of the West Indies, Mona campus, Jamaica, West Indies, [2]University of Arkansas for Medical Sciences, Little Rock, AR, United States

Chapter Outline

25.1 INTRODUCTION

Pharmacokinetics, derived from the Greek words *pharmakon* (drug) and *kinetikos* (movement), is used to describe the absorption, distribution, metabolism, and excretion of a compound. Although preclinical studies require the determination of acceptable *in vitro* activity and pharmacokinetics in at least two animal species, pharmacokinetic studies must be performed in man to correlate blood concentrations with particular biological effects. Knowledge of disposition *in vivo* is required to tailor modifications in order to eventually derive semisynthetic drugs. Pharmacokinetic studies of natural products are challenging because they typically involve the administration of complex mixtures of substances, in many instances of unknown components. Some Chinese medicines including Danshen (*Salvia miltiorrhiza*), Kang-lai-te (*Coix lachryma*), and *Ginkgo bilboa* have been evaluated in randomized controlled clinical trials [1]. Many pharmacokinetic parameters, particularly of poisons, have not yet been determined as even *in vitro* studies are hindered by the extreme toxicity of some poisons, such as palytoxin [2].

25.2 ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Absorption and distribution are dependent on physicochemical properties. Absorption from the site of administration into the systemic circulatory system climaxes with a maximum concentration in the blood. Distribution usually occurs via diffusion through pores after which compounds are subject to metabolism and elimination, predominantly by the liver and kidneys, respectively. In addition, a number of transporter proteins play a role in the transcellular absorption, distribution, or elimination. Of note, the solute carriers (SLC), and the ATP-binding cassette (ABC) transporters, as described in detail in Chapter 27, (Drug Metabolism), play a major role in absorption, distribution, and excretion. Many factors such as genetics, disease, age, lifestyle, and diet play a role in pharmacokinetics, and those influencing metabolism are discussed in that chapter.

Concentration-time profiles following intravenous administration of a compound often demonstrate an exponential decline in blood concentrations with a half-life or $t_{½}$, the time taken for the concentration to be reduced by half. Examples of half-lives and other pharmacokinetic parameters of compounds are detailed in Table 25.1 and 25.2, for plants and microbes respectively. Drug administration following oral administration results in a distinctive absorption phase as well as phases where elimination predominate (Fig. 25.1). Compounds such as gentamicin, which is taken up into muscle, kidney, and bone, may exhibit multicompartmental models based on the number and timing of distribution equilibria and release from tissues. For example, in a two-compartmental model, the first phase, or α phase, is a result of both distribution to other parts of the body and elimination, which produces a sharp decline in blood concentration. In the second, or β phase, distribution equilibrium among the various tissues and fluids has been achieved and elimination from the body predominates (Fig. 25.1).

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TABLE 25.1 Pharmacokinetic Properties of Selected Compounds Present in Plants

Source	Compound	Properties/Uses	Typical Route	$t_{1/2el}$ (h)	V_D (L/kg)	F (%)	CL (mL/min/kg)[a]	F[b]	pK_a
Andrographis paniculata	Andrographolide	Antiinflammatory, immunostimulant, liver complaints, fever, anticancer	po	3.9 (0.8–15)	0.8	–	3.9	0.55	12.3
Atropa belladonna	Scopolamine Atropine	Antimuscarinic	po, sc, iv, td oph po, sc im, iv, oph	2–6 2–3	1.4–2.0 2.3–3.6	11–48 -	70 ± 23 mL/min[b] 660 mL/min[b]	0.10 0.18	8.2 9.8
Boswellia serrata	11-Keto-Boswellic Acid	Rheumatism, nervous diseases, antiinflammatory	po	6.0	2.4	–	5.0	–	4.6
Cannabis sativa	THC	Antiemetic, appetite increase, analgesic	po, in	20–57	4–14	6	14	0.97	10.6
Coffea arabica	Caffeine	Stimulant	po	2.3–12	0.4–0.6	~100	1.75	0.35	0.8
Chondrodendron tomentosum	Tubocurarine	Neuromuscular blocking agent, anaesthetic adjunct	iv	2.5–3.9	0.28–0.56	N/A	56 mL/min	0.44–0.51	8.1, 9.1, cation
Cinchona succirubra	Quinine	Antimalarial	po	3–15	1.8–3.0	39–73	1.2–4	0.9	4.3, 8.4
Digitalis lanata	Digoxin Digitoxin	Cardiac glycoside	po, iv	30–45 96–240	5.1–7.4 41	67–100 90	2.8 0.011 [b]	0.2 0.97	–
Ephedra sinica	Ephedrine Pseudoephedrine	CNS stimulant, decongestant, bronchodilator, pressor	in, po, iv	4–10 3–16	2.6–3.1 2-3	~85 ~100	383–500 mL/min –	– 0.2	9.6 9.4
Erythroxylon coca	Cocaine	Local anaesthetic, vasoconstrictor	oph, in	0.7–1.5	1.6–2.7	33	20–45	0.92	8.6
Galanthus and Narcissus genera	Galantamine	Cholinesterase inhibitor	po	5–8	2.4–3.0	80–100	5.7	0.18	–
Hypericum perforatum	Hypericin	Antibiotic, antiviral	po	15–58	1–3	14	20–52 mL/min	–	7
Nicotiana tabacum	Nicotine	Smoking cessation	td, po, in	0.4–1.4	1.0	<20	920–2430 mL/min	0.05	7.8, 3.0
Papaver somniferum	Morphine	Narcotic analgesic	po, iv	1.3–6.7	2–5	15–60	21	0.35	8.1
Vinca rosea	Vincristine Vinblastine	Antineoplastic	iv	8–67 12–48	1–3 20–50	N/A N/A	1.8–3.7 12.3	0.75 0.75	5.0, 7.4 5.4,7.4

[a] *Units of mL/min/kg for plasma/total/systematic clearance unless otherwise specified.*

[b] *Renal clearance. N/A, not administered orally or absorbed very poorly so total bioavailability unknown or not detectable; iv, intravenous; po, oral; im, intramuscular; sc, subcutaneous; td, transdermal; oph, ophthalmic; in, intranasal.*

TABLE 25.2 Pharmacokinetic Properties of Selected Compounds Produced by Microbes

Source	Compound	Properties/Uses	Typical Route	$t_{1/2el}$ (h)	V_D (L/kg)	F (%)	CL (mL/min/kg)[a]	F[b]	pK_a
Amycolatopsis orientalis	Vancomycin	Antibiotic (narrow spectrum)	po, iv	2.6–7.8	0.3–0.7	N/A	0.5–67 mL/min	0.3–0.5	2.7, 7.5, 8.6, 9.3, 10.2,11.9
Amycolatopsis rifamycinica	Rifampicin derived from Rifamycin B	Antibiotic (broad spectrum)	iv	2.3–4	0.93–1.6	90–95	1.4–6.4	0.8	6.9, 7.53
Artemisia annua	Artemisinin	Antimalarial	po, im, iv, r s	1.9–4.3	V_D/F[b] = 22.8	30	6667 mL/min	0.64	2.8
Micromonospora purpurea	Gentamycin	Antibiotic (broad spectrum)	iv, im ith, oph	1–3	0.28	N/A po ~100 im <2 lung	1.31 11.7–200 mL/min	<0.1	8.2
Penicillium griseofulvum	Griseofulvin	Antifungal	po	9–33	0.6–1.6	50–70	–	0.84	–
Penicillium notatum	Penicillin G	Antibiotic (broad spectrum)	im, iv	0.7–0.9	0.2	30	–	0.4-0.6	2.8
Streptomyces griseus	Streptomycin	Antibiotic (broad spectrum)	im	1.9–4.7	0.2–0.4	84–88 im	1.2	0.3–0.35	10.88, 11.9
Streptomyces hygroscopicus	Rapamycin/ sirolimus	Immunosuppressant	po	57–63	12	20	50–55 mL/min	0.92	9.96

[a] *Units of mL/min/kg for plasma/total/systematic clearance unless otherwise specified.*
[b] *F = bioavailability. N/A, not administered orally or absorbed very poorly so total bioavailability unknown or not detectable; iv, intravenous; po, oral; im, intramuscular; oph, ophthalmic; ith, intrathecal, r; rectal.*

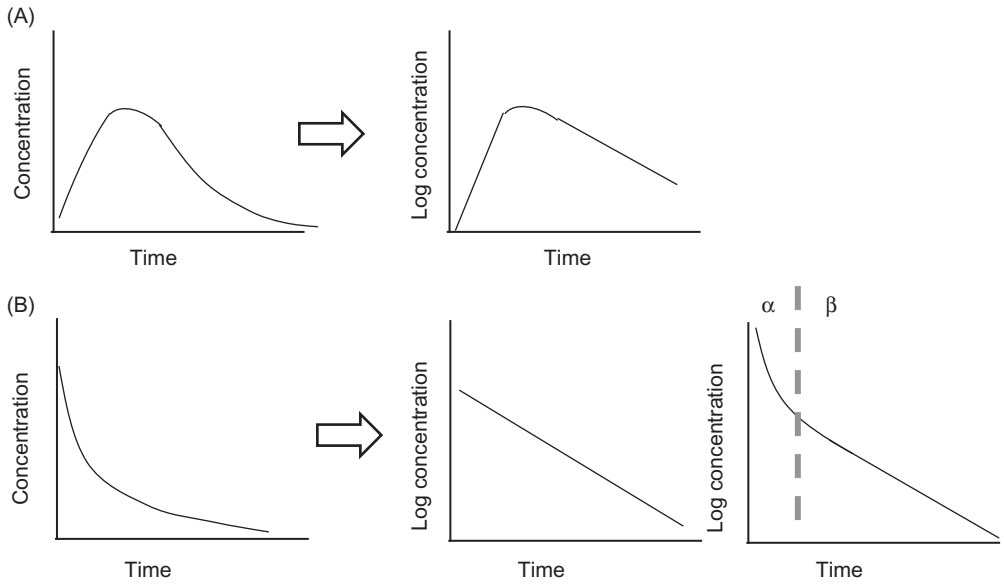

FIGURE 25.1 **Typical concentration–time profiles** (A) following oral administration and (B) intravenous administration. The concentrations are transformed to a semilogarithmic scale (right panels) to aid in the determination of pharmacokinetic parameters. Following intravenous administration it may be possible to determine two distinct phases, α and β.

25.2.1 Absorption

Following oral administration, absorbtion typically occurs via passive diffusion of the unionized form. Therefore, the pK_a or pK_b and the pH of the surrounding environment and target tissues play a major role in the extent of passive absorption. Quinidine, an antimalarial from the genus *Cinchona*, has two pK_b's, of 5 and 8.6 [3]. In the environment of the stomach, pH 2, the ionized form would be favored. However, if a patient was taking antacid to treat an ulcer, the stomach pH would increase, fewer molecules would be ionized leading to increased passive absorption.

Other compounds may either undergo facilitated transport along a concentration gradient or active transport.

Facilitated transport mechanisms include SLC transporters from organic cation transporter (OCT) and organic anion transporter (OAT) families. Digoxin, rifampicin, penicillin G and salicylate are examples of substrates of the OAT superfamily and quinine and quinidine of the OCT superfamily. Active efflux transporters belonging to the P-glycoprotein (P-gp) family can pump drug back into the lumen against the concentration gradient, which reduces drug absorption and promotes urinary and biliary elimination. Vinca alkaloids such as vinblastine, vincristine, vindesine, and vinorelbine are substrates of P-gp, which accounts for resistance to these compounds in some individuals. The activity of transporters can modify the volume of distribution (V_D). For example, inhibition of efflux and uptake transporters located in the liver have a potential to decrease V_D [4].

Phytochemicals can be administered by routes which avoid "first-pass metabolism," a principle discussed in Chapter 27, (Drug Metabolism). Flavonoids, such as iso-orientin, which exist in many plants and are responsible for anti-oxidation, antiinflammation, anticancer, and antidiabetic effects are thought to undergo extensive first-pass metabolism [5]. The poor permeability and extensive metabolism of polyphenols and tanshinones present in the Chinese medicine Danshen (*S. miltiorrhiza*) has led to intravenous preparations of their less hydrophobic derivatives for heart disease and angina [1]. It is important to know the bioavailability of a compound by comparing the fraction reaching the systemic circulation following oral and intravenous administration either using a washing out period in between or by administering the intravenous dose as a stable isotope-labeled variant. When this is performed in animal experiments it has the additional bonus of halving the animals required, halving the time for experiments to be completed, and eliminating the requirement of a washout period between doses.

Artemisin, an antimalarial phytochemical extracted from *Artemisia annua*, is an example of a compound that can be dosed via multiple routes—intravenous, intramuscular, oral, or rectal [6]. When administered orally, the bioavailability of artemisin is 30% as a result of first-pass metabolism. Another natural compound having a significant first-pass effect is morphine and therefore it is administered intravenously. Penicillin G cannot be administered orally as it degrades in

the acidity of the stomach. Quaternary amines, such as the muscle relaxant tubocurarine from *Chondrodendron tomentosum*, and large molecules, such as mistletoe leptins, that are used in supportive cancer treatment typically require injection as they cannot be absorbed. Conopeptides such as the analgesics Ziconotide and Contulakin-G from *Conus geographus* are large, charged molecules which do not readily penetrate membrane barriers and are digested by peptidases following oral or intravenous administration. Accordingly, these labile compounds must be administered intrathecally, into spinal fluid, to be effective [7,8].

Improvements to oral bioavailability can be made by altering the vehicle. One popular approach for lipid-soluble drugs involves the formation of a "phytosome" where the active principles of the plant are bound to phospholipids comprising one hydrophilic head and two hydrophobic tails. This has been employed in formulations of *Ginkgo biloba*, milk thistle, grape seed, green tea, hawthorn, and ginseng [9]. Lipophilic soft gelatin capsules containing the natural surfactant lecithin, rather than commercial hard shell capsules without surfactant, have been effective in improving, by approximately threefold, the bioavailabilities of hyperforin and hypericin, the active principles of *Hypericum perforatum* (St. John's Wort) [10]. Similarly, the oral bioavailability of silybin, from milk thistle, was improved by producing a lipophilic silybin-phospatidylcholine complex called Silipide. Maximum plasma concentrations of Ginkgolide A, Ginkgolide B, and Bilobalide from *G. bilboa* extracts approximately doubled when administered as a phospholipid complex rather than as their free form [11]. Oligoethylene glycol chains, membrane carriers, and lipophilic derivatives of natural products are all subjects of investigation to improve solubility and absorption [12]. In addition, pH-sensitive nanoparticles, such as for andrographolide from *Andrographis paniculata*, have been developed to increase bioavailability, while allowing the drug to be released only at the tumor [13].

In vitro studies such as those involving Caco-2 permeability models can be used to predict absorption of compounds. This model incorporates a human cell line of colorectal epithelial adenocarcinoma cells. The absorption is dependent on many parameters such as the octanol−water partition coefficient, molecular weight (MW), number of hydrogen-bond acceptors, and number of hydrogen-bond donors [14]. Lipinski's "rule of 5" are a series of rules that are violated if either the logP of a compound is greater than 5, the MW is above 500, there are more than 10 hydrogen bond acceptors or there are greater than 5 hydrogen bond donors. If a molecule has two or more violations, absorption/permeation is likely to be poor. Of all the components of *Daphne genkwa*, a Traditional Chinese Medicine used for its diuretic, antitussive, expectorant, abortifacient, and antitumor properties, those compounds with both a fatty acid chain and a benzene group were poorly absorbed whereas those without such a fatty acid chain, yuanhuafine and yuanhuapine, were more easily absorbed [14].

25.2.2 Distribution

As with absorption, xenobiotic distribution is dependent on many physicochemical properties of the molecule such as degree of ionization, lipophilicity, and MW. For example, tissue distribution of the methylxanthine theophylline is lower than that of the more lipid soluble methylxanthine, caffeine [15]. Plasma protein binding is a factor that can reduce the extent of distribution by blood to other sites. Acidic drugs bind predominantly to albumin whereas basic drugs bind to sites on β-globulin and α_1-acid glycoprotein. Amatoxins from the *Amanita*, *Galerina*, and *Lepiota* genera of mushrooms do not display protein binding properties and a larger fraction can be distributed around the body. Conversely, the anticancer drug paclitaxel (taxol) from the bark of the pacific Yew, *Taxus brevifolia*, has a protein-bound fraction of 0.89−0.98 [3]. The moderately lipophilic and volatile substance camphor shows intermediate plasma binding with a bound fraction of 0.61. Plasma binding can be influenced by disease as seen for polyphenols in Type II diabetes as excess glucose competes with polyphenols for binding to plasma proteins. Furthermore, the glycation of proteins weakens noncovalent forces between plasma proteins and polyphenols [12]. Compounds may also demonstrate different extents of distribution into red blood cells. For example, as very little tetrahydrocannabinol is distributed into red blood cells, concentrations in plasma are almost double compared to whole blood and a greater proportion is available for distribution [16].

Distribution is often defined in terms of compartmental models (Fig. 25.2). For example, if the reduction of concentration in a tissue or fluid is accompanied by an increase in other tissues in equilibrium with each other, this would result in the presence of two compartments. Andrographolide from *Andrographis paniculata* used for infectious diseases, such as influenza, exhibits a two-compartmental model in humans. The same compound administered at the same dose is also able to exhibit one- and two-compartmental models in different subjects. Following the administration of andrographolide to 15 subjects, 4 individuals exhibited one-compartmental models while the remainder exhibited two-compartmental models [17]. The number of compartments determined also depends on the time allowed for

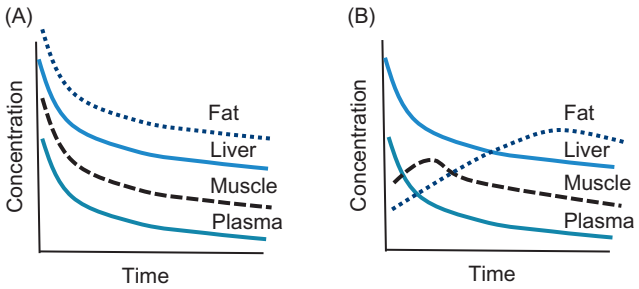

FIGURE 25.2 Compartmental models. Example of (A) a concentration−time profile of a single compartmental model where the drug is distributed into the central compartment (blood and liver) following immediate equilibrium between plasma and liver and maintains a constant ratio; (B) An illustration of a three-compartmental model with a central and two peripheral compartments (muscle and liver).

sampling. For instance, the compounds vincristine, vinblastine, and vindesine exhibit three-compartmental models which only become apparent when plasma concentrations are monitored for 48 h and beyond [18].

Compounds which are distributed in the circulating blood are distributed in an average volume of 5 L which has a plasma volume of approximately 3 L. The total volume of water in an average adult is approximately 42 L, of which 25 L is considered intracellular water while the remainder comprises extracellular water including plasma [16]. The apparent V_D in L/kg describes the extent of distribution throughout the body. Compounds with a low V_D those that have a high blood concentration, often display high plasma protein binding and an increased potential for competition. Conversely those with a high V_D are those with a low blood concentration because they have high tissue-binding properties. If a compound is administered orally, the apparent V_D is corrected by multiplying by the bioavailability. In many instances the bioavailability is unknown and the symbol for apparent V_D is depicted as V_D/F. Most compounds have a V_D of between 0.1 and 10 L/kg which represents a proportion in the total body water compartment of 40 to 0.4% respectively [16]. Digoxin has a high V_D, of 5.1−7.4 L/kg, and therefore the therapeutic concentration is low at 0.9−2 ng/mL. Digitoxin, which is derived from the same plant and structurally very similar has a V_D of 41 L/kg as a result of the absence of a single hydroxyl moiety. Galantamine from the *Galanthus* and *Narcissus* genera (daffodils) is used to treat degenerative dementias such as Alzheimer's and is another example of a drug that has a high V_D because of its low protein binding and high bioavailability [19].

Many compounds exhibit preferential distribution to particular organs. For example, puerarin, derived from the root of the kudzu *Pueraria lobota*, is used in Traditional Chinese Medicine to benefit individuals with cardiovascular, neurological, and hyperglycemic disorders. In rat models, it distributes into the kidney and pancreas, supporting its role in improving the diabetic condition [20]. However, the highest concentrations were found to be present in the lung and efforts are now focused on ascertaining benefits to this organ. Lipophilic molecules such as 10-hydroxycamptothecin and camptothecin, indole alkaloids from *Camptotheca acuminate*, are distributed into tumor cells, kidneys, bone marrow, and enterohepatic system in mice and thus have potential in the treatment of various cancers [21]. In rats, the antimalarial artemisinin displays preferential distribution into the intestine, followed by brain and then kidney and liver [6]. Although the kidney accounts for 0.8% of body weight, 8.1% of the administered dose of hydroxytyrosol, the antioxidant present in olive oil was found in the kidney 5 min after distribution [22]. The animal poison tetrodotoxin from the *Tetraodontidae* species, such as the Japanese puffer fish, has been shown to be absorbed quickly following subcutaneous injection in animal models but concentrates in the liver and kidneys within 2 h of its injection [23].

There is some evidence that administering natural products as a phospholipid complex improves distribution to certain tissues. For example, the Chinese medicine puerarin exhibits higher tissue distribution in the heart, lung, and brain when administered as a complex with a soy phospholipid [24]. Various other approaches have been used to target natural products to certain tissues. For example, tumor-targeted therapies have relied on the synthesis of natural product−antibody conjugates. This was demonstrated in mice administered the ribsome inactivating protein, gelonin, gelonin bound to a monoclonal antibody that recognizes glycoprotein present on human melanoma cells [25].

25.2.3 Metabolism

Metabolizing enzymes are discussed at length in Chapter 27, (Drug Metabolism), of this book and are illustrated in Fig. 25.3. Phase I reactions include oxidation (e.g. hydroxylation, dealkylation, deamination) reduction, hydrolysis, or hydration, in addition to more minor pathways such as carboxylation, cyclization, isomerization, dimerization, and

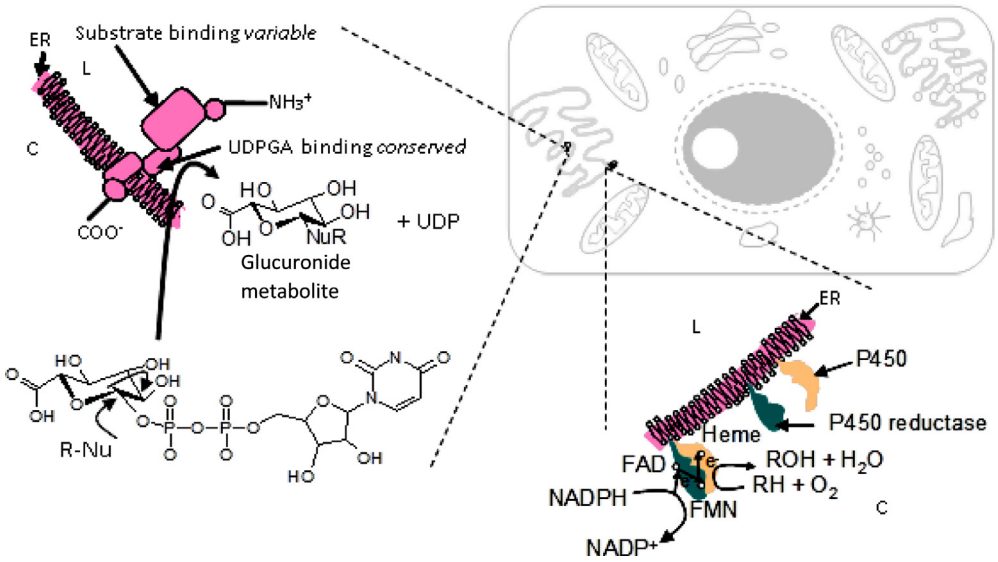

FIGURE 25.3 **The subcellular location of CYP450s and UGTs in the membrane of the endoplasmic reticulum (ER) of the liver.** The active site of UGTs are located in the lumen (L) and the active site of the CYP450s are on the side of the cytoplasm (C). Coupling of CYP450s with CYP450 reductase containing flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) results in phase I metabolism following electron transfer through NADPH, FAD, FMN on the reductase, to the heme moiety on the CYP450 enzyme, to the substrate molecule. UGTs require UDPGA as a cosubstrate. Nucleophilic attack by the substrate (containing for example hydroxyl or amine groups) results in the formation of a glucuronide conjugate and UDP.

transamidation. Some metabolites are significant for their activity. For example, vinblastine, an antineoplastic, is deacetylated to a pharmacologically active metabolite. As well as liver enzymes, plasma esterases mediate the hydrolysis of drugs such as heroin and cocaine. Anaerobic metabolic pathways, such as hydrolysis and reduction, and bacterial metabolism occur in the mucosal lining of the gastrointestinal tract. The metabolism of flavonoids involves hydrolysis by lactase phlorizin hydrolase and degradation into phenolic acids by bacteria [12]. For compounds that exhibit less than optimal metabolism resulting in prolonged or reduced activity, semisynthetic analogues can be tailored to produce altered half-lives. For example the half-life of the cancer drug sirolimus is 60 h compared to 30 h for the semisynthetic analogue, everolimus.

Phase II reactions include glucuronide, sulfate, amino acid and glutathione conjugation, glycosidation, methylation, and acetylation. Glucuronide conjugates are typically inactive, although morphine-6-glucuronide has been shown to be an active metabolite. Glucuronide conjugates can be recirculated in the body by enterohepatic circulation through the activity of uridine 5'-diphospho-glucuronosyltransferases (UGTs), sulfotransferases, and efflux transporters, and deconjugation by microbial enzymes, resulting in prolonged drug activity and excretion [26]. For example, in animal studies, berberine, palmatine, and jatrorrhizine, the pharmacologically active constituents of the *Coptidis rhizoma* and *Evodiae fructus* combination in Traditional Chinese Medicine exhibited three peaks in a time−concentration profile indicating repeated recycling of these compounds into the plasma [27].

The following examples draw on a selection of compounds with varied uses, structures, physicochemical properties, and metabolism [3].

25.2.3.1 Cannabis

The metabolism of the active principle of cannabis, tetrahydrocannabinol is shown in Fig. 25.4. The two monohydroxy compounds, 11-hydroxy-THC and 8-beta-hydroxy-THC, are active but minor metabolites. About 40% of the dose is eliminated in the feces and 30% in the urine in which conjugates (such as glucuronides) of 11-carboxy-THC predominate.

25.2.3.2 Salicylic Acid

Salicylic acid is predominantly metabolized to its conjugates as shown in Fig. 25.5. The administration of aspirin (acetylsalicylic acid) as a prodrug is more commonly employed. This results in hydrolysis by liver and blood esterases to salicylic acid which accounts for the pharmacological activity.

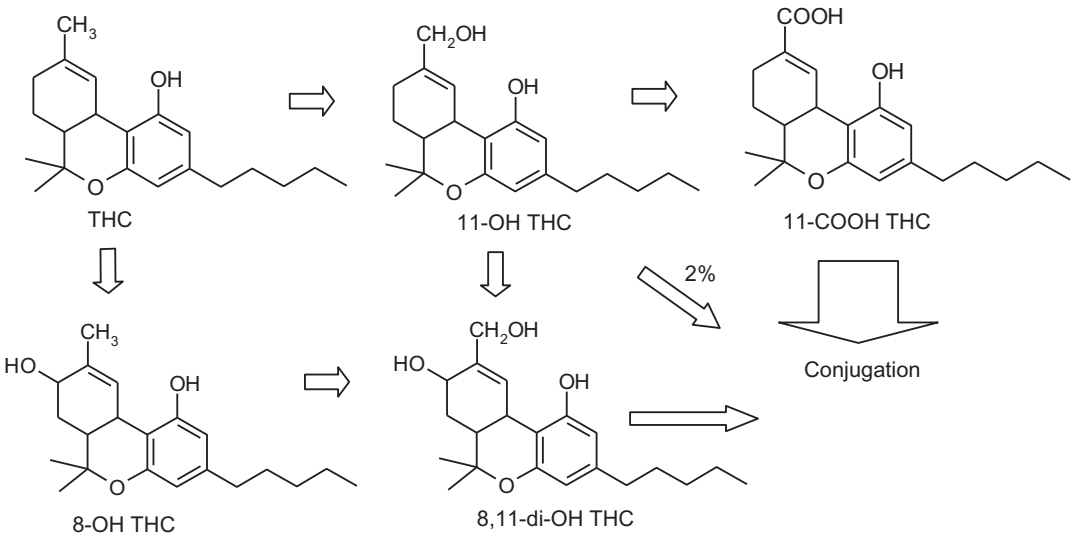

FIGURE 25.4 **Metabolism of tetrahydrocannabinol.** Most of the dose is metabolized to conjugates of 11-COOH THC but 2% can be found as conjugates of 11-OH THC in the urine.

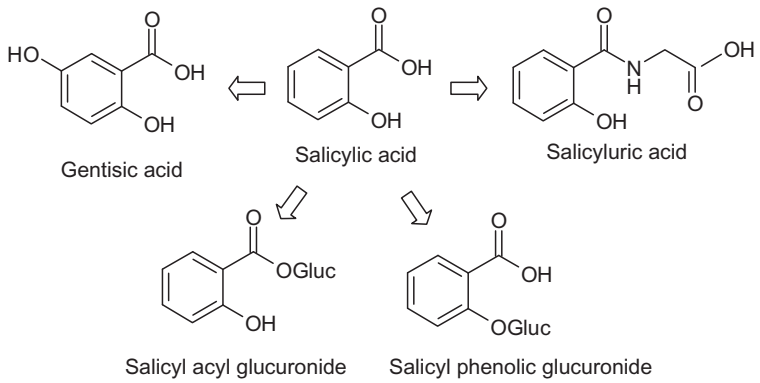

FIGURE 25.5 **Metabolism of salicylic acid.** The major metabolite is salicyluric acid followed by salicyl phenolic glucuronide.

25.2.3.3 Griseofulvin

The metabolism of the antibiotic derived from *Penicillium griseofulvin* is displayed in Fig. 25.6. Griseofulvin has a highly variable bioavailability which has been attributed to its poor solubility in water. Urinary metabolites account for 50% of the dose whereas 36% is eliminated in feces within 5 days.

25.2.3.4 Galantamine

Galantamine, the reversible cholinesterase inhibitor from *Galanthus woronowii* (a daffodil species), shows large polymorphisms in metabolism with some individuals eliminating up to 40% of the dose as galantamine from deficiency in O-desmethylation. Other individuals form metabolites as shown in Fig. 25.7. One feature of galantamine metabolism is that the compound undergoes epimerization at the 6-OH position resulting in many isomers.

25.2.3.5 Artemisinin

The active principle of *Artemisia annua* is a compound having a different structure compared to classical antimalarial drugs as a result of its endoperoxide bridge. Urinary metabolites (Fig. 25.8) are inactive due to the absence of this moiety. The metabolism is due primarily to the enzyme CYP2B6 and also CYP3A4.

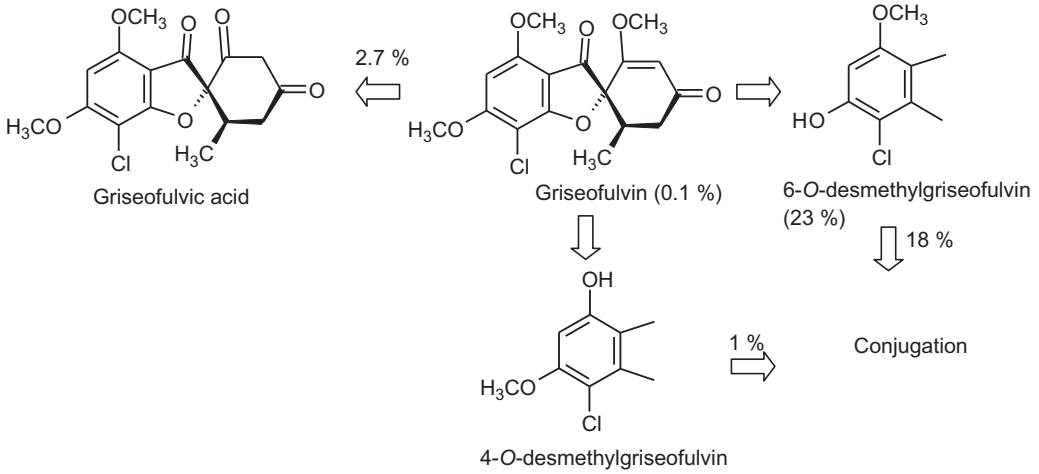

FIGURE 25.6 Metabolism of griseofulvin. The major metabolites are free 6-*O*-desmethylgriseofulvin followed by conjugated 6-*O*-desmethylgriseofulvin.

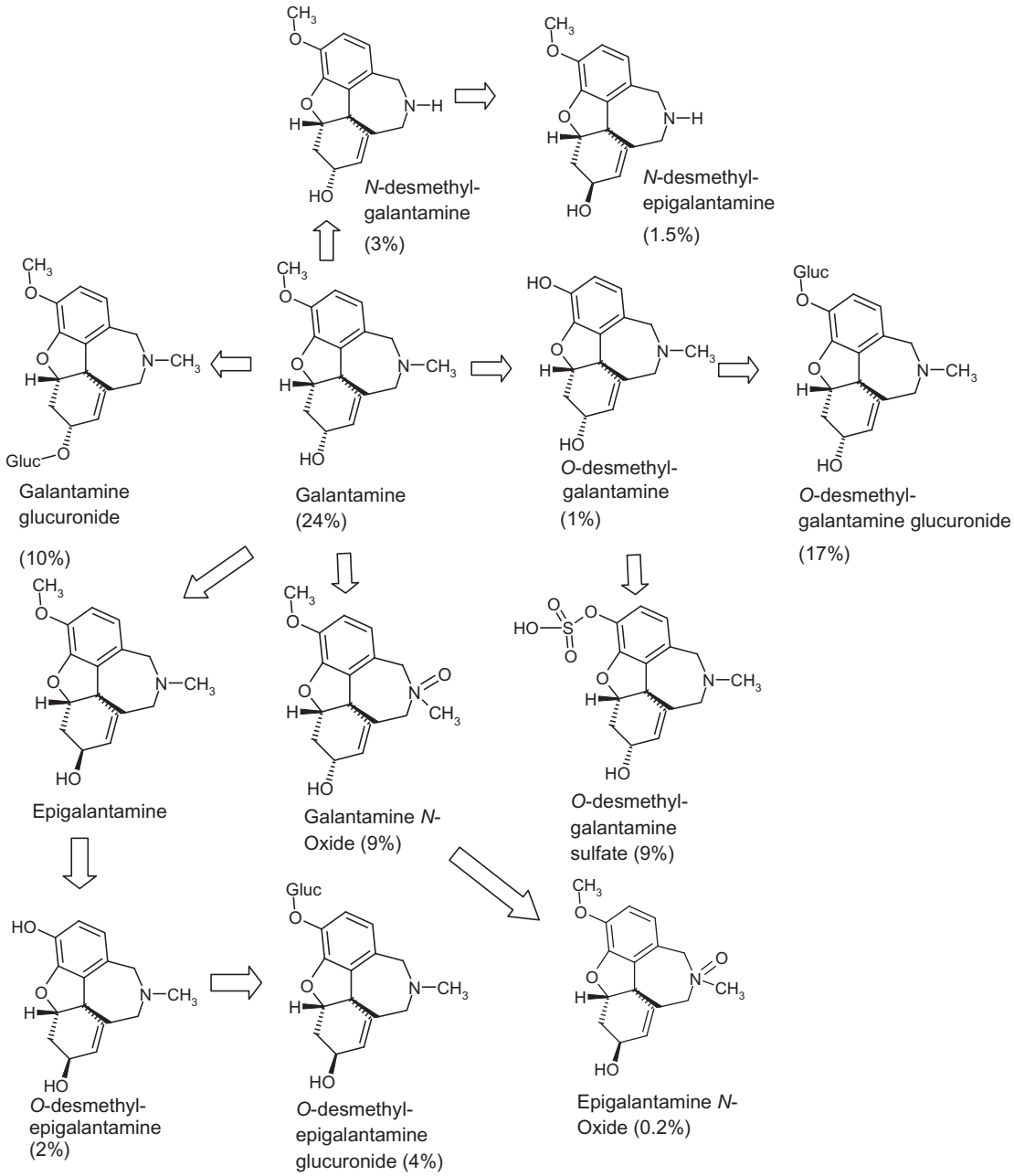

FIGURE 25.7 Metabolism of galantamine. Metabolism is extensive in genetically predisposed individuals with epimerization resulting in the production of many isomers of metabolites.

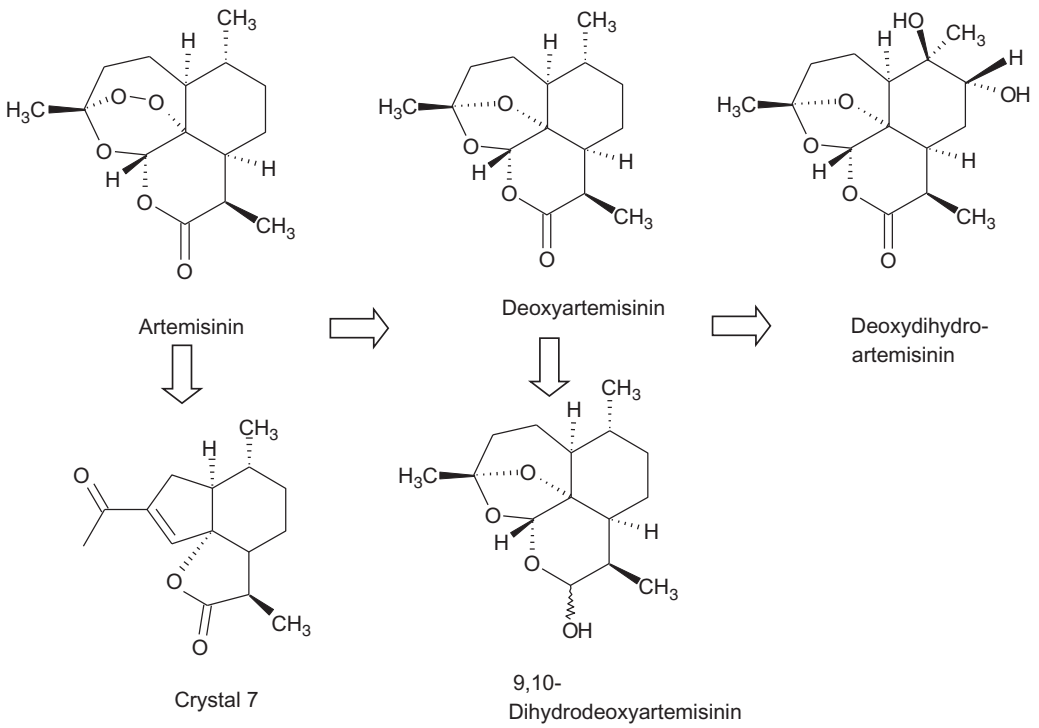

FIGURE 25.8 Metabolism of artemisinin. Metabolism involves deactivation by removal of the endoperoxide structure, primarily by CYP 2B6. The metabolites have been identified but the pathway is yet to be ascertained.

25.2.4 Excretion

Most compounds are filtered by the kidney and eliminated from the body via the urine or feces. There are three principal mechanisms of renal excretion: filtration, secretion, and tubular reabsorption. The filtration rate across a typical renal nephron of the kidney is 100−130 mL/min in young, healthy individuals. Tubular reabsorption is pH-dependent which is apparent for weak bases and acids with pK_a's and pK_b's in the physiological pH range. The pH of the urine varies between 4.5 and 7.5 and alters with diet. Thus, salicylic acid is more likely to be excreted into alkali urine and morphine in acidic urine when the ionized water-soluble forms prevail. Excretion can be manipulated with alkali diuresis with a sodium bicarbonate injection. For example, about 70−80% of ephedrine is typically excreted unchanged and 4% as norephedrine, whereas alkalinization results in excretion of a lower proportion of ephedrine of 22−35% and more norephedrine at 11−24% [3]. Filtration on the other hand is dependent on MW with protein-bound drugs and compounds with a MW of greater than 500 not amenable to filtration.

Some ions are subject to active tubular secretion into the kidney from the blood via various uptake and efflux transporters in the proximal convoluted tubule of the kidney. For example, penicillin secretion is a carrier-mediated process. It is also subject to competition for the carrier protein by other weak acids, which is the principle behind administering it with probenicid to prolong the effects of penicillin. Salicylates and glucuronide metabolites are also subject to secretion via OATs, and morphine is secreted by cation transporters. Atropine is subject to tubular secretion resulting in a high renal clearance [28].

Biliary excretion is an important route of elimination and may cause enterohepatic circulation to occur by conjugation and intestinal deconjugation. Invasive techniques used to directly study biliary excretion in healthy subjects have been applied to digoxin, antibiotics, and quercetin [29]. Transporters involved in biliary excretion include those in the ABC B (BSFP and MDR1) and ABC C (MRP1 and 2) families [30]. Escin, the anti-inflammatory mixture of triterpenic saponins present in the seeds of horse chestnut (*Aesculus hippocastanum*) is an example of a compound that is predominantly (66% of the dose) eliminated into bile in animal models [11]. In rat models, up to 20% of morphine-3-glucuronide is eliminated into the bile for which the transporter MRP 3 has been implicated [31]. Vinca alkaloids, such as vinblastine, vincristine, vindesine, and vinorelbine, are eliminated into bile and feces in rodents, dogs, and humans [24]. Other compounds detected in significant quantities are metabolites of THC [32], ergot alkaloids such as dihydroergocristine, dihydroergotamine, and ergotamine [33], and flavonolignans in milk thistle

extract. In the latter case, percentages of dose eliminated vary from 21% to 100%, the primary transporter responsible being ABC C2 (MRP 2) [34].

The $t_{1/2}$ is used to express the rate of elimination of a compound exhibiting first-order kinetics. Some medicines contain several compounds of differing half-lives. For example, the Chinese medicine Shakuyakukanzoto, consisting of *Glycyrrhizae* (liquorice) and *Paeoniae* (peony), which is used to relieve muscular cramps, has compounds with half-lives ranging from 1.7 h (paeoniflorin) to 15 h (glycycoumarin). This gives rise to a medicine which has both immediate efficacy and sustained efficacy in relieving muscular cramps and sustaining analgesia, with synergistic effects of at least six components with varying pharmacokinetic profiles [35].

Clearance is the volume of blood or plasma cleared of drug per unit time by all clearing organs and is often expressed in units of mL/min/kg. It is used to describe the processes of metabolism and excretion by the liver, kidney, and other clearing organs. The clearance of a particular organ is governed by its extraction ratio, E, which is the proportion of compound cleared from the plasma by the organ during its passage through the organ. E is a function of organ blood flow, metabolic enzyme or transporter activity, and protein binding. The sum of individual organ clearances account for the total body clearance. A compound with a large V_D will persist in the body so clearance is sometimes expressed as a fraction of the V_D to give an indication of persistence in the body (CL/V_D), a term which is also an expression of the elimination rate constant. A high renal clearance would be indicative of active secretion as well as filtration and a comparatively low reabsorption rate. A high hepatic clearance would be expected for compounds which are extensively metabolized in the liver.

25.3 DETERMINATION OF PHARMACOKINETIC PARAMETERS

In the previous sections, several parameters were qualitatively described that are used to determine the pharmacokinetic properties of natural products. In this section, their graphical representation is considered for the scenario of the consumption of a single dose.

25.3.1 Half-Life

For the majority of compounds, the rate of elimination, in units of reciprocal time or h^{-1} is directly proportional to its concentration. These are said to exhibit first order elimination kinetics. Under these conditions, $t_{1/2} = 0.693/k$ or $(0.693 \times V_D)/CL$. The elimination rate constant, k, for compounds following linear kinetics is $k = CL/V_D$. The $t_{1/2}$, expressed in units of hours or minutes, can be determined by plotting the concentration−time profiles of compounds from pharmacokinetic experiments. Often, the data are transformed to a semilogarithmic scale (as displayed in Fig. 25.9A) and the negative slope of the resulting line can be used to determine the elimination rate constant (k) (Fig. 25.9B).

25.3.1.1 α and β Phases

Following intravenous administration, a logarithmic transformation may result in the identification of a biexponential curve which can be broken down into two components. In the α phase, distribution is the major factor determining the decrease of a compound in plasma, and in the β phase, elimination is the major contributor to the decrease in plasma concentration (Fig. 25.9C). The exponential functions are combined to provide a depiction of the plasma concentration−time profile of the agent. Three-compartmental models, which include an γ phase, and one-compartmental models may also become apparent based on the shape of the concentration-time profile.

25.3.1.2 Apparent V_D

The semilogarithmic graph can also be used to determine the original concentration at time 0. Typically the concentration at time 0 is not known as there is a lag phase between administration and equilibration, and equilibration and sample collection. However, if equilibrium is reached immediately following intravenous administration, extrapolation of concentration to time 0 (Fig. 25.9C) will be a close approximation to the concentration present immediately after administration. One approach of estimating the apparent V_D is to divide the dose (in mg/kg) by the initial concentration C_0 (in mg/L) (Table 25.1).

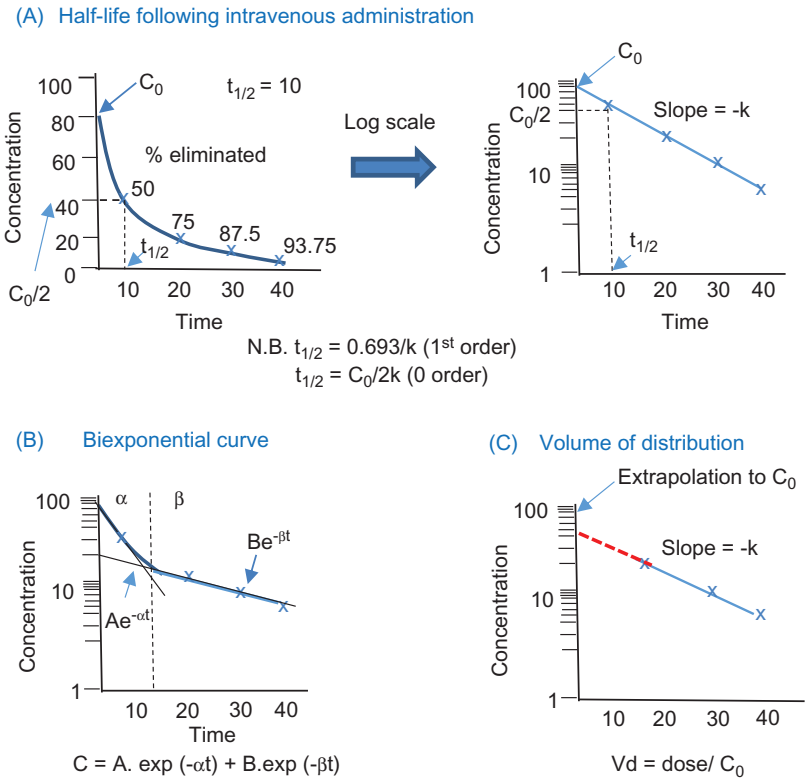

FIGURE 25.9 Determination of the pharmacokinetic properties following intravenous administration (A) half-life $t_{1/2}$, initial concentration C_0 and rate constant k; (B) absorption and elimination phases and their exponential terms; and (C) extrapolation to C_0 to determine the volume of distribution V_D of a compound.

25.3.1.3 Elimination rate constant (k) and absorption rate constant (k_a)

Following oral or intravenous administration, the elimination rate constant can be calculated from the terminal portion of the concentration−time curve (the β or postabsorption phase) by plotting the data on a semilogarithmic graph (compare Figs. 25.9A and 25.10A). This forms the term $\exp(-kt)$ but in order to determine the contribution of the other exponential term, $\exp(-k_a t)$ from which the absorption rate constant can be calculated, the extrapolated line is subtracted from concentration− time profile. This is known as the method of residuals (Table 25.2).

25.3.1.4 T_{max} and C_{max}

Following administration, the time and plasma concentration at which the compound reaches its maximum are known as T_{max} and C_{max}, respectively. They are related to the dose, clearance, apparent V_D, and absorption rate constant (k_a) as shown in Fig. 25.10B.

25.3.1.5 Clearance

Clearance can be calculated using the relationship $CL = k \times V_D$ and also from V_D and $t_{1/2}$ as shown in Fig. 25.10. It can also be expressed as $CL = (F \times \text{Dose})/\text{AUC}$, where F is bioavailability and AUC is the area under the curve for a plasma concentration−time profile, if these parameters are known.

25.3.1.6 Plasma/serum concentration

The overall concentration following a single dose in a one-compartmental model is a function of two exponential terms containing the elimination rate constant and absorption rate constant as shown in Fig. 25.10D.

25.4 PHARMACOKINETICS AND DRUG INTERACTIONS

Multiple maladies and the requirement of combination therapy for some diseases, such as cancer and AIDS, can result in multidrug use. In many developing countries, the habit of self-medicating with medicinal plants while on a prescription medicine is highly prevalent. In Jamaica, for example, 80% of pharmacy patrons on prescription medicines were

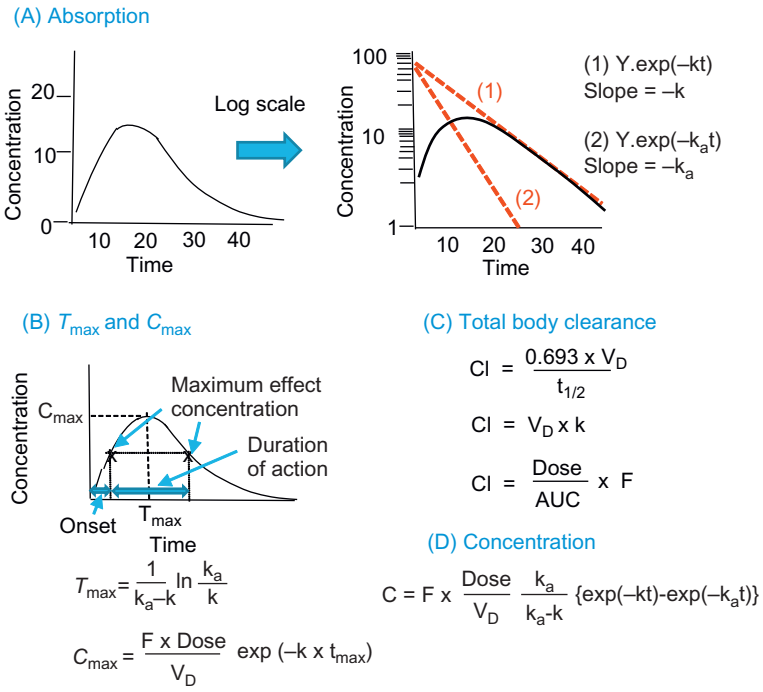

FIGURE 25.10 Determination of pharmacokinetic parameters following oral administration. (A) Calculation of the absorption rate constant using the method of residuals: Extrapolation of terminal portion of time-concentration curve, i.e., exp(−k*t*) contribution of *Y* term and deduction from curve gives the contribution of *Y*. exp(−$k_a t$), the exponential term describing absorption; (B) Determination of T_{max} and C_{max} from the time−concentration profile and using k, k_a, bioavailability, *F*, dose, and volume of distribution, V_D, to calculate these parameters. (C) Calculation of clearance using V_D, $t_{1/2}$, and k; and (D) the relationship of the terms to concentration following an oral dose.

also using medicinal plants [36,37] and worryingly only 20% of physicians were aware of their patients' habits. The use of multiple medicines lends itself commonly to metabolism-based interactions, whereby one drug alters the pharmacokinetics of another, which may lead to an untoward clinical effect. The US Food and Drug Administration report that two million serious adverse drug reactions (ADRs) occur each year, leading to 10,000 deaths, making ADRs the fourth leading cause of death in the United States [38].

In a recent review of ADRs reported globally through the WHO VigiBase database (Table 25.3), pharmacokinetic interactions were mainly attributed to CYP inhibitions, with CYP3A4 being the most commonly reported metabolic pathway. The reported ADRs often involved significant threats to patients' safety and were specifically linked to drug combinations that involved known high-risk drugs [39]. Figures for drug−medicinal plant interactions are hard to find, particularly because adversities tend not to be attributed to the use of plants, given the general assumption that natural products are safe for use under any circumstance.

As well as cytochrome P450 (CYP) enzymes, other drug-metabolizing enzymes (as described in chapter 27: Drug Metabolism) and drug transporters such as ABC, (e.g., P-gp, multidrug-associated resistance proteins), and SLC families, (e.g. organic anion transporting polypeptides and OATs), have also been shown to be play a role in drug interactions in many disease states. Phytochemicals found in several plants are also known to influence both CYPs and transporters. Furanocoumarins present in *Rutacea* species (e.g., grapefruit) inhibit CYP3A4 and ABC transporters and isoquinolines in *Hydrastis canadensis* (goldenseal) have the potential to irreversibly inhibit CYP3A4 and CYP2D6. Hyperforin in *H. perforatum* (St. John's Wort) is an inducer of CYPs, UGTs, and ABC transporters which is exacerbated by its potential for accumulation due to its bioavailability and moderate half-life of 8−12 h [40].

Potent inhibitors of CYP enzymes in clinical practice, of both of pharmaceutical and natural origin, raise safety concerns associated with drug interactions. Inhibitors CYPs 3A4, 2C9, 2D6 and 1A2 raise concern in that order [41]. The induction of CYP enzymes via the transcriptional regulators, such as the nuclear receptors pregnane X receptor, constitutive androstane receptor, and aryl hydrocarbon receptor, which regulate many CYPs, UGTs, SULTs, GSTs, and ABC transporters [42] can increase the rate of elimination of drug substrates. CYP induction and inhibition have been assessed by (1) in vitro models involving microsomes (human liver microsomes, heterologously expressed enzymes), hepatocytes, and liver slices; (2) in vivo animal models, although species differentiation makes the extrapolation less possible; (3) clinical evaluations; and (4) case reports (Fig. 25.11).

In Table 25.4, a number of well-documented cases of medicinal plant and food drug pharmacokinetic based interactions are summarized. Such potential drug interactions tables are now becoming widely accessible for physicians and

TABLE 25.3 Most Frequently Reported Drug–Drug Interactions Involving CYP Enzymes

Drug A	Drug B	Interaction Type	Clinical Impact
Gemfibrozil (lipid regulating)	Cerivastatin (cholesterol lowering)	CYP2C8 inhibition	Rhabdomyolysis leading to renal failure, myalgia
Isoniazid (antituberculosis)	Rifampicin (antituberculosis)	CYP3A4 induction	Hepatotoxicity, hepatitis, jaundice
Warfarin (anticoagulant)	Roxithromycin (antibiotic)	CYP3A4/2C9 inhibition	Decreased prothrombin levels, increased INR, hematura
	Miconazole (antifungal)	CYP3A4/2C9 inhibition	Decreased prothrombin levels, increased INR, hematura
	Celecoxib (NSAID)	CYP3A4/2C9 inhibition	Decreased prothrombin levels, increased INR, hematura

Reported in the WHO Global Individual Case Safety Report (ICSR) database, VigiBase, in the past 20 years, January 1990 to February 2010 [39].

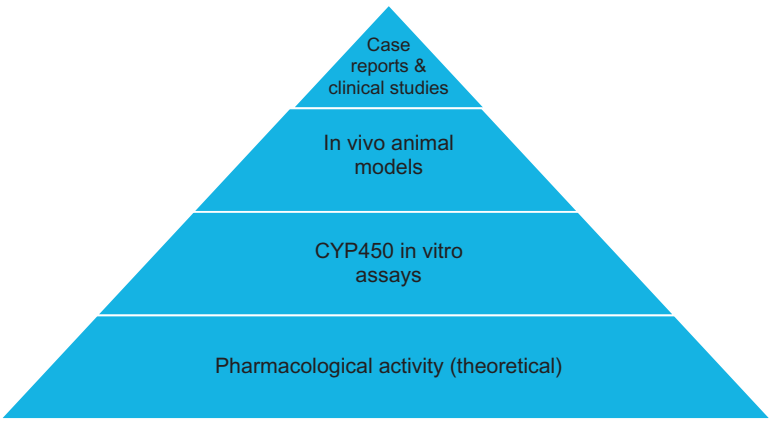

FIGURE 25.11 Risk assessment for potential medicinal plant–drug interactions. Case reports, which are detailed and validated with positive rechallenge and published clinical studies, offer the highest level of evidence. Cytochrome P450 (CYP450) *in vitro* assays provide a useful first-stage screening, while pharmacological activity alone provides theoretical evidence, and as such may be considered to represent the lowest level of evidence.

patients, helping to mitigate potential ADRs worldwide. It should also be noted that drug disposition is dependent on genetic variants of metabolizing enzymes. For example, the extent of effects of Ginkgo (*G. biloba*) and garlic (*Allium sativum*) on disposition of other drugs is dependent on the *CYP2C19* allele and disposition of compounds in milk thistle (*Silybum marianum*) is dependent on the CYP2C9 metabolizer status [42].

25.5 CONCLUSION AND FUTURE WORK

Determination of pharmacokinetic properties involves the administration of the compound to animal species and humans. It involves the determination of the primary pharmacokinetic parameters clearance, apparent V_D, and bioavailability, as well as the secondary parameters elimination half-life, T_{max} and C_{max}. In the future, novel approaches (Table 25.5) may assist in overcoming challenges, namely the presence of mixtures and large number of potentially active principles and the need for reliable animal and in vitro models, associated with performing pharmacokinetic studies on natural medicinal products.

TABLE 25.4 Summary Table of Well-Documented Medicinal Plant–Drug and Food–Drug Interactions

Pharmaceutical Drug (Class/Trade Name)	Potential Interaction	Evidence	Risk Assessment
***Aloe vera*, Sinkle Bible (*Aloe vera*) (Inner Gel)**			
Multiple medications	Examined for drug level increases	No evidence of CYP inhibition from in vitro data (CYPs 3A4, 2D6)	Very low level of risk—monitor
Multiple medications	Examined for inhibitory impact of permeability glycoprotein (P-gp) mediated transport (digoxin)	No evidence of P-gp inhibition from in vitro data	Very low level of risk—monitor
Bitter Melon, Cerasee (*Momordica charantia*) (Vine Aqueous Extract)			
Multiple medications	Examined for drug level increases	No evidence for CYP inhibition from in vitro data (CYPs 1A2, 2C19, 2C9, 2D6, 3A4)	Very low level of risk—monitor
P-gp substrates, e.g., digoxin, everolimus letairis, topotecan, vinblastine	Inhibits P-gp and can increase the interacellular concentration and toxicity of substrate drugs	Extracts may increase bioavailability and efficacy of certain chemotherapy agents—*in vitro d*ata	Potential role in enhancing the bioavailability and efficacy of chemotherapy drugs
Echinacea (*Echinacea angustifolia* & *Echinacea purpurea*)			
HIV protease inhibitors, e.g., darunavir	May decrease drug levels	*Clinical study E. purpurea* taken by HIV-infected patients. No overall effect, but some patients showed a decrease by as much as 40%. All study patients maintained an undetectable viral load	Low level of risk—monitor
Immunosuppressant medicines, e.g., cyclosporine	May decrease drug effectiveness	*Theoretical* concern based on the immune enhancing activity of Echinacea. No adverse events reported	Contraindicated
Midazolam	Decreases drug levels when drug is administered intravenously	*Clinical study* (*E. purpurea* root, 1.6 g/day)	Medium level of risk—monitor (when drug administered intravenously)
Garlic (*Allium sativum*)			
HIV protease inhibitors, e.g., saquinavir	Decreased drug levels	Two *clinical studies* (garlic extract, standardized for allicin content) with healthy volunteers showed large variability but in one study the decrease (15%) was not significant	Medium level of risk—monitor
Grapefruit (*Citrus paradisi*) (To Date Approximately 43 Drugs Have Been ID'd That Interact and Can Lead to Serious ADRs)			
Antiarrhythmic drugs, e.g., amiodarone	Increased drug levels	*Case reports*: ventricular tachycardia	Contraindicated
Immunosuppressant medicines, e.g., tacrolimus	Increased drug levels	*Case reports*: nephrotoxicity	Contraindicated
Statins, e.g., simvastatin, lovastatin, atrovastatin	Increased drug levels	*Case reports* and *clinical studies* with reports of rhabdomyolysis	Contraindicated
St. John's Wort (*Hypericum perforatum*)			
Amitriptyline	Decreases drug levels	Clinical study	Medium level of risk—monitor
Calcium channel antagonists, e.g., nifedipine, verapamil	Decreases drug levels	Nifedipine: *Clinical study* Verapamil: *Clinical study*	Contraindicated

(*Continued*)

TABLE 25.4 (Continued)

Cancer chemotherapeutic drugs, e.g., irinotecan, imatinib	Decreases drug levels	*Clinical studies*	Contraindicated
Immunosuppressant medicines, e.g., cyclosporin, tacrolimus	Decreases drug levels	Cyclosporin: *Case reports* and *Clinical studies* Tacrolimus: *Case reports* and *Clinical studies*	Contraindicated
Soursop Leaf (*Annona muricata*) (Aqueous Extract)			
Multiple medications	Examined for drug level increases	No evidence for CYP inhibition from in vitro data (CYPs 1A2, 2C19, 2D6, 3A4)	Very low risk—monitor
Turmeric (*Curcuma longa*)			
Talinolol	May decrease drug levels	*Clinical study*: 300 mg/day curcumin with healthy volunteers	Low level of risk—monitor at high doses of curcumin ≥300 mg/day

TABLE 25.5 Recent Advances in Pharmacokinetics

Approach	Principle	Example Compounds
Integrated pharmacokinetics	Mixtures analyzed by weighting area under curve (AUC) of all similar constituents derived from the same herb and determining an integrated concentration.	Chinese medicine Huang–Lian–Jie–Du–Tang. This contains four herbs that includes the bioactive components berberine, palmatine, baicalin, baicalein, and geniposide.
Reversed pharmacokinetics	"Clinic to laboratory" approach where metabolism and pharmacokinetics performed before knowing the active component. Targets are revealed simultaneously for different compounds and physiological models developed for extrapolation to target and mechanism of action.	Ginseng and berberine
Automated dosing and sampling and miniaturization	Automated dosing, sampling, and fraction collection automated and sample size reduced to 10 μL minimizing number of animals required as each can be studied at a greater number of time points. Animals are mobile minimizing stress-induced physiological changes.	Isoliquiritigenin
Ex vivo approaches	Drug–drug interactions studied by incubating human biological fluids from individuals administered the compound with cytochrome P450 enzymes and their standard probe substrates rather than nonphysiological buffers to better represent the physiological conditions in clinical studies.	Silybin A and B in milk thistle interactions with tolbutamide; hydrastine and berberine in goldenseal (*Hydrastis canadensis*) with midazolam.
In silico pharmacokinetics	Computer-based approaches to eliminate compounds with unwanted pharmacokinetic profiles. Physically relevant descriptors used such as "total solvent accessible molecular surface" and "the number of likely metabolic reactions" to perform absorption, distribution, metabolism, elimination and toxicity predictions, and generate a drug-likeness parameter. Reduces labor/cost in finding hits.	Quercetin, rutin, naringenin, and naringin. Another study showed that of 1859 compounds from 224 species from the forests of Cameroon, half of the compounds had properties falling within the range of common drugs.

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Chapter 26

Pharmacodynamics—A Pharmacognosy Perspective

J.E. Campbell[1] and D. Cohall[2]

[1]*The University of the West Indies, Mona, Jamaica,* [2]*The University of the West Indies, Cave Hill, Barbados*

Chapter Outline

Objectives:

Upon completion of this chapter, you should be able to:

- Define drug targets.
- Describe the basic and molecular basis for drug and drug target interactions.
- Identify the mechanisms for cellular effects of the drug-receptor interaction.
- Define the terms adverse drug reaction, adverse drug event, polypharmacy.
- Discuss the impact of adverse drug reactions and the need for pharmacovigilance.
- Discuss the importance of drug–drug and drug–herb interactions.

26.1 DEFINITIONS

Pharmacodynamics is defined as the response of the body to the drug. It refers to the relationship between drug concentration at the site of action and any resulting effects namely, the intensity and time course of the effect and adverse effects.

Pharmacodynamics is affected by receptor binding and sensitivity, postreceptor effects, and chemical interactions.

Both pharmacodynamics and pharmacokinetics explain the drug's effects, which is the relationship between the dose and response. The pharmacologic response depends on the drug binding to its target. The concentration of the drug at the receptor site influences the drug's effect. A drug's pharmacodynamics can be affected by physiologic changes due to disease, genetic mutations, aging, or other drugs. These changes occur because of the ability of the disorders to change receptor binding, alter the level of binding proteins, or decrease receptor sensitivity.

Pharmacognosy is the study of drugs derived from natural sources. The content of this chapter emphasizes pharmacodynamics and mechanisms by which substances, primarily from natural sources, effect changes directly or indirectly on living systems.

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26.2 DRUG TARGETS

26.2.1 Introduction to Drug Targets

In the introductory chapter we discussed that drugs are characterized as substances which bring about changes in physiological systems [1]. Medicines were defined as one or more drugs given to produce a desirous effect [1]. We also addressed the concept that these characterized substances also can effect changes in biological systems in their natural states as observed in herbal and other natural forms of medicine as well as in their comodified and synthetic forms.

In this chapter, we will discuss how drugs interact with specific targets in biological systems to bring about changes at the cellular level to effect changes. Drug targets are macromolecular components of cells and tissues which interact with drugs and, in some cases, endogenous substances, to effect physiological changes [2]. To bring about these changes, the introductory chapter on Pharmacokinetics mentioned that drugs and these endogenous substances are distributed after their point of origin, to facilitate interaction with the molecular targets. We also discussed that cellular transport mechanisms were integral in facilitating the movement and subsequent interaction of drugs with their respective molecular targets [3].

These molecular targets are mainly polypeptides in structure but there are other macromolecules, such as nucleic acids, primarily deoxyribonucleic acids, which are targeted by drugs for cancer, immunological, and antiinfective chemotherapy. Not much will be mentioned about nonpolypeptide targets but essentially these targets are explored for the disruption of cell division process which eventually effects cell death via apoptosis and other cell death mechanisms.

The protein drug targets which will be discussed are classified below.

26.2.1.1 Membrane Carrier Proteins

Membrane carrier proteins are important transmembrane polypeptide molecules which facilitate the movement of charged and polar molecules and ions across the lipid bilayer structure of the cell membranes [4]. Carrier proteins are usually found in tissues which function extensively in the absorption and excretion of molecules. Therefore, these can be found extensively in the digestive tract and the kidneys [5–7]. Carrier proteins are also important structural and functional protein molecules which play an important role in facilitated diffusion and active transport processes. These processes are two of the mechanisms introduced in the chapter on Pharmacokinetics which facilitate the distribution of drugs and other molecules to their respective drug targets (Fig. 26.1).

Transmembrane carrier proteins undergo conformation changes upon the binding of polar molecules and ions at their respective binding sites on the carrier protein which results in the facilitated movement of the molecules and ions across the cell membrane. Drugs interact with carrier proteins by occupying the binding sites of the polar molecules and ions or by affixing themselves to allosteric sites to modulate the movement of the polar molecules and ions across the cell membrane which will result in an effect [7]. Reserpine, an indole alkaloid derived from the roots of *Rauwolfia serpentine,* is known to block the vesicular monoamine transporter carrier protein and prevents the storage of catecholamine neurotransmitters.

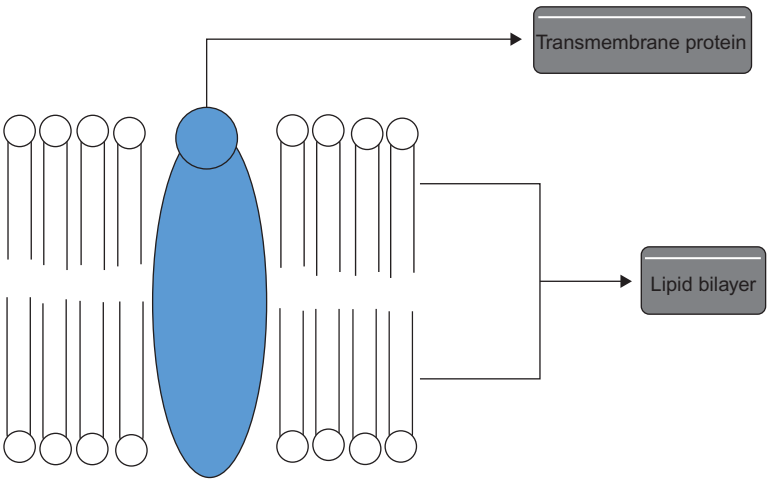

FIGURE 26.1 Transmembrane protein spanning the lipid bilayer of the cell membrane. The macromolecule can be both structural and functional.

Some carrier proteins can also function in the dual movement of molecules and ions across the cell membrane especially for the movement of organic molecules. These carrier proteins are categorized as symport and antiport carriers [4]. The pairing and binding of these molecules and ions are integral to the function of the carrier proteins (Fig. 26.2).

26.2.1.1.1 Symport Carriers

Symport carrier proteins facilitate the movement of polar molecules and/or ions on the extracellular or intracellular side of the cell membrane [8]. The Na-K-2Cl carrier protein is a notable example of a symport cotransporter. It plays a vital role in salt secretion in the secretory epithelia cells along with renal salt reabsorption. Another notable example is the Na^+-dependent glucose transporter which is active in the gastric mucosa and in the renal tubules.

26.2.1.1.2 Antiport Carriers

Antiport carrier proteins facilitate the movement of polar molecules and/or ions in opposite directions across the cell membrane [8]. The antiporter carrier protein can be illustrated with the Na^+/Ca^{2+} exchanger. This system is used by many cells to remove cytoplasmic calcium by the exchange of a Ca^{2+} ion for three Na^+ ions for the regulation of the cytosolic Ca^{2+} level.

26.2.1.2 Ion Channels

Conceptually, ion channels are quite similar to carrier proteins by the facilitation of the movement of polar ions across the cell membrane [8,9]. These drug targets are more predominantly associated with excitable cells and tissues in contrast to the carrier proteins. Ion channels have been shown to facilitate or modulate the transmission of nerve impulses in the nervous and neuroendocrine systems as well as generate other stimuli effecting smooth and striated muscle contraction [8,9] (Fig. 26.3).

Ion channels are categorized into main categories: voltage-gated ion channels and ligand-gated ion channels. Quite notably, substances such as tubucararine, an alkaloid of plant origin, produces its muscle relaxant effect by antagonizing the binding of the neurotransmitter acetylcholine (Ach) on the ligand-gated receptor, nicotinic Ach receptor, thus inhibiting the influx of sodium ions through the receptor's associated ion channel.

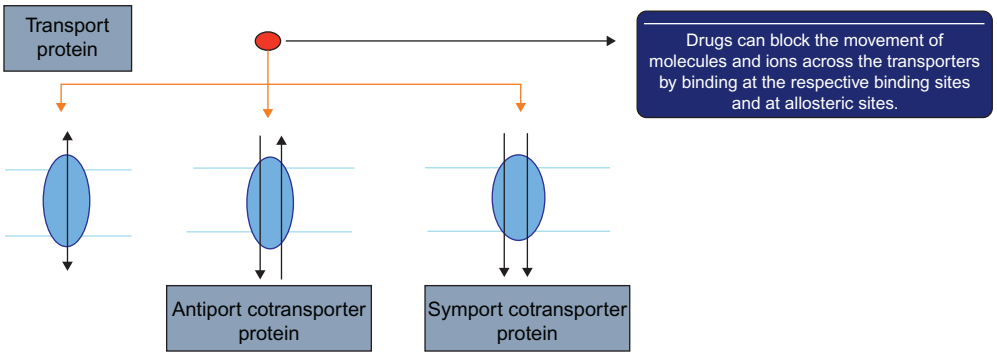

FIGURE 26.2 Carrier proteins can also function as cotransport proteins. Antiport and symport carriers are characterized. Drugs interact with these carrier proteins by binding to the binding sites of the polar molecules and ions and also by binding at allosteric sites.

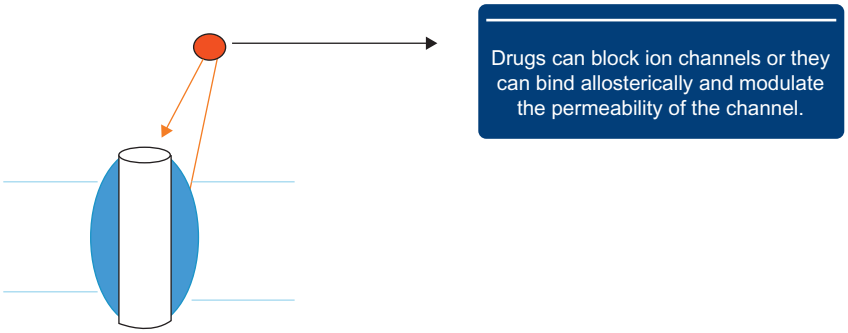

FIGURE 26.3 Ion channels open upon electrical stimuli or via a ligand-gated mechanism. Drugs can inhibit the permeability of ions through these channels by blocking the channel or binding at allosteric sites on the protein.

26.2.1.2.1 Voltage-Gated Ion Channel

These are protein channels which open when the cell membrane is polarized. They play an active role in generation and transmission of electrical excitability [9]. There are two main types of voltage-gated channels which have shown physiological and pharmacological relevance in biomedical studies.

26.2.1.2.2 Voltage-Gated Calcium Channels

Voltage-gated calcium channels have been characterized in nerve terminals, cardiac muscle, and also vascular smooth muscle, where they play important roles in nerve transmission, automaticity, and vascular smooth muscle contraction [10]. Upon opening, there is an influx in calcium ions which further polarizes the tissue and can lead to the development of electrical excitability and other secondary processes.

26.2.1.2.3 Voltage-Gated Sodium Channels

Voltage-gated sodium channels have been characterized in nerve and cardiac tissue and play an important role in neurotransmission, especially relating to the induction of local anesthesia and automaticity of the cardiac myocytes [11]. Upon opening, there is an influx of sodium ions which further polarizes the tissue and can lead to the development of electrical excitability.

26.2.1.2.4 Ligand-Gated Ion Channels

Ligand-gated ion channels incorporate a receptor which has to be bound by a ligand for the channel to open [9]. The nicotinic Ach receptor and gamma-aminobutyric acid (GABA) receptors are two popular types of ligand-gated ion channels characterized and play an active role neurotransmission. The nicotinic Ach receptor is known to produce excitatory effects while GABA receptor is known to produce inhibitory effects.

26.2.1.3 Enzymes

Enzymes are important drug targets and have been found to be quite effective for the screening of natural inhibitors from plants and other sources for novel drug compounds [12]. These are functional proteins which have catalytic activity within cells and tissues. Inhibitors of enzymes are molecules which have similar structure and stereochemistry to the enzyme's known substrate; however, they are not catalyzed to form products from the enzymatic reactions. These inhibitors can be categorized as reversible or irreversible based on the strength of their chemical interaction at the binding sites of the enzyme. Drugs can also modulate enzyme activity allosterically. Cholinesterases, extracellular membrane enzymes, facilitate the recycling of the nerve transmitter, Ach, and also enable the continuous transmission of nerve impulses across the neuromuscular junction [13]. Inhibitors of this enzyme have been used clinically as muscle relaxants and also have been shown to manifest as toxic chemicals which can lead to paralysis and other symptoms associated to Ach crisis. A useful and clinically relevant example of an enzyme inhibitor derived from natural sources is the cardiac glycoside digoxin, similar to digitoxin from the foxglove plant, which inhibits sodium/potassium adenosine triphosphatase (Na/K−ATPase) and is used in heart failure and to treat cardiac arrhythmias (Fig. 26.4).

26.2.1.4 Receptors

Receptors can be described as protein targets which can be bound and activated by endogenous substances [14]. These endogenous substances can be but are not limited to hormones, neurotransmitters, signal transduction

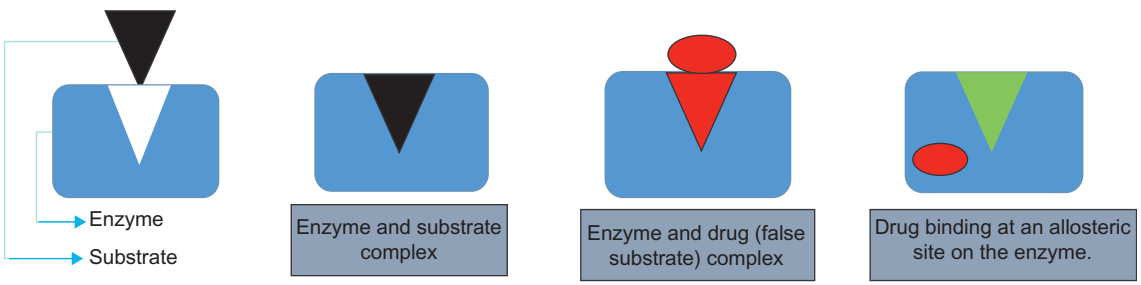

FIGURE 26.4 Inhibitors of enzymes can bind at the respective binding sites of the substrates and also at allosteric sites.

intermediates, and cytokines [14]. These drug targets are usually transmembrane proteins, notwithstanding that intracellular receptors also exist and have important roles as drug targets. Receptors tend to have an extensive signal transduction pathway associated with them to elicit their cellular effects.

Before we delve into the specific types of receptors, there are some basic concepts which should be known about receptors and their respective ligands. It is generally understood that receptors have to be bound to elicit any response [15]. The binding of drugs to their respective receptors depend on the activated and inactivated states of receptors. There are drugs or substances which can lead to receptor activation upon binding and there are also drugs or substances which can prevent receptors from activation. The former substances are known as receptor *agonists* and the latter are termed as receptor *antagonists* [16]. Both classes of drugs bind differentially to *the two state activation model of receptors*. Agonists have a greater affinity to the activated state of the receptor, while antagonists tend to bind more readily to inactivated receptors. Drugs or substances must be selective to receptors to enable binding to form a receptor−ligand complex. Selectivity is governed by the affinity of the drug or substance to the receptor. The greater the affinity of the drug or substance to the receptor, the greater the binding of the two molecules [15].

26.2.1.4.1 Receptor Agonists

Agonist will bind to receptors and cause receptor activation. This activation is governed by efficacy. Efficacy is the ability of the drug or substance to bring about a response from the binding of the receptor and is usually between 0 and 1. *Full receptor agonists* can bring about maximal tissue response upon 100% occupancy of their respective receptors except in the case of spare receptors dealt with in Section 26.2.1.4.3. However, *partial receptor agonists* cannot bring about maximal tissue response even when there is 100% receptor occupancy of their respective receptors [16]. There are also *inverse agonists* which are known to decrease the intrinsic activation which is an attribute of some receptors such as cannabinoid receptors and GABA receptors [17]. The potency of an agonist drug is a function of the drug's efficacy and its affinity to the receptor [16].

26.2.1.4.2 Receptor Antagonists

In the classical description, a receptor antagonist will bind to the receptor and prevent the binding of the agonist to the receptor and thus prevent the activation of the receptor [16]. This classic description can be described as competitive antagonism and can be further categorized to irreversible or reversible competitive antagonism. As the name suggests, reversible competitive antagonism is surmountable with an increase in the concentration of the agonist; however, this is not the case with the irreversible competitive antagonism [14]. Atropine, an alkaloid, is a prototypic reversible competitive cholinergic antagonist from the plant *Atropa belladonna*, which is selective for muscarinic cholinergic receptors.

In understanding drug interactions, we have expanded our understanding of antagonism to identifying other forms of antagonism [14,18]. These other forms are described below:

Chemical antagonism—a chemical interaction of the agonist and the antagonist which affects the ability of the agonist from binding to its pharmacological target.

Pharmacokinetic antagonism—the ability of the antagonist to affect any of the pharmacokinetic processes which results in a reduction in the concentration of the agonist at its respective pharmacological targets.

Physiological antagonism—occurs when two agonists acts independently to bring about opposite effects which oppose each other.

Noncompetitive antagonism—the binding of the antagonist at an allosteric site on the receptor or at a specific location of the signal transduction pathway associated with the receptor rendering cellular effects of the receptor's activation. Thus, a noncompetitive antagonist maintains receptors in the inactivate state.

26.2.1.4.3 Concept of Spare Receptors

Spare receptors addresses specific types of receptors which can bring about maximal tissue response when activated by an agonist without having 100% receptor occupancy. In some instances the percentage of receptors that needs to be bound is less than 1% [18]. Interestingly, noncompetitive and irreversible antagonism can affect the potency of an agonist interacting with spare receptors, unlike other receptors without this reserve capacity. This phenomenon of spare receptors is observed more frequently among drugs which elicit smooth muscle contractions.

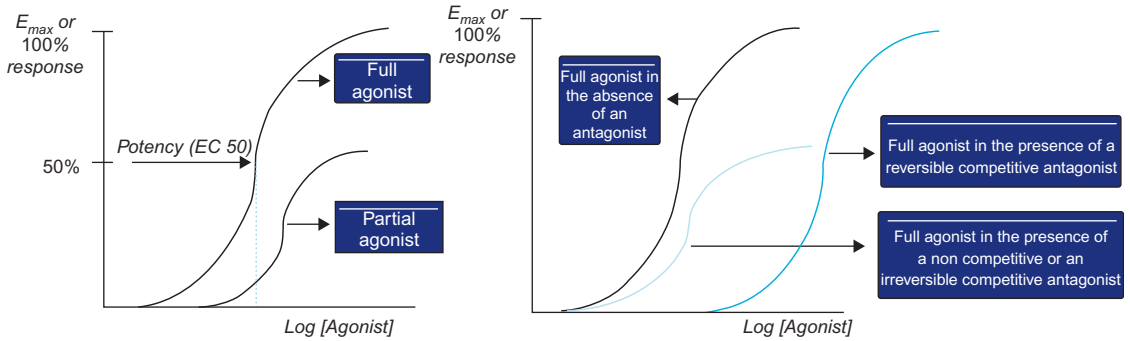

FIGURE 26.5 Drug dose–response curves for receptor agonists in the presence and absence of competitive and noncompetitive antagonists.

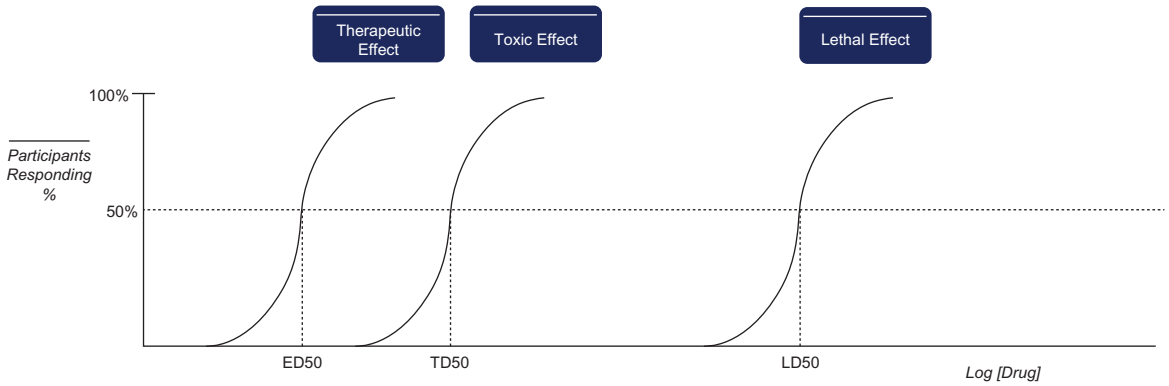

FIGURE 26.6 Quantal dose–response curves outlining the ED50, TD50, and LD50 doses by extrapolation.

26.2.1.4.4 Drug Dose–Response Relationships

Drug dose–response relationships are important in assessing the efficacy and potency of drugs. They are also useful for the interpretation of drug and receptor interactions. There are two types of drug dose–response relationships, namely, the graded dose–response and the quantal dose–response relationships. In the graded dose–response relationship, the tissue will respond to the administered drug until it reaches maximal response as the drug concentration is increased. The occupancy of the receptors by the drugs also plays a critical role in determining the response and is stated to be proportional. A drug's potency can be derived from a graded dose–response curve [19]. The EC50 is the concentration which brings about 50% of the maximal tissue response and is used to determine the potency of drugs (Fig. 26.5).

Quantal dose–response curves describe responses in a noncontinuous way and are usually used for the determination of toxic, therapeutic, and lethal doses of drugs during development, specifically, TD50, ED50, and LD50 values [18,20]. The concept is to generalize a result to a population, rather than to examine the graded effect of different drug doses on a single individual or experimental specimen.

TD50 is the dose which 50% of the participants showed a toxic response. ED50 is the effective dose which 50% of the participants received the therapeutic effect and is also used to determine the potency of drugs. LD50 is the dose which causes death in 50% of the participants. The ratio of the TD50 and ED50 is used to determine the therapeutic index of a drug, which is a numerical index of the drug's safety and, generally, is ≥ 1. Drugs or substances with a therapeutic index of 1 are considered to be toxic [18]. That is, the dose which will brings about the therapeutic effect in 50% of participants may also elicit a toxic effect. Drugs such as warfarin, an anticoagulant, and a coumarin derivative of the sweet clover plant are known to have low therapeutic index in comparison to aspirin, a salicylate also of plant origin which has a high index (Fig. 26.6).

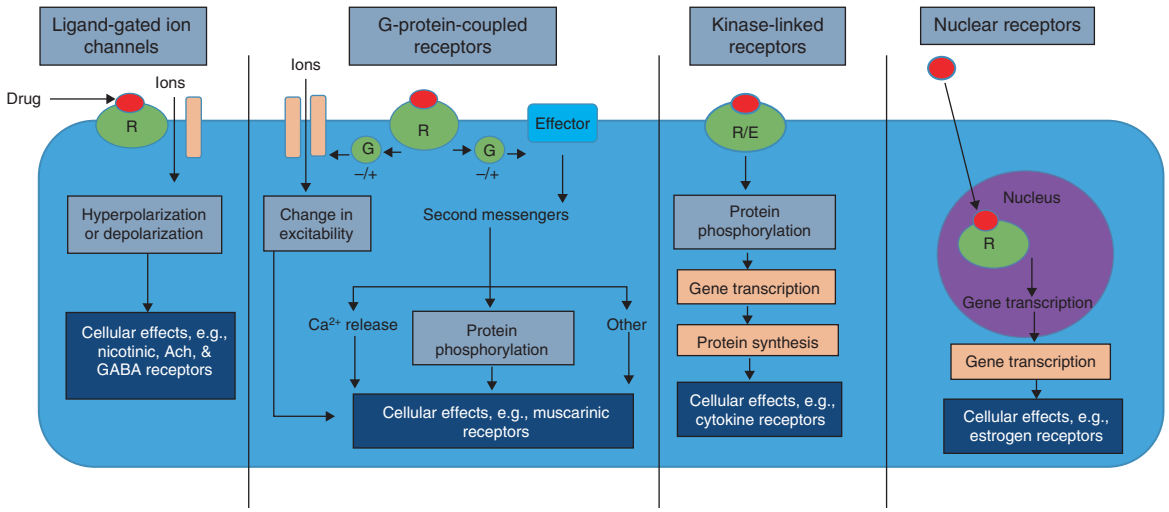

FIGURE 26.7 The four main types of receptors and their signal transduction mechanisms which lead to the cellular response after receptor activation. Abbreviations: *R*, receptor; *R/E*, receptor/enzyme; *Ach*, acetylcholine.

26.2.1.5 Receptor Categories and Signal Transduction Mechanisms (Fig. 26.7)

26.2.1.5.1 Ligand-Gated Ion Channel Receptors

Ligand-gated ion channel receptors were mentioned in Section 3.3 as ion channels coupled with ligand binding domains in the extracellular domain of the receptor. The structure is usually an oligomeric assembly of subunits surrounded by a central pore [14]. These receptors are usually implicated where neurotransmitters such as Ach and gamma aminobutyric acid act. For example, the nicotinic Ach receptor has a significant role in cholinergic transmission in the central and peripheral nervous system. Upon the binding of Ach to its binding sites, the channel becomes permeable to sodium and potassium ions with a net influx of sodium causing depolarization which leads to an electrical excitatory effect [21]. In contrast to the nicotinic Ach receptors, the gamma aminobutyric acid receptors in the central nervous system elicit inhibitory effects on nerve transmission. Upon the binding of the neurotransmitter to the receptor, the ion channel becomes permeable to chloride ions. The net influx of these ions causes hyperpolarization which leads to the inhibitory effects on nerve transmission [22].

26.2.1.5.2 G-Protein–Coupled Receptors

The G protein receptors are membrane receptors comprised of seven spanning alpha helices and coupled with intracellular effector systems via the G protein. The G protein is described as a molecular switch which alternates between the inactive guanosine diphosphate and active guanosine triphosphate (GTP)-bound states. The switching of these states effects downstream cellular processes [14].

These receptors are the largest subset of receptors and include receptors for many hormones and slow neurotransmitters. Examples of these receptors are the muscarinic cholinergic and adrenergic receptors. The receptors have three subunits, namely, alpha, beta, and gamma, and the alpha subunit has been associated with the GTPase activity which is critical for the activated state of the receptor. There are five main classes of the alpha subunits, namely, Gαs, Gαi, Gαo, Gαq, and G12/13 [14,23]. These five classes of the alpha subunits interact with various effector molecules to generate second messengers which facilitate the signal transduction pathways. Gαs is known to activate Ca^{2+} channels and also activates adenylyl cyclase. The ionotropic and chronotropic effects of the heart upon the binding of the Beta-1 adrenergic receptor by catecholamines are based on the activation of the adenylyl cyclase effector and cAMP, the second messenger molecule. Gαq is known to activate phospholipase C which cleaves membrane phospholipid phosphatidylinositol-4, 5-biphosphate (PIP2) into diacylglycerol and inositol-1, 4, 5-triphosphate (IP3). IP3, the second messenger molecule, leads to the release of Ca^{2+} ions from the sarcoplasm reticulum. This effector mechanism is the primary cause of smooth muscle contraction. Gαi is known to activate K^{+} channels and inhibit adenylyl cyclase. Gαo is known to inhibit Ca^{2+} channels. G12/13 is known to have diverse ion transporter interactions [14,24].

26.2.1.5.3 Kinase-Linked and Related Receptors

Kinase-linked receptors respond primarily to protein mediators. They are comprised of an extracellular ligand binding domain which is linked to an intracellular domain by a single transmembrane helix. The intracellular domain is sometimes enzymatic with protein kinase or guanylyl cyclase activity [25]. These receptors are known to have significant effects in cell division, growth, apoptosis, differentiation, inflammation, tissue repair, and immune responses [14,18]. Cyclosporine and Tacrolimus are immunophilin inhibitors derived from fungal sources and produce their immunosuppressive effects by inhibiting mechanisms associated with these types of receptors. The main types of receptors in the category are described below.

Receptor Tyrosine Kinases These include receptors for hormones and growth factors. They can transduce signals by phosphorylating tyrosine residues on the cytoplasmic tail of the receptor. Examples of these receptors are the insulin and epidermal growth factor receptors.

Tyrosine Phosphatase These receptors can be found on the immune cells where they regulate cell activation. As the phosphatases are known to dephosphorylate molecules, receptor tyrosine phosphatases remove phosphate groups from specific tyrosine residues.

Cytokine Receptors These receptors lack the intrinsic tyrosine kinase activity but associate with a cytosolic kinase when bound by its respective ligand. The ligands for the receptor are cytokines, e.g., interferons, and colony-stimulating factors involved in immunological responses.

Serine/Threonine Kinases The members of this kinase related receptor are the transforming growth factor family. Upon activation, these receptors stimulate the growth of normal cells, especially endothelial cells and also cancer cells.

Guanylyl Cyclase-Linked Receptor These are similar to the RTKs and they exert their effects by the production of cGMP.

26.2.1.5.4 Nuclear Receptors

The nuclear receptors are a family of receptors that regulate gene transcription and are otherwise considered ligand activated transcription factors [26]. These receptors respond to lipid and hormonal signals and may be categorized into two main classes. Class 1 comprises receptors for steroid hormones, e.g., estrogen and androgen receptors [14]. Class 2 receptors generally respond to lipid and hormonal stimuli. The receptors which respond to lipid stimuli can be exemplified with the proliferator activated receptor which recognizes fatty acids and the liver oxysterol receptor that functions as a cholesterol sensor in the cell [27]. Receptors which respond to hormonal stimuli are the thyroid hormone, vitamin D, and retinoic acid receptor [14].

26.2.1.6 Tolerance and Tachyphylaxis

Section 5.5 introduced us to the types of receptors and their signal transduction mechanisms which bring about their cellular effects. Comprehending those mechanisms will provide insight to the concept of drug tolerance. Drug tolerance can be described as the gradual diminishing effects of drugs when given continuously over a specified period of time with durations lasting days to weeks. On the other hand, tachyphylaxis is a rapid developing tolerance which usually occurs within the course of minutes. Refractoriness is also used to describe this phenomenon, primarily as it relates to the loss of therapeutic efficacy [18]. Both of these effects are primarily due to receptor desensitization and can be illustrated primarily among the G protein−coupled receptors. There are many mechanisms which contribute to desensitization which include but are not limited to receptor phosphorylation, receptor sequestration, exhaustion of signal transduction mediators, and receptor degradation [28]. As one would imagine, there are exceptions to the concept of desensitization. These are primarily drugs which bring about their effects by not binding to receptors such as osmotic diuretic agents or drugs which bind to cell adhesion receptors.

26.2.2 Concluding Remarks—Drug Targets

So far from the reading, we have been convinced that drug targets form the basis of our understanding of how chemical substances effect physiological changes. The intricacies of the interactions between drugs, in their natural states or

modified forms, and their targets provide a deeper understanding of the successes and pitfalls of the drug discovery pipeline. Ultimately, the advances in medicine and, more specifically, in pharmacology have been bolstered by our understanding of the mechanisms of these molecular targets upon which drugs of various classes act and it sets the platform for the further discovery of safer and more efficacious drugs.

26.3 ADVERSE DRUG REACTIONS

According to the World Health Organization, an adverse drug reaction (ADR) is "a response to a drug which is noxious and unintended and occurs at doses normally used in man for prophylaxis, diagnosis or therapy of disease, or for the modification of physiological function" [29]. Another definition of the term ADR has been proposed that is "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" [30].

An adverse event (ADE) is defined as an unfavorable outcome that occurs while a patient is taking a drug, but which may or may not be caused by the drug [29]. An ADR has been described as an ADE with a causal link to the drug [31].

26.3.1 The Impact of ADRs

ADRs are ranked as one of the top 10 causes of morbidity, mortality, and illness in the developed world [32,33]. ADRs are documented in the United States to claim 100,000 to 218,000 lives annually and are the third leading cause of death after heart disease and cancer [34–36]. However the burden of the problem may actually be underestimated, as in many instances, ADRs are not suspected, thereby leading to underreporting [37,38]. ADRs represent a vast economic burden in terms of health care costs and contribute to a significant percentage of hospital admissions; they are regarded as a major public health problem [39–41,31].

26.3.2 Pharmacovigilance

Prior to approval, most drugs will only have been tested for short-term safety and efficacy on a limited number of carefully selected individuals [42]. In some cases as few as 500 subjects and seldom more than 5000 will have received the drug prior to its release [43].

To identify an ADR that occurs in 1 in 10,000 patients, at least 30,000 patients need to be treated with the drug [44]. Consequently, the limited numbers of persons involved in premarketing clinical trials do not facilitate good estimation of the ADR profile of a drug. Additionally, the controlled environment of premarketing clinical trials bears very little resemblance to how the drug is used in larger populations. It is after release, when the drug is used in more patients having a variety of concurrent diseases and who may be taking other drugs, that limitations to its use become evident. These limitations result from a paucity of long-term safety data, underrepresentation of certain populations in clinical trials, and inadequate information regarding off label use.[1] Furthermore the regular use of surrogate endpoints can give misleading information about the effects of drugs in comparison to usage in actual patients [46,47]. It is also during the postapproval phase that, previously unidentified ADRs (many manifesting years after the release of a drug) may occur [48]. This can be illustrated by two examples. Rhabdomyolysis is a serious but uncommon adverse effect of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins). However there have been reports of rhabdomyolysis occurring as a result of the interaction between azithromycin and various statins [49].

26.3.3 The Assessment of ADRs—Severity and Seriousness

The International Committee on Harmonization has distinguished serious from severe ADRs. A definition of severe is related to a grading of the degree of the reaction; a definition of serious is related to the outcome of the reaction [29]. Severity of reaction is defined as *severe* (potentially life-threatening, causes permanent damage, or requires intensive medical care); *moderate* (requires a change in drug therapy or specific treatment to prevent a further adverse outcome, symptoms resolved in more 24 h, caused a hospital admission to a nonintensive medical care unit); or *minor* (requires no therapy or antidote to event, symptoms resolve in less than 24 h, does not contribute to prolonging length of stay) [50].

1. The use of a drug in a manner different from that approved [45].

The WHO has defined a serious ADE or reaction as any untoward medical occurrence that at any dose: results in death; requires inpatient hospitalization or prolongation of existing hospitalization; results in persistent or significant disability/incapacity; is life-threatening.

Using the WHO definition of serious, Lazarou et al. [32] estimated the incidence of serious ADRs to be 6.7%. In one of the few prospective studies that have measured the incidence of serious ADRs in a general practice setting, Lacoste-Roussillon et al. [51] reported 13 validated serious ADRs resulting in an incidence density of 10.2 per 1000 days of practice.

In a retrospective chart review of 437 ADRs occurring in a university hospital, 24% of the reactions were considered severe to life-threatening [52]. These studies have highlighted the fact that serious and severe ADRs are of significant public health concern.

26.3.4 Polypharmacy

There is an increased risk of the development of ADRs with the number of drugs ingested [53–55]. Polypharmacy has been described "as the long-term[2] simultaneous use of two or more drugs" [55]. Polypharmacy is considered minor (2–3 drugs), moderate (4–5 drugs), or major (> 5 drugs) [54].

However this definition does not account for the appropriate or inappropriate use of drugs. That is, it does not account for the inclusion of "as-needed medications" (e.g., a short course of antibiotics), over-the-counter medications, topical drugs, ophthalmic drops, vitamin supplements, and herbal preparations [56]. Polypharmacy is prevalent in the elderly and has a significant impact on their health [56,57].

The practice of polypharmacy adds to the overall costs of drugs and increases the risk of development of adverse reactions to not only a single drug, but also as a result of interactions with other drugs, herbs, and food.

26.3.5 Drug Interactions

26.3.5.1 Drug–Drug Interactions

Drug–drug interactions (DDIs) occur when one drug interferes with the pharmacological activity of another [58]. These interactions can result in decreased effectiveness and/or increased toxicity. Additionally they may result in the development of ADRs, morbidity, hospitalizations and death [59]. DDIs constitute only a small proportion of ADRs; however, they are important because they are often predictable and therefore avoidable or manageable [58,60,61].

The frequency of DDI is related to the age of the patient and polypharmacy. As the number of medications taken by a patient increases, the risk of DDIs in that patient increases. In fact the risk of DDIs can increase from 6% in patients taking two drugs to 50% in those taking five drugs and 100% in those taking 10 drugs [62].

Although DDIs occur frequently in normal drug therapy, there is variation in the clinical significance of the interactions [59]. Additionally many of the DDIs that are potentially harmful only occur in a small number of patients with the severity of the interaction varying from one patient to another [61]. The classification of potential DDIs (pDDIs) on the basis of severity is important in the assessment of the possible impact of the DDI. The severity of pDDIs can be classified into different types as follows:

Contraindicated: The drug-combination is contraindicated for concurrent use.
Major: If there is risk of death and/or medical intervention is required to prevent or minimize serious negative outcome.
Moderate: The effect of interaction can deteriorate a patient's condition and require alteration of therapy.
Minor: Little effects are produced that do not impair therapeutic outcome and there is no need of any major change in therapy [60].

26.3.5.2 Drug–Herb Interactions

Many herbs and prescription drugs are therapeutic at one dose and toxic at another. The concurrent use of herbs may mimic, magnify, or oppose the effect of drugs [63]. The use of herbal and dietary supplements is extremely common. In the Caribbean use of natural products is extensive with a survey indicating that 100% of households use herbs in both rural and urban settings in Jamaica. [64]. In a survey of adults in the United States, who regularly take prescription

2. Long term is defined as 480 days or more in 2 years.

medication, 18.4% reported the concurrent use of at least one herbal product or high-dose vitamin. The majority of those persons (61.5%) did not disclose such use to their physicians. [65]. Of interest are the findings of a survey by Barnes et al. of 515 users of herbal remedies in the United Kingdom. The results demonstrated that 26% of those surveyed would consult their doctor for a serious ADR associated with a conventional over-the-counter medicine, but not for a similar reaction to an herbal remedy [66].

Research has revealed that a large proportion of persons on prescription medicines for diabetes, hypertension, and gastrointestinal disorders were comedicating with medicinal herbs [67].

Delgoda et al. [68] in a study of 306 adults and 60 children found that 80.6% of the adults and 75.6% of the children were engaged in the concomitant use of herbs and drugs. Among persons indicating such practices, the most commonly cited reasons for the concurrent use of prescription medications and herbal preparations was the belief that there was no harm in taking both and the belief that prescription medicine alone was not an adequate cure. The trend of not informing the physician of the practice of combining prescription and herbal medications persists with only 18% of respondents who practiced such comedication indicating that their doctors knew of their use of herbal preparations.

As more persons engage in the concomitant use of herbs and drugs, there has been increased reporting of drug−herb interactions—a reflection of the increasing world consumption of herbal remedies as medications [69].

Research has demonstrated that the concomitant use of red yeast rice and drugs can potentially cause undesirable herb−drug interactions [70]. Common examples of drug−herb interactions include the interactions between the HMG CoA reductase inhibitors and Red Yeast Rice resulting in rhabdomyolysis [71] and Azithromycin and Red Yeast Rice resulting in rhabdomyolysis [72].

Other reports include the interaction between warfarin and garlic (*Allium sativum*) resulting in increased international normalized ratio, INR [73]; warfarin and Dong quai (resulting in increased INR [73]; grapefruit and amiodarone resulting in ventricular tachycardia [74].

26.4 CONCLUDING REMARKS

Drugs have changed the way in which diseases are treated. Despite all the advantages of pharmacotherapy, evidence continues to mount that adverse reactions are a recognized hazard of drug therapy. ADRs are a common, frequently preventable, cause of illness, disability, and death. There is an increased risk of the development of ADRs with the number of drugs ingested. Many herbs and prescription drugs are therapeutic at one dose and toxic at another. The concurrent use of herbs may mimic, magnify, or oppose the effect of drugs.

26.5 QUESTIONS

1. Define the term “drug target.” Outline the four polypeptide drug targets with examples of each.
2. Identify the four main types of receptors and describe the signal transduction mechanisms which effect responses upon the receptors’ activation.
3. Differentiate between graded drug dose responses versus the quantal dose responses.
4. Define tachyphylaxis.
5. Define the terms adverse drug reaction and adverse drug event.
6. Give two definitions of the term "polypharmacy."
7. What is the difference between severe and serious adverse drug reactions?
8. Give three examples of drug−herb interactions.

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Chapter 27

Drug Metabolism

L.A. Stanley
Consultant in Investigative Toxicology, Linlithgow, United Kingdom

Chapter Outline

Learning Objectives

- To understand the role of metabolism in the disposition of drugs and other xenobiotics.
- To know the difference between metabolic activation and detoxification and appreciate the need for an appropriate balance between these processes.
- To be able to explain the phases of xenobiotic metabolism and identify the enzymes and transporters which mediate them.

27.1 INTRODUCTION

Metabolism, the "M" part of ADME (Absorption, Distribution, Metabolism, Excretion) is central to the excretion of lipophilic compounds. Unlike water-soluble polar compounds, which are readily excreted, these tend to be sequestered in body fat. Even if they are excreted into the gut or kidney tubules, they are rapidly reabsorbed. Metabolism to more polar compounds makes them easier to excrete and enhances their elimination from the body.

27.1.1 Clearance

The clearance of a xenobiotic represents the efficiency of its elimination from the body. Clearance can be determined by measuring plasma concentrations at various time points after an intravenous dose and calculating the area under the plasma concentration−time curve (AUC; Fig. 27.1; Eq. (27.1)).

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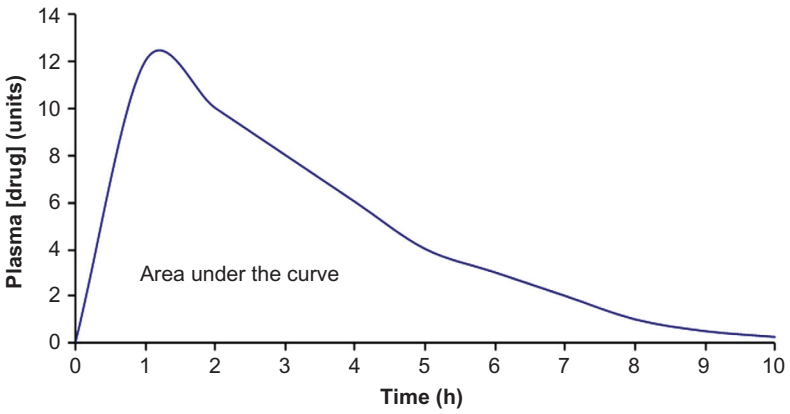

FIGURE 27.1 Representative plasma concentration–time relationship after a single oral dose of a hypothetical drug.

$$\text{Clearance } (L/h) = \frac{\text{Dose (mg)}}{\text{AUC (mg} \times \text{h}/L)} \tag{27.1}$$

The major routes of xenobiotic elimination from the body are excretion as unchanged compound via the kidneys and elimination by metabolism in the liver. The proportion excreted unchanged via the kidneys can be calculated by measuring the amount of intact compound in the urine and can range from near zero (e.g., morphine) to near 1.00 (e.g., penicillin).

Total clearance is the sum of all the clearance processes in the body; it approximates to the sum of the two main processes involved, hepatic clearance (metabolism) and renal clearance (excretion).

27.1.2 First-Pass Metabolism

A xenobiotic which is absorbed via the intestine enters the portal circulation and is delivered straight to the liver. Only after it has passed through the liver does it enter the systemic circulation. Metabolism of the xenobiotic in the liver and intestinal wall before it reaches the systemic circulation is called first-pass metabolism.

It is important to understand first-pass metabolism because it determines the effectiveness of many orally administered drugs; if first-pass metabolism adversely affects therapeutic performance, it may be necessary to create alternative formulations (e.g., skin patches). In addition, variations in first-pass metabolism can affect therapeutic responses and contribute to drug–drug interactions. It is particularly important to be careful when administering oral therapy to patients with compromised liver function because first-pass metabolism may be affected by liver disease.

27.1.3 Bioavailability

When a drug is administered intravenously, it enters the systemic circulation directly. The proportion of an orally administered drug which reaches the systemic circulation is termed its bioavailability and is a function of the opposing processes of absorption and clearance. Oral bioavailability can be calculated from the AUCs of the drug following oral and intravenous administration (Eq. (27.2)):

$$\text{Bioavailability} = \frac{\text{AUC (oral)}}{\text{AUC (intravenous)}} \tag{27.2}$$

The oral absorption and bioavailability of a drug are influenced by its solubility: very hydrophilic drugs are absorbed poorly because they cannot easily cross the plasmalemma. Conversely, very hydrophobic drugs are also absorbed poorly because they are insoluble in aqueous body fluids. Drugs which are unstable at the acid pH of the stomach, or are degraded by digestive enzymes, also tend to have poor oral bioavailability and the absorption and bioavailability of a drug may also be affected by its formulation.

If a drug is absorbed efficiently, first-pass clearance in the liver is likely to be a major determinant of bioavailability. Some drugs undergo extensive metabolism during passage through the liver. Consequently, their bioavailability is near zero (0%): they are called flow limited and this can lead to problems caused by hepatic first-pass metabolism. Others are metabolized very inefficiently by the liver: their hepatic clearance is minimal and their bioavailability is near 1.00 (100%).

27.2 FUNCTION OF XENOBIOTIC-METABOLIZING ENZYMES

The primary function of xenobiotic-metabolizing enzymes is to render hydrophobic chemicals more hydrophilic so that they can be excreted (Fig. 27.2). However, this often involves the generation of highly reactive intermediates. Xenobiotic-metabolizing enzymes can therefore be thought of as double-edged swords, because a single enzyme can mediate both activation and detoxification. If detoxification outweighs metabolic activation, the drug will be removed safely from the body. If metabolic activation outweighs detoxification, the consequence may be protein or DNA binding leading to cytotoxicity, DNA damage, or other toxic effects.

The process of xenobiotic metabolism is conventionally divided into four phases (Fig. 27.3). Of these, strictly speaking only Phases I and II involve xenobiotic-metabolizing "enzymes," although the transporters which mediate Phases "0" and "III" are also often described in terms of substrates and activity.

- "Phase 0": Passage across the plasmalemma and is mediated by influx (uptake) transporters.
- Phase I: Introduction of a reactive group into the molecule.
- Phase II: Transfer of polar groups onto the products of Phase I metabolism.
- "Phase III": Transport of water-soluble Phase I and II metabolites out of the cell and is mediated by efflux transporters.

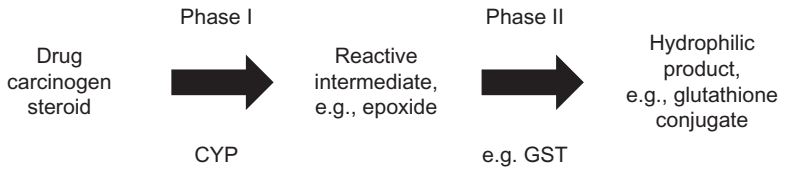

FIGURE 27.2 The role of xenobiotic-metabolizing enzymes in metabolic activation and detoxification.

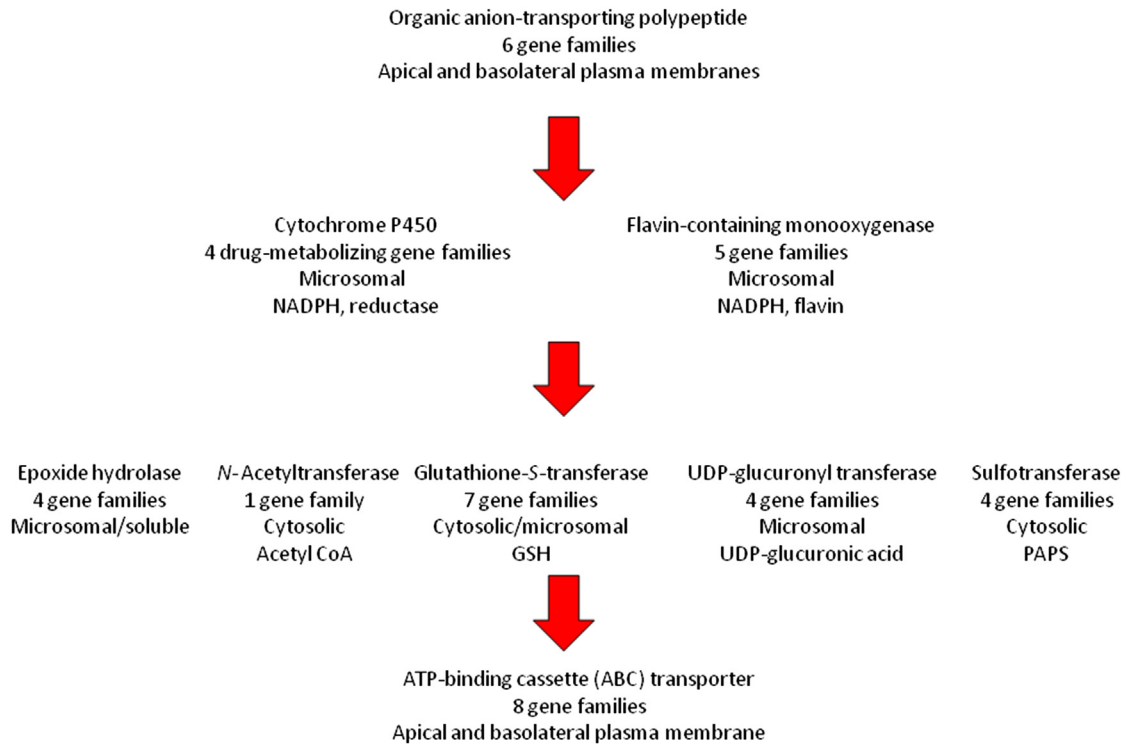

FIGURE 27.3 Key xenobiotic-metabolizing enzyme families. Details (updated January 2015) of the key enzyme/transporter families involved in xenobiotic metabolism.

27.3 PHARMACOGENETIC VARIATION

Interindividual variability is a recurring concept in xenobiotic metabolism. It is often linked to disease susceptibility and differences in drug bioavailability, even between individuals of the same weight who are taking the same dose of drug.

Pharmacogenetic variation is determined by genes, each of which is found at a specific chromosomal locus. The most prevalent genetic variants are known as polymorphisms. A polymorphism is conventionally defined as a sequence variant at a particular locus which is present in $\geq 1\%$ of the population. An understanding of genetic polymorphisms facilitates the evaluation of variability in pharmacological and toxic responses (Fig. 27.4) and may make it possible to personalize therapy and identify/protect susceptible populations.

A polymorphism may alter the amino acid sequence of the protein encoded by a particular gene; alternatively, some polymorphisms affect splicing or the control of transcription. Others have no direct effect on the genes in which they are found but still exert biological consequences, possibly via genetic linkage to functional polymorphisms elsewhere in the genome. The complete absence of a gene (null genotype) may also be classified as a polymorphism. Polymorphisms can always, in principle, be detected at the level of nucleotide sequence and those which affect expressed proteins may also be identified in terms of their effects on amino acid sequence, mRNA/protein expression, and/or biological function.

27.3.1 Genotyping and Phenotyping

An individual's genetic characteristics define his/her genotype; the term phenotype refers to the biological expression of this genotype. Accordingly, genotyping involves examining the altered nucleotide sequences of polymorphic variants in the DNA itself while phenotyping involves measuring biological functions such as enzyme activity.

In the pharmacogenetic context, the principle of phenotyping is to measure the extent of metabolism of a "probe" drug *in vivo* or *in vitro* to identify altered activity due either to the existence of variants of an enzyme which have intrinsic differences in activity or to differential expression of a uniformly active enzyme. If the observed variability in activity is due to differences in expression, this may also be characterized by determining the level of gene expression at the protein or mRNA level.

The detection of polymorphisms at the DNA level is called genotyping. An individual's genotype can be used to predict his/her phenotype if a clear correlation between genotype and phenotype has been demonstrated; however, genotyping can only ever be a surrogate for biological phenotype because it only provides an indirect measure of activity.

27.3.2 Single Nucleotide Polymorphisms

The vast majority (99.5%) of the human genome is common to everybody; most differences between individuals are due to single nucleotide polymorphisms (SNPs), which may account for as much of 90% of human genetic variation [1].

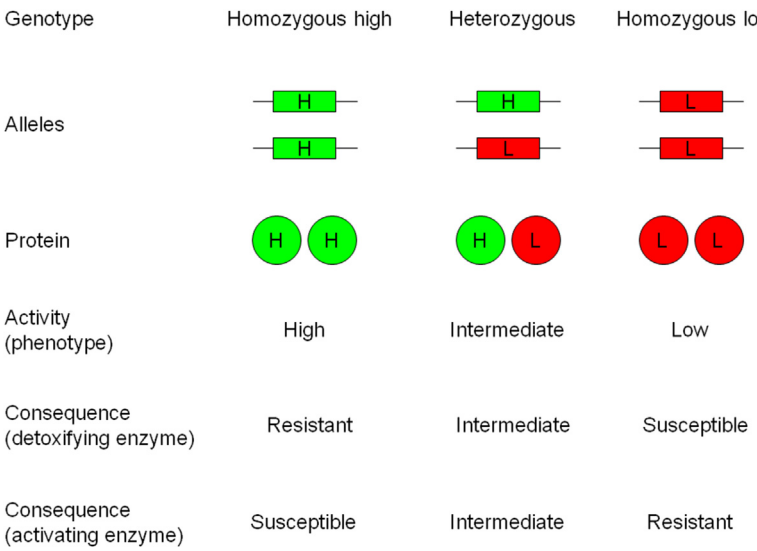

FIGURE 27.4 Genotype and phenotype and their consequences. Possible consequences of polymorphism in a hypothetical xenobiotic-metabolizing enzyme with both high-activity and low-activity variants. *Reprinted from Stanley LA, Toxicogenetics. In: Greim H, Snyder R, editors.* Toxicology and risk assessment: a comprehensive introduction *(ISBN: 978-0-470-86893-5) with the permission of John Wiley and Sons Ltd.*

On average there is about one SNP every 300 bases in the human genome, but they are not evenly distributed: there are SNP hotspots with much higher densities of SNPs than in other regions.

Probably only about 2000 SNPs cause actual amino acid changes. These nonsynonymous SNPs alter the amino acid sequence of the cognate protein and may affect its function. Synonymous SNPs do not affect the coding information, although if located within a regulatory region they can affect responses to xenobiotics. Most SNPs are located in non-coding/nonregulatory regions of the genome and are functionally silent.

27.4 INDUCTION

Many xenobiotic-metabolizing enzymes are subject to induction, i.e., increased expression in response to xenobiotics which may or may not be substrates. This often involves transcriptional activation. Inducing agents activate nuclear receptors either by direct binding or indirectly. Following heterodimerization with additional transcription factors, these bind to specific enhancer or response elements and upregulate gene expression. Inducing agents include flavonoids in cruciferous vegetables and polycyclic aromatic hydrocarbons (PAHs) in barbecued food. Drugs (e.g., phenobarbital) can also be enzyme inducers, as can environmental pollutants (e.g., dioxins), occupational exposures (e.g., acrylonitrile), and herbal remedies (e.g., St. John's Wort).

27.5 NOMENCLATURE

One of the key features of mammalian xenobiotic metabolism is its complexity. This led to complex nomenclature systems which were originally based on the inducibility, substrate specificity, protein chemistry, and antibody cross-reactivity of purified enzymes. The nomenclature is, however, now based on cloned genes, permitting unequivocal classification based on DNA sequence. Luckily for those of us who worked on xenobiotic-metabolizing enzymes in the days before cloning, the two usually correspond!

27.6 PHASE 0

27.6.1 Hepatic Uptake Transporters

The organic anion-transporting polypeptides (OATPs) comprise six families, the most important for drug uptake being the OATP1A, OATP1B, and OATP2B1 families [2]. The substrates of OATP1A include bile acids, estrogen derivatives, peptides, and drugs, while OATP1B1 and OATP1B3 also transport conjugated and unconjugated bilirubin, thyroid hormones, and drugs (e.g., digoxin). The functions of the OATP2B family still require clarification.

27.6.1.1 Example: Role of OATP1B1 in the Disposition of Methotrexate

A 25-fold decrease in hepatic uptake of methotrexate, which is transported by human OATP1B1/murine Oatp1b1, is observed in Oatp1a/1b null mice, while OATP1B1-humanized mice exhibit increased hepatic uptake and decreased plasma concentrations [3]. This suggests a mechanism for the increased plasma methotrexate concentrations and attenuated gastrointestinal toxicity observed in patients carrying low activity variants of OATP1B1: reduced liver uptake and diminished biliary excretion of methotrexate may reduce direct intestinal exposure and hence toxicity.

27.7 PHASE I

Phase I metabolism involves flavin-containing monooxygenases, monoamine oxidases, cyclooxygenases, dihydrodiol dehydrogenases, NAD(P)H:quinone oxidoreductases, alcohol dehydrogenases, and aldehyde dehydrogenases, but this section focuses on polymorphic cytochrome P450 (CYP) enzymes, 50−60 kD heme-thiolate monooxygenases with broad substrate specificity in oxidative xenobiotic metabolism.

CYPs are intrinsic membrane proteins located in the endoplasmic reticulum which mediate the terminal oxidation stage of the microsomal electron transport chain, generating reactive intermediates, such as epoxides [4].

In order to catalyze the monooxygenase reaction, CYPs require the electron-donating proteins cytochrome b_5 and NADPH:CYP oxidoreductase. In contrast to the multiplicity of CYPs, mammals have only one NADPH:CYP oxidoreductase gene, and all murine CYP activity can effectively be abolished by conditional deletion of this single gene [5].

Monooxygenation is essential to the detoxification of many xenobiotics; however, in some cases CYP enzymes generate intermediates that are more toxic than the original substrate and require detoxification by Phase II enzymes.

If this process is inefficient, reactive metabolites can bind to proteins or DNA leading to cytotoxicity and/or mutations.

Not all CYPs are polymorphic but polymorphism is an important feature of this multigene family [6]. Polymorphisms can affect therapeutic responsiveness and influence the consequences of occupational exposure and accidental ingestion, although it is debatable whether they are relevant at low (environmental) exposures.

Mammals have many CYP gene families, each containing numerous genes, but a few enzymes belonging to the CYP1, CYP2, CYP3, and CYP4 families account for the metabolism of most drugs and xenobiotics [6].

27.7.1 The CYP1 Family

The CYP1 family comprises three functional genes organized into two subfamilies, the highly conserved CYP1A subfamily and the more distantly related CYP1B subfamily.

Many PAHs are potent carcinogens which may be either activated or detoxified by CYP1A1. The regulation of CYP1A gene expression is therefore important in determining susceptibility to chemical carcinogenesis, and has been the subject of intense research ever since the discovery that PAHs upregulate their own metabolism [7]. Polychlorinated biphenyls and flavones also induce this activity. The major PAH-inducible CYP, now known as CYP1A1, is highly active in the metabolism of PAHs and model substrates (e.g., 7-ethoxyresorufin).

In rodents, PAHs upregulate Cyp1A gene expression via a regulatory system involving the aryl hydrocarbon receptor (AhR), which mediates the effects of PAHs by a mechanism similar to that by which steroid hormone receptors regulate steroid-responsive gene expression. Briefly, the highly lipophilic PAH molecule crosses the plasmalemma and binds tightly to the AhR, which then forms a heterodimer with the AhR nuclear translocator, moves to the nucleus, binds to regulatory sequences in the 5′ flanking regions of target genes, and activates transcription.

The physiological role of the AhR system is still subject to debate. One possibility is that it arose to deal with combustion products and plant toxins; however, since many naturally occurring compounds are activated to potent carcinogens by AhR-inducible CYPs it is hard to see what overall benefit such a system may confer. Another possibility is that the AhR coordinates aspects of the immune system. Immunologists have taken an interest in this receptor since its role in T-cell differentiation was identified in 2008, leading to the identification of the tryptophan breakdown product kynurenine as the first endogenous AhR ligand [8]. In addition, the AhR may play a role in normal development, since AhR ligands also induce the expression of important enzymes including alcohol dehydrogenases and phospholipase A2.

27.7.1.1 Example: CYP1A1 and Dioxin

Dioxin is a persistent environmental pollutant produced during combustion and industrial processes. It induces CYP1A1 via the AhR and is a potent carcinogen in rodents; however, its carcinogenicity in humans remains the subject of intense debate. The only toxic effect to be unequivocally linked with dioxin in humans is the skin condition chloracne. The best known example of this is Ukrainian president Viktor Yushchenko, who became ill during the 2004 election campaign and was diagnosed with dioxin poisoning. Even 2 years later, disfiguration due to chloracne could still be clearly seen (Fig. 27.5). Ironically, Mr. Yushchenko has made a significant contribution to dioxin research because the well-documented nature of his poisoning and the high dose he received have allowed researchers to characterize the toxicokinetics of this long-lived compound in detail [9]. Fortunately, however, Mr. Yushchenko and other people exposed to high levels of dioxin show no evidence of increased risk of cancer, although the WHO classifies dioxin as a known human carcinogen.

The second, closely related, member of the CYP1A gene family, CYP1A2, is expressed constitutively in human liver. Several nongenetic factors influence its hepatic expression and/or *in vivo* activity: it is inducible by β-naphthoflavone and isosafrole in rodents and, in humans, by cigarette smoke [10].

Unlike CYP1A1, which prefers planar aromatic hydrocarbons, CYP1A2 metabolizes aromatic amines and heterocyclic compounds, mediating the metabolic activation of industrial combustion products and carcinogens found in chargrilled food. Polymorphisms in the CYP1A family have limited impact on xenobiotic metabolism, although interindividual variability in CYP1A2 may affect susceptibility to colorectal cancer.

The third member of the CYP1 superfamily, CYP1B1, mediates the 4-hydroxylation of PAHs, aromatic amines, and steroid hormones. It can also mediate the metabolic inactivation of structurally diverse anticancer drugs. Like CYP1A1 and CYP1A2, CYP1B1 is regulated by the AhR.

FIGURE 27.5 Viktor Yushchenko with chloracne from dioxin poisoning. *"Viktor Yuschenko" by Muumi. Licensed under CC BY-SA 3.0 via Wikimedia Commons.*

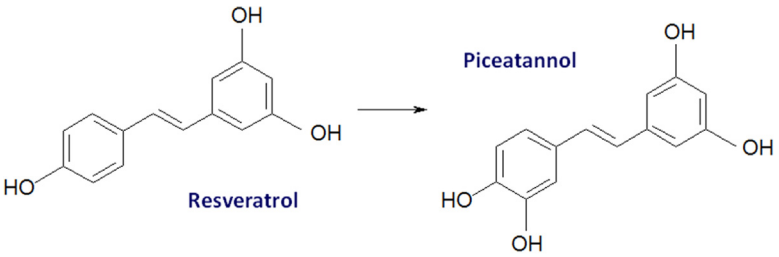

FIGURE 27.6 Conversion of resveratrol to piceatannol.

27.7.1.2 Example: Chemoprevention by CYP1B1

Solid human tumors express high levels of CYP1B1 which contrasts with a lack of expression in histologically normal tissues [11]. This observation is now being exploited in oncology; although most published studies assume that CYP1B1 acts as a carcinogen-activating enzyme like CYP1A1, it can activate the phytoalexin resveratrol to the potent protein kinase inhibitor piceatannol (Fig. 27.6) [12], with potential implications for cancer chemoprevention and therapy.

27.7.2 The CYP2 Family

Of the several CYP2 subfamilies, the CYP2B subfamily contains genes which are highly inducible by barbiturates, whereas others (CYP2A and CYP2C) exhibit higher constitutive expression but are only marginally inducible. Many pharmacologically important CYP2 genes are highly polymorphic.

The CYP2A enzyme family is expressed constitutively in liver. It mediates testosterone 7α-hydroxylation in the rat but metabolizes only a few drugs (e.g., coumarin, nicotine) in humans. Little is known about its physiological function, although human CYP2A6 is the major isoform involved in the oxidative inactivation of nicotine [13]. Polymorphism at the CYP2A6 locus can be associated with either increased or decreased expression and activity.

The major barbiturate-inducible CYP in rat liver, CYP2B1, is regulated via the constitutive androstane receptor (CAR), which is related to the pregnane-X-receptor (PXR) and the steroid, retinoid, and thyroid hormone receptors. The basic mechanism by which CAR and PXR activate transcription is a conventional one, involving xenobiotic binding, heterodimerization (with the retinoid-X-receptor), and interactions with the 5′ regulatory sequences of target genes, but the ligand responsiveness of CAR is complex. This constitutively active receptor is regulated both by agonists which upregulate its activity (e.g., 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, TCPOBOP) and inverse agonists which downregulate it (e.g., androstanol) [14].

Members of the CYP2B family catalyze benzphetamine *N*-demethylation, 7-pentoxyresorufin depentylation, and aldrin epoxidation. The main human isoform is CYP2B6, which metabolizes environmental chemicals, chemotherapeutic agents, antiretrovirals, antidepressants, anesthetics, the antimalarial artemisinin, and the μ-opioid agonist methadone.

Polymorphisms in CYP2B6 can be clinically important; e.g., CYP2B6 4-hydroxylates the prodrug cyclophosphamide, ultimately generating the cytotoxic metabolites phosphoramide mustard and acrolein, and polymorphic variation can have a marked impact on the success of cyclophosphamide chemotherapy [15].

Encoded on chromosome 10, the human CYP2C subfamily comprises four highly homologous enzymes: CYP2C8, CYP2C9, CYP2C19, and the inefficiently translated CYP2C18, which does not contribute significantly to xenobiotic metabolism.

The polymorphic enzyme CYP2C8 metabolizes antidiabetic agents, antimalarials, the antiarrhythmic amiodarone, and the natural product anticancer drug paclitaxel. The consequences of polymorphism in CYP2C8 are currently under investigation [16].

Diclofenac and tolbutamide are commonly used to phenotype CYP2C9, which accepts weakly acidic substances including the anticoagulant warfarin, anticonvulsants, angiotensin receptor blockers, oral antidiabetic agents, and most nonsteroidal antiinflammatory drugs.

The first CYP2C enzyme to be discovered was actually CYP2C19, which is responsible for the inactivating metabolism of proton pump inhibitors and metabolic activation of the anticoagulant clopidogrel. It has also a prominent role in antidepressant metabolism, while its endogenous substrates include progesterone and melatonin [6]. Like CYP2C9, CYP2C19 is highly polymorphic, resulting in the *S*-mephenytoin poor and extensive metabolizer phenotypes identified in the early 1980s [17].

As indicated above, the CYP2C subfamily is highly polymorphic and, since all its members are involved in the metabolism of key therapeutic agents, this is often clinically significant.

27.7.2.1 Example: CYP2C9 and Warfarin Metabolism

Warfarin, a natural product anticoagulant administered as racemic mixture of *R*- and *S*-stereoisomers, acts by antagonizing the vitamin K cycle [18]. Individuals' responses to treatment with warfarin vary enormously and it can sometimes take months to identify an appropriate dose. Excessive bleeding occurs if the initiating dose is too high, while control of clotting is ineffective if the dose is too low. *S*-warfarin is 3−5 times more potent than *R*-warfarin and is metabolized in the human liver via CYP2C9, generating harmless 6- and 7-hydroxy metabolites which are excreted in the urine. Approximately 40% of the Caucasian population carry one or both of the functionally defective variants CYP2C9*2 and CYP2C9*3; they are likely to need lower doses of warfarin and have an increased risk of bleeding complications during therapy. It is now possible to use demographic characteristics, CYP2C9 genotype, and control of the vitamin K cycle for warfarin dose setting [19], and the FDA recommends dose reduction in individuals known to have a variant CYP2C9 allele.

The only known member of the human CYP2D subfamily is CYP2D6, originally identified as the polymorphic enzyme debrisoquine 4-hydroxylase which, when defective, causes a dramatic hypotensive response to debrisoquine [20]. Despite its poor expression in the liver, CYP2D6 is responsible for the metabolism of 15−25% of all drugs, including antiarrhythmics, antidepressants, antipsychotics, β-blockers, opioid analgesics, and anticancer drugs [21].

Thanks to its wide spectrum of genetic variants (from null alleles to several-fold gene amplification), variable expression at the protein level, and extraordinarily broad substrate selectivity, CYP2D6 shows the greatest impact of genetic polymorphism among all major xenobiotic-metabolizing CYPs. Individuals are now classified as poor, intermediate, efficient, or ultrarapid metabolizers [21]. Poor metabolizers (5−10% of the Caucasian population) have significantly altered metabolism of several major drug classes. This can lead to failure of detoxification and adverse drug reactions, while extensive and ultrarapid metabolizers may exhibit poor therapeutic responsiveness to CYP2D6 substrates because of rapid clearance. As a consequence, the development of drug candidates which are metabolized by CYP2D6 is usually discontinued.

The major ethanol-inducible CYP, CYP2E1, encoded on chromosome 10, is the only member of the CYP2E subfamily. Its expression is induced by ethanol and other small organic molecules via complex mechanisms involving transcriptional, translational, and posttranslational effects and is modulated in conditions including diabetes and nonalcoholic liver disease (in which it is believed to play a pathophysiological role). The substrate preference of CYP2E1 is for low molecular weight molecules, including industrial chemicals, environmental toxicants, procarcinogens anesthetics, and drugs (e.g., acetaminophen). There is little evidence that polymorphic variation in CYP2E1 has any clinical significance.

27.7.3 The CYP3A Family

The human CYP3 subfamily, located on chromosome 7, comprises four genes (CYP3A4, CYP3A5, CYP3A7, and CYP3A43). These are inducible by glucocorticoids via PXR, whose mechanism of action is similar to that of conventional nuclear receptors except that, instead of binding a narrow range of ligands with very high affinity, it binds a wide range of ligands with relatively low affinity. This pattern of ligand responsiveness, which exhibits marked species differences between humans and rodents, may have evolved in order to deal with the vast number of exogenous chemicals to which organisms are exposed.

The predominant CYP isoform in human liver is CYP3A4, which can account for up to 50% of total hepatic CYP expression and metabolizes immunosuppressants, macrolide antibiotics, benzodiazepines, statins, antidepressants, opioids, and anticancer drugs. It is also involved in endogenous steroid catabolism. Midazolam, erythromycin, alprazolam, and dextromethorphan are commonly used to phenotype human CYP3A activity while its *in vitro* model substrates include testosterone, 7-benzyloxyresorufin, and benzoquinoline.

The expression of CYP3A4 is highly variable between individuals, but the question of whether this is due to adventitious exposure to inducing agents or to genetic regulation remains unresolved. Some drugs (e.g., ketoconazole) and dietary components (e.g., bergamottins found in grapefruit juice) inhibit CYP3A4, while others (e.g., rifampicin) induce its expression. Both induction and inhibition can lead to drug−drug interactions.

In individuals who express appreciable amounts of CYP3A5 (only 5−10% of Caucasians, but $\geq$60% of Africans and African-Americans), CYP3A5 could contribute significantly to the metabolism of xenobiotics, particularly those which are better substrates for CYP3A5 than CYP3A4 (e.g., tacrolimus).

The CYP3A7 isoform is preferentially expressed in fetal liver and, although expression shifts to CYP3A4 after birth, it continues to be expressed in some adult livers and in the intestine. The clinical significance of the CYP3A7 polymorphism remains unclear.

27.7.4 The CYP4A Family

Peroxisome proliferators, so-called because they induce a spectacular increase in the number of peroxisomes within hepatocytes, include phthalate plasticizers and fibrate-based hypolipidemic agents. They represent a unique class of nongenotoxic hepatocarcinogens in rodents [22] and also induce a family of CYP enzymes, the CYP4A family, which catalyzes ω-hydroxylation of lauric acid and important eicosanoids such as the arachidonic acid metabolite 20-hydroxyeicosatetraenoic acid.

Expression of CYP4A family members is induced via peroxisome proliferator-activated receptor α (PPARα), which mediates diverse responses to peroxisome proliferators and, along with the related receptors PPARγ and PPARδ, plays a key role in lipid homeostasis [23]. Important toxicological questions surround PPARα because it mediates peroxisome proliferator-induced hepatocarcinogenesis, at least in rodents. The relevance of this phenomenon, and the role therein of PPARα, has been the subject of intense debate for many years because human cells appear to be resistant to the *in vitro* effects of PPARα ligands, raising questions regarding the validity of species−species extrapolation when assessing potential risks due to peroxisome proliferators.

27.8 PHASE II

Phase II xenobiotic metabolism involves conjugation to hydrophilic moieties, typically leading to more water-soluble and therefore more readily excretable compounds. Phase II enzyme families include the glutathione *S*-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), sulfotransferases (STs), and *N*-acetyltransferases (NATs). Epoxide hydrolases (EHs), which convert epoxides to dihydrodiols, are also classified as Phase II enzymes since they act on the products of CYP-mediated Phase I metabolism.

27.8.1 Glutathione *S*-transferases

Many reactive electrophiles form conjugates with the tripeptide glutathione (GSH: Fig. 27.7) which is present in cells at concentrations up to 10 mM. These conjugation reactions are catalyzed by cytosolic GSTs found mainly in the liver, lung, and kidney, although strong electrophiles may also react nonenzymatically with GSH. The resulting conjugates are degraded to *N*-acetylated cysteine thioethers (mercapturic acids), which are subsequently excreted. Generation of

diversity among GSTs is achieved by the existence of homo- or heterodimers of 25−27 kD subunits, which leads to a wide range of specificities.

The generic model substrate for GSTs is 1-chloro-2,4-dinitrobenzene; they also metabolize carcinogens, anticancer drugs, and organophosphorous insecticides. The peroxidase activity of GSTs helps to protect cells against oxidative stress, but GSTs can also mediate the metabolic activation of some compounds (e.g., hexachlorobutadiene), leading to toxicity.

The cytosolic GSTs were originally named using the Greek symbols α, μ, ο, π, σ, τ, and ζ. They are now classified using the superfamily code GST and the same initial as the original Greek family name, i.e., GSTA (human members GSTA1−5); GSTM (GSTM1−5); GSTO (GSTO1, GSTO2); GSTP (one member, GSTP1, in humans); GSTS (one member, GSTS1, in humans); GSTT (GSTT1, GSTT2); and GSTZ (GSTZ1) [24].

The microsomal GSTs (MGST1, MGST2, and MGST3) are distinct from the cytosolic forms, with which they have low sequence homology, suggesting that this is the result of convergent evolution. Microsomal GSTs metabolize α-hexachlorocyclohexane and are activated by *N*-ethylmaleimide.

27.8.1.1 Example: GST Gene Expression and Cancer

The distribution of GST expression can determine the tissue specificity of carcinogens; e.g., arylmethylsulfate metabolites of arylmethanols are substrates for rodent Gstt2 (formerly $Y_{rs}-Y_{rs}$), which is expressed in liver but not skin; they are, correspondingly, carcinogenic in rat skin but not liver [25].

27.8.1.2 Example: GSTP as a Tumor Marker

The GSTP isoenzyme is normally a fetal form, but when cells become transformed they start to express it again, possibly due to upregulation by the *fos/jun* oncogene complex. In rodent hepatocarcinogenicity studies, GSTP-positive foci (Fig. 27.8) are considered to be preneoplastic lesions; they indicate that initiation has occurred and the promotion stage of carcinogenesis is under way. In clinical medicine, GSTP can be used as an immunohistochemical tumor marker [26], although it cannot be used as a blood test because it is expressed in red blood cells.

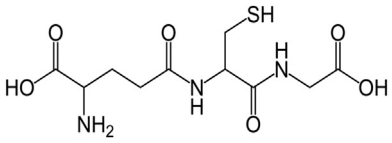

FIGURE 27.7 Structure of glutathione.

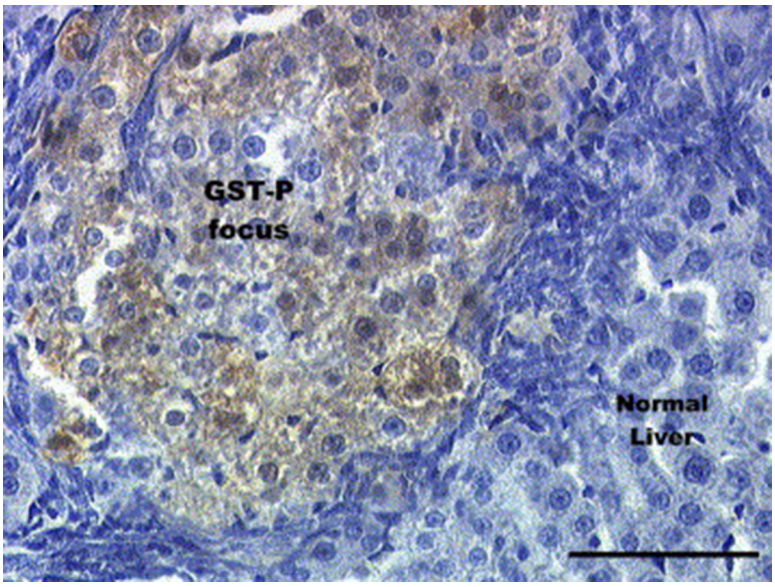

FIGURE 27.8 Photomicrograph of a GST-P positive preneoplastic nodule. *Reprinted from Gonzalez de Mejia et al. Inhibition of liver carcinogenesis in Wistar rats by consumption of an aqueous extract from leaves of* Ardisia compressa*. Food Chem Toxicol 2004;42:509−16 with permission from Elsevier.*

27.8.2 UDP-Glucuronosyltransferases

These membrane-bound 50−56 kD isoenzymes catalyze the transfer of D-glucuronic acid from Uridine 5′-diphospho (UDP)-glucuronic acid to aliphatic and aromatic alcohols, carboxylic acids, amines, hydroxylamines, amides, and thiols. They generate *O*-, *N*-, *S*-, and *C*-glucuronides by forming a β-glycosidic bond between the xenobiotic and the glucuronic acid and are essential in endogenous homeostasis for the glucuronidation of bilirubin, steroids, and thyroid hormone. Xenobiotic UGT substrates include phenols, anthraquinones, carcinogen metabolites, and synthetic steroids.

Xenobiotic-metabolizing UGTs comprise two subfamilies: UGT1 contains a single gene whereas UGT2 is a multigene family. They exemplify different ways of generating diversity [27]: in the case of UGT2, diversity is generated by the conventional mechanism of having multiple individual genes, but diversity in the UGT1 family is generated by an unusual mechanism involving alternative mRNA splicing. There is only one UGT1 gene (*UGT1A1*, located on chromosome 2), but this encodes both phenol and bilirubin UGTs. The specificity-determining region of the gene is encoded by exon 1. There are many different exon 1 sequences, any of which can be spliced to exons 2−5, thus generating enzymes with different specificities from a single gene.

27.8.2.1 Example: Crigler-Najjar Syndrome

Crigler-Najjar (CN) Syndrome is a congenital metabolic disorder caused by UGT1A1 deficiency, which leads to defective glucuronidation and severe hyperbilirubinemia. It has two forms, Types 1 and 2 (Arias Syndrome). A milder condition, Gilbert's Syndrome, also results from *UGT1A1* mutations. The commonest defect found in CN Type 1 sufferers is a 13 bp deletion in exon 2 [28]. Since there is only one copy of each of exons 2−5, this cannot be compensated for by redundancy, unlike defects in exon 1. Treatment for CN Type 1 requires regular phototherapy throughout life, although liver transplantation has been successful in some patients. Taking advantage of the phenomenon of induction, upregulation of UGT1A1 by treatment with phenobarbital is sometimes used to manage the milder form, Arias Syndrome.

27.8.3 Sulfotransferases

Sulfotransferases and UGTs frequently metabolize the same substrates and may cooperate in generating conjugates which are excreted from the liver into the blood, making them available for renal clearance. The human genome contains four Sulfotransferase gene families (SULT1, SULT2, SULT4, and SULT6) with ≥ 14 members encoding cytosolic 32−36 kD enzymes which use 3′-phosphoadenosine-5′-phosphosulfate as a sulfate donor [29]. They metabolize endogenous biogenic amines and mediate the sulfation of dehydroepiandrosterone and progesterone in the adrenals, after which the sulfated forms are secreted and transported to sites of estrogen/androgen synthesis. This may regulate hormonal activity in target tissues since the sulfated forms are less potent than the unconjugated forms, presumably due to weaker receptor binding properties. Synthetic steroids (e.g., 4-hydroxytamoxifen) are also Sulfotransferase substrates; this may influence the susceptibility of estrogen-receptor positive breast tumors to antiestrogen therapy [30].

The large hydrophobic binding sites of Sulfotransferases, which can accommodate up to three aromatic rings, confer broad substrate specificity. This is another means by which diversity in xenobiotic metabolism is generated. The sulfonates produced tend to be ionized at physiological pH, increasing water solubility and resulting in detoxification, although Sulfotransferases can mediate the metabolic activation of some mutagens and procarginogens [31].

27.8.4 *N*-Acetyltransferases

Both detoxification via *N*-acetylation (e.g., of aromatic amines) and metabolic activation by *O*-acetylation (e.g., of hydroxylamines) are mediated by NATs. The substrates of these 31 kD cytosolic enzymes include aromatic amines, hydrazines, hydrazides, sulfonamides, some aliphatic primary amines, and hydroxylamines.

Humans have two functional NAT genes (*NAT*1 and *NAT*2) found, along with the pseudogene *NAT*p, on chromosome 8 [32]. Human NATs were among the first xenobiotic-metabolizing enzymes to be identified as polymorphic [33,34].

Human NAT1 was originally known as the "monomorphic" NAT, although it is actually highly polymorphic. It metabolizes *p*-aminobenzoic acid, 4-aminosalicylic acid, and 2-aminofluorene, as well as endogenous substrates such as *p*-aminobenzoyl glutamate. Like its murine homologue (confusingly called Nat2), it has a wide tissue distribution, being expressed in all epithelial tissues and in red blood cells. It can catalyze the direct hydrolysis of acetyl Coenzyme A in the presence of folate [35] and may play a role in homeostasis.

Human NAT2 (previously known as "polymorphic" NAT) behaves more like a "typical" xenobiotic-metabolizing enzyme, being expressed mainly in the liver and small intestine. Its substrates include sulfamethazine, isoniazid, caffeine, and 2-aminofluorene. Humans are classified as fast or slow acetylators according to their NAT2 phenotype (some studies also identify an intermediate group).

While individuals can readily be classified as rapid or slow acetylators according to their *NAT*2 genotype/phenotype, the genotype/phenotype correlation for *NAT*1 is less clear-cut. Part of the problem is that this gene is both genetically polymorphic, having around 30 allelic variants, and subject to regulation at the protein level. Interestingly, cell biology studies have shown that some of the variant alleles fail to fold correctly and accumulate in aggresomes, where they are targeted for ubiquitinylation and proteolysis [36].

27.8.5 Epoxide Hydrolases

Xenobiotic-derived epoxides are formed by the monooxygenation of carbon−carbon double bonds in olefins or aromatic ring systems. These very reactive species are converted to dihydrodiols by EHs, 49 kD enzymes which thereby protect cells from the formation of both DNA and protein adducts. The hydrolysis of an epoxide usually results in increased water solubility and elimination of toxic potential; however, this is not always the case.

27.8.5.1 Example: Metabolic Activation of Benzo(a)pyrene

EHs usually contribute to the detoxification of reactive epoxide intermediates, including PAH metabolites. However, they can also participate in metabolic activation, as exemplified by the metabolism of benzo(*a*)pyrene [37] (Fig. 27.9). While many CYP-derived PAH epoxides are good substrates for hydrolysis, bay region diol epoxides display potent genotoxicity resulting from two-stage metabolic activation. Benzo(*a*)pyrene is converted to benzo(*a*)pyrene-7,8-epoxide by CYP1A1 and/or CYP1B1; this then undergoes regioselective EH-mediated hydrolysis. This would normally lead to detoxification, but benzo(*a*)pyrene-7,8-dihydrodiol is a good substrate for secondary epoxidation at the 9,10-position, yielding the ultimate carcinogen benzo(*a*)pyrene-7,8-dihydrodiol-9,10-epoxide.

Microsomal EH (mEH; EPHX1) and soluble EH (sEH; EPXH2) are the most important isoforms involved in xenobiotic metabolism [38,39]. Additional isoforms also exist, but these do not generally metabolize xenobiotics.

The human mEH gene is located on the long arm of chromosome 1. Two coding region polymorphisms (Tyr113His and His139Arg) have been identified; the resulting protein variants have different stabilities, suggesting a potential impact on mEH activity *in vivo*. Human mEH expression is inducible via the transcription factors *Nrf*2 and CAR by compounds including phenobarbital and *N*-acetylaminofluorene, and alternative noncoding exon 1 sequences found in mammalian genomes permit tissue and cell type-specific expression.

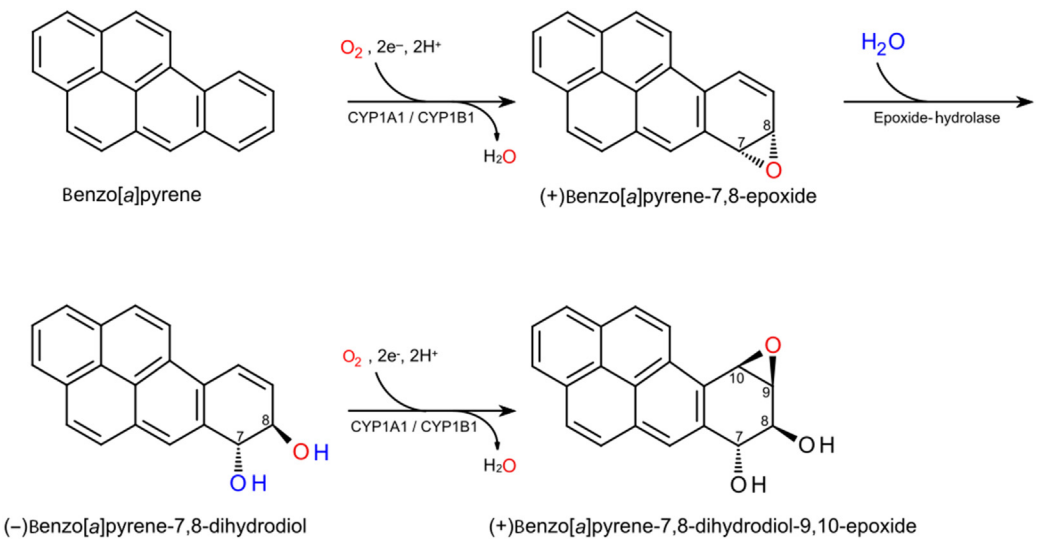

FIGURE 27.9 Metabolism of benzo(*a*)pyrene yielding benzo(*a*)pyren-7,8-diol-9,10-epoxide.

Many of the xenobiotic epoxides degraded by mEH are toxins, mutagens, and carcinogens, while others are drug metabolites; the broad substrate selectivity and distribution of mEH provides systemic defense against such compounds, so inhibition of mEH by therapeutic agents is considered to be undesirable.

The human sEH (EPHX2) locus on chromosome 8 exhibits various polymorphisms which affect its coding sequence and/or enzymatic activity. The substrate selectivity of sEH complements that of mEH in that it hydrolyzes trans-substituted epoxides. Its main function seems to be the turnover of lipid-derived epoxides, which have diverse functions in the control of blood pressure, inflammation, cell proliferation, and nociception. In recent years, sEH has been identified as a promising therapeutic target for the treatment of hypertension and other diseases.

Many organs express sEH, mainly in the cytosol, although peroxisomal expression does occur in some organs. In rodents, peroxisome proliferators (e.g., fibrates, glitazones) induce sEH expression via both PPARα and PPARγ [40].

27.9 POLYMORPHISMS IN XENOBIOTIC-METABOLIZING ENZYMES

There is now overwhelming evidence that polymorphic xenobiotic metabolism is associated with differential responses to drugs, carcinogens, and industrial chemicals.

27.9.1 Metabolic Polymorphisms and Acute Toxicity

27.9.1.1 Example: Occupational exposure to acrylonitrile

Acrylonitrile is both acutely toxic and carcinogenic. It is metabolized by CYP2E1 forming a mutagenic epoxide, cyanoethylene oxide [41] (Fig. 27.10). Further metabolism generates the acute toxin cyanide, either via EH or by rearrangement and hydride transfer. Both acrylonitrile itself and cyanoethylene oxide also undergo GST-mediated reactions with tissue thiols. The acute toxicity of acrylonitrile is considered to be due to a combination of GSH depletion and cyanide generation.

Two cases of acrylonitrile intoxication illustrate this point [42]. Two individuals, one with low and one with high GST activity, were accidentally exposed to acrylonitrile. The person with low activity experienced headache, nausea, and vomiting; furthermore, the level of hydrocyanic acid in his blood entered the lethal range, although he fortunately recovered following treatment with the antidote, *N*-acetylcysteine. This substantiated the hypothesis that, particularly in individuals with low GST activity, acrylonitrile toxicity is gated by GSH depletion. If insufficient GSH is available for conjugation (or the activity of GST is too low), free acrylonitrile can enter the CYP2E1-mediated oxidative pathway, leading to toxicity.

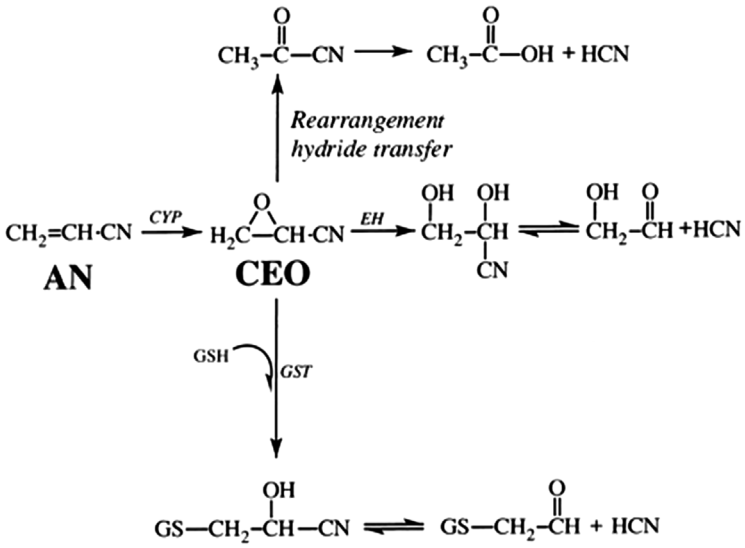

FIGURE 27.10 A proposed scheme showing the role of CYP and epoxide hydrolase enzymes in the metabolism of acrylonitrile to cyanide. *Reprinted from Wang et al. Cytochrome P450 2E1 (CYP2E1) is essential for acrylonitrile metabolism to cyanide: comparative studies using CYP2E1-null and wild-type mice. Drug Metab Dispos 2002;30:911–7 with permission from ASPET.*

27.9.2 Polymorphic Xenobiotic-Metabolizing Enzymes and Cancer

Over the last 30 years a huge amount of effort has been devoted to evaluating the impact of polymorphic xenobiotic metabolism on cancer susceptibility with a view to identifying potentially vulnerable individuals. The simplest paradigm for this approach is aromatic amine-induced bladder cancer.

27.9.2.1 Example: Metabolic Polymorphisms and Bladder Cancer

Bladder cancer is associated with occupational exposure to aromatic amines. A 1982 study found that 22/23 (95.7%) dye factory employees (or ex-employees) with bladder cancer were slow acetylators, compared with only ~60% of controls [43]. Although subsequent studies found lower slow acetylator frequencies among bladder cancer patients [44], the observation that slow acetylators have an increased risk of bladder cancer is now well established, and has been confirmed by meta-analysis [45]. This effect seems to be specific to smokers.

The role of NAT2 should not be considered in isolation, since other activating and detoxifying enzymes in the liver contribute to susceptibility by competing for the aromatic amine substrate, leading to the generation of metabolites which are subsequently delivered to the bladder [46] (Fig. 27.11). Low levels of NAT2-mediated *N*-acetylation allow hepatic CYP1A2-mediated *N*-hydroxylation to predominate. Following further metabolism by UGTs and transport to the bladder, hydrolysis regenerates a reactive *N*-hydroxylamine which can be *O*-acetylated by NAT1 leading to the initiation of carcinogenesis. There is evidence that individuals with both slow NAT2 and rapid CYP1A2 phenotypes have a further increase in their risk of bladder cancer if they smoke [47]. Meta-analysis does not, however, support a key role for NAT1 genotype [45], even though it is NAT1 rather than NAT2 which is expressed in urothelial cells.

It is unusual for studies on toxicogenetic factors in cancer to yield such clear-cut results. Attempts to elucidate the role of metabolic polymorphisms in colorectal cancer illustrate some of the difficulties in obtaining definitive answers.

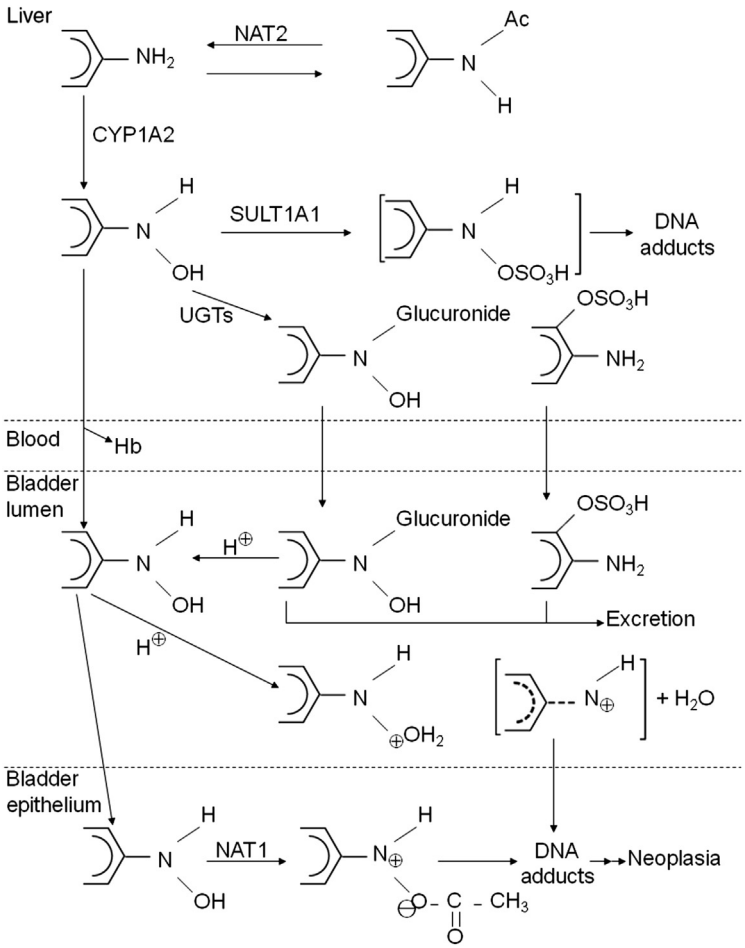

FIGURE 27.11 Pathways of aromatic amine metabolism in the liver and bladder. *Reprinted from Stanley LA. Toxicogenetics. In: Greim H, Snyder R, editors. Toxicology and risk assessment: a comprehensive introduction (ISBN: 978-0-470-86893-5) with the permission of John Wiley and Sons Ltd.*

27.9.2.2 Example: Heterocyclic Amine-Induced Colorectal Cancer

An individual's risk of colorectal cancer is modified by numerous lifestyle and hereditary factors. In particular, people who frequently consume well-done red meat are regularly exposed to heterocyclic amines generated during cooking; these are thought to mediate carcinogenesis in the colon. The carcinogen 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]-pyridine is particularly significant in this context [48].

The pathway of heterocyclic amine metabolism [48] (Fig. 27.12) suggests that the rapid NAT2 phenotype might confer increased colorectal cancer risk, especially when combined with the rapid CYP1A2 phenotype and regular consumption of well-done red meat. Early phenotyping studies suggested that this was the case, at least in smokers [49], but the results of genotyping studies have been contradictory [50–52] and meta-analysis does not clearly link NAT2 genotype with colorectal cancer susceptibility [53].

27.10 CONSEQUENCES OF PHASES I AND II METABOLISM

The main consequence of Phase I metabolism is the generation of reactive intermediates. In the absence of adequate Phase II metabolism these may bind to DNA (forming nucleotide adducts and possibly acting as carcinogens) or to proteins (forming peptide adducts and possibly acting as immunogens). In the presence of active Phase II enzymes the consequences may be further activation (e.g., formation of diol epoxides), which can lead to toxicity, or formation of hydrophilic products ready to be excreted by efflux transporters—"Phase III."

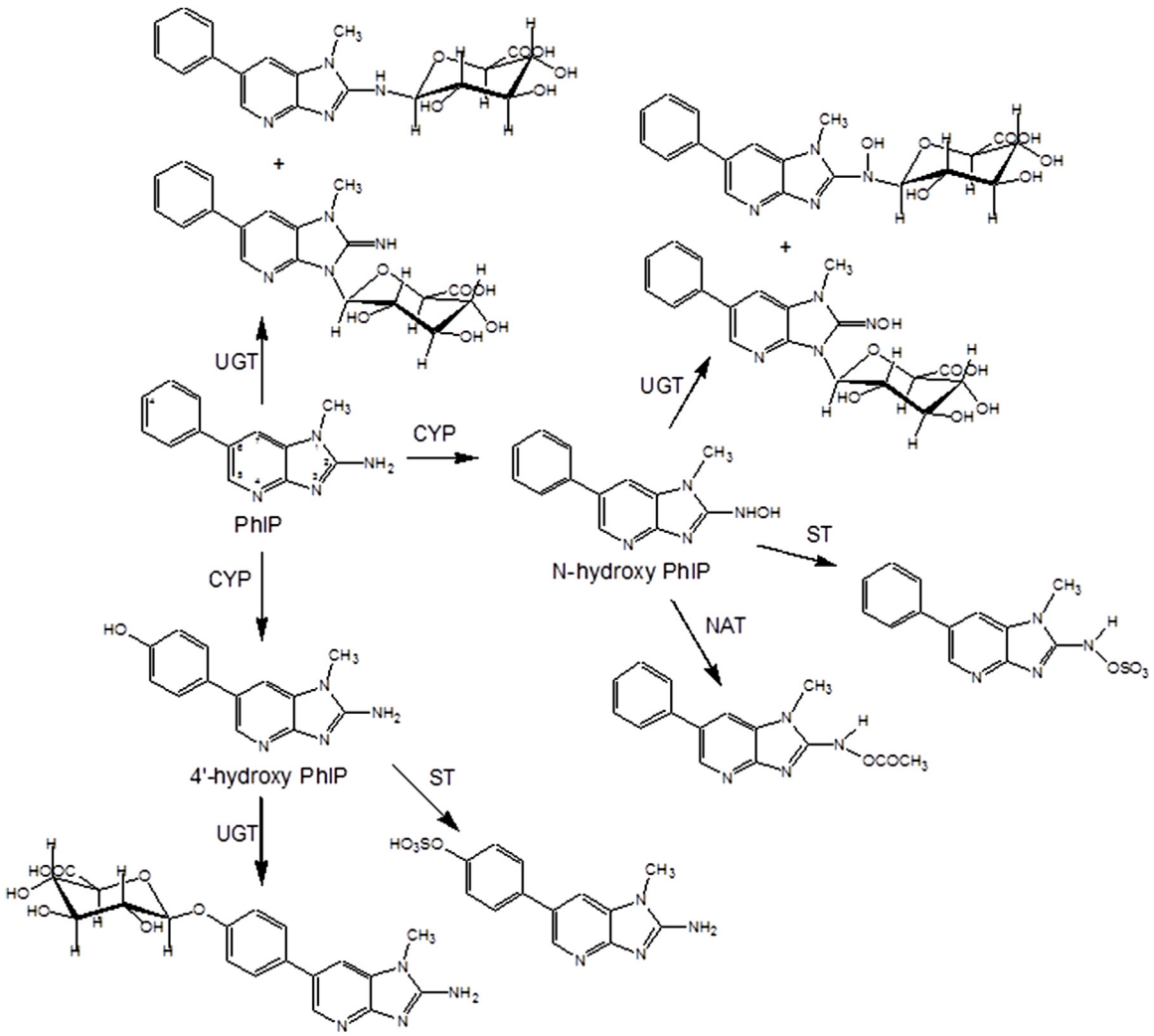

FIGURE 27.12 Pathways of heterocyclic amine metabolism. *Reprinted from Stanley LA. Toxicogenetics. In: Greim H, Snyder R. Toxicology and risk assessment: a comprehensive introduction (ISBN: 978-0-470-86893-5) with the permission of John Wiley and Sons Ltd.*

27.11 PHASE III

In xenobiotic metabolism, "Phase III" is the export of compounds from cells by energy-dependent transporters [54]. However, calling this "Phase III metabolism" is, strictly speaking, erroneous because it describes the transport of molecules across membranes without altering their chemical structure; also, it implies that this is towards the end of the ADME process whereas drug transporters play a key role in intestinal absorption, which is a function of the balance between transporter-mediated influx and efflux in intestinal epithelial cells. Drugs that diffuse passively across the apical membranes of intestinal epithelial cells are often substrates for transporters which extrude them back into the intestinal lumen. These transporters can also mediate active transport of compounds from the blood to the intestinal lumen. If, however, compounds get past this line of defense, hepatic and renal extraction becomes significant, so expression of transporters in the liver and kidney is also important.

27.11.1 The ABC Superfamily

The ATP-binding cassette (ABC) superfamily includes a variety of membrane transporters, receptors, and ion channels.

27.11.1.1 P-Glycoprotein/MDR1

As its name suggests, the first drug transporter identified, multidrug resistance protein 1 (MDR1), was discovered as a result of its ability to confer drug resistance on cancer cells. This key member of the ABCB1 family will be referred to here by its original name, P-glycoprotein, for reasons which will become apparent when we discuss its murine homologues. It is found in the apical membranes of various normal polarized cell types including intestinal mucosal cells (facing the intestinal lumen), hepatocytes (bile duct-facing membrane), renal proximal tubular cells (brush borders), and capillary endothelial cells of the brain and testis; in other words, in key locations involved the uptake and excretion of xenobiotics. For example, expression at the tip of the intestinal villus means that P-glycoprotein is ideally placed to control xenobiotic absorption from the intestinal lumen.

The pattern of P-glycoprotein expression and its known role in drug resistance are consistent with its normal function in the removal of potentially toxic substances from susceptible tissues. It also has postulated functions in hormone and lipid transport, reproduction, cellular immunity, and the regulation of cell volume. In addition, its presence at the maternal−fetal interface of the placenta suggests a role in protection of the fetus.

The substrate selectivity of P-glycoprotein encompasses molecules ranging in size from 300 to 2000 Da. It seems to have a preference for molecules containing a basic N atom and two planar aromatic domains, though this is not an absolute requirement, and it mediates the ATP-dependent transport of lipophilic amphipathic drugs, particularly cations.

Mice have two *Mdr* genes, *Mdr*1a and *Mdr*1b, which, between them, seem to cover the same functions as the single human *MDR1* gene. In particular, Mdr1a shares many of the functions of human MDR1. The functions of Mdr1b seem to be more limited, but it must have some importance as it has been conserved during evolution. *Mdr*1a and *Mdr*1b null mouse models have been used to study murine P-glycoprotein function.

27.11.1.2 MDR-Associated Proteins: MRP1 and MRP2

The second important family of ABC transporters, ABCC1, includes MRP1 and MRP2.

The MRP1 protein is expressed at high levels on the basolateral membranes of polarized epithelial cells in the kidney (distal tubule and glomeruli), intestine, brain, lung, testis, placental endothelial cells, and liver (lower levels). It is thought to protect cells by extruding substances into the blood for subsequent elimination via the intestine or kidneys and is the primary transporter for endogenous GSH, glucuronide, and sulfate conjugates. It can also transport unconjugated anticancer drugs via a cotransport mechanism with GSH.

The ATP-dependent transport of anionic drugs and metabolites, including sulfates, glucuronides (including bilirubin glucuronides), and GSH conjugates is mediated by MRP2, which is located on the apical membranes of hepatocytes, intestinal mucosal cells, kidney proximal tubular cells, and syncytiotrophoblast cells. This orientation allows MRP2 to drive xenobiotic excretion into bile, feces, and urine and limit xenobiotic intake into protected compartments.

Humans and mice with deficiencies in MRP2/Mrp2, including the inherited disorder Dubin-Johnson syndrome, exhibit impaired biliary secretion of GSH, GSH conjugates, and bilirubin glucuronides associated with conjugated hyperbilirubinemia and pigment deposition in the liver.

27.11.2 Consequences of Altered Drug Transporter Function

One of the drivers of research into the effects of drug transporters on systemic pharmacokinetics is the need to improve plasma concentrations of potent drugs which have very poor bioavailability. This is important in veterinary settings as well as in human medicine: P-glycoprotein substrates have been reported to cause problems in Collies and other canine breeds that carry *MDR1* mutations (http://www.vetmed.wsu.edu/depts-vcpl/drugs.aspx).

Transporter null and humanized mice have been used to study the consequences of altered drug transporter function in various contexts [54]. The loss of *Mdr*1a in mice often has its most striking effects in terms of changes in the permeability of the blood−brain barrier (BBB), the most important barrier between the central nervous system and the systemic circulation. The BBB comprises a monolayer of endothelial cells connected by complex tight junctions which form a physical barrier to large hydrophilic compounds, though it may be crossed by small lipophilic compounds which can enter cells by passive diffusion. Its endothelial cells express an array of metabolic enzymes and efflux transporters, thus forming a biochemical barrier to compound uptake.

27.11.2.1 Example: Altered Susceptibility to Ivermectin in Mdr*1a Null Mice*

Interest in the susceptibility to xenobiotics of mice lacking a functional *Mdr*1 gene arose when naturally Mdr1-deficient CF-1 mice were sprayed with ivermectin to treat a mite infestation. The null mice were 50−100 times more sensitive to the adverse effects of ivermectin, which is normally nontoxic, and the concentration of ivermectin in their brains following oral dosing was 90 times higher than in wild-type littermates [55]. The mice also exhibited slower clearance and increased tissue concentrations when dosed with the anticancer drug vinblastine.

This generated considerable interest because it suggested the possibility of manipulating the access of drugs to the brain. Further studies [56] supported the concept that many relatively hydrophobic drugs are excluded from the brain via secretion back into the blood by P-glycoprotein at the BBB.

27.12 IMPORTANCE OF RECENT DEVELOPMENTS

Until recently we depended on results from animal experiments to understand xenobiotic metabolism; however, numerous *in vitro* and *in silico* methods are now available for predicting xenobiotic disposition. These cannot, individually, provide a complete picture of this complex process; however, by integrating data from *in vitro* studies and *in silico* models effective predictions can be made.

Furthermore, advances in molecular biology and the development of humanized models now enable us to study the *in vivo* functions of human xenobiotic-metabolizing enzymes directly. This is important because there are many differences in xenobiotic metabolism between animals and humans. As better humanized models become available, their use is likely to extend both backwards into the process of drug discovery (verifying bioavailability prior to candidate selection) and forwards into the clinical phase (explaining observations made during clinical trials). These models will enable us to predict responses to drugs and xenobiotics more confidently, but they must be characterized carefully and used judiciously: the traditionalist would still argue that the problem with a humanized mouse is that it is neither a human nor a mouse!

27.12.1 The Future

The term “drug-metabolizing enzyme” conventionally refers to the enzymes which metabolize aromatic and aliphatic organic compounds; however, as the range of substances used as “drugs” is extended, many more enzymes need to be considered. What additional enzyme families should be included in order to allow us to understand the disposition of the many novel plant products which are now being developed for therapeutic use?

27.13 REVIEW QUESTIONS

1. How might changes in the rate of hepatic xenobiotic metabolism affect the bioavailability of an orally administered drug?
2. What are the possible consequences of CYP-mediated metabolism of an aromatic organic chemical?
3. Can you rationalize the fact that NAT2 fast acetylators appear to have an increased risk of colon cancer but be protected from bladder cancer?

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Chapter 28

Biotechnology

I.I. Amarakoon, C.-L. Hamilton, S.A. Mitchell, P.F. Tennant and M.E. Roye
University of the West Indies, Mona, Kingston 7, Jamaica

Chapter Outline

Learning Objectives

- Examine the definition of, and relate a brief history of biotechnology.
- Discuss the similarities between biotechnology, bioengineering and biomedical engineering.
- Present some of the major developments in the four main areas of biotechnology: industrial, marine, medicinal, and agricultural.
- Offer a brief overview of the standard techniques of recombinant DNA technology, nucleic: extraction, separation, transfer, detection, sequencing, amplification, and cloning.

28.1 DEFINITION

The term "biotechnology" dates back to 1919, when it was first coined by the Hungarian engineer Karl Erkey [1]. At that time, biotechnology encompassed the use of living organisms for the production of new products from raw materials of biological origin. Hence the name consisting of a combination of the Greek words: bios—life; techno—technical; and logos—study. Since then the definition of biotechnology has been redeveloped a number of times [2], but the most widely accepted definition was given by the Organization for Economic Cooperation and Development (OECD) in 1981 [3]. The OECD defines biotechnology as "the application of scientific and engineering principles to the processing of materials by biological agents."

For much of the 20th century, the term "biotechnology" continued to be broadly used in reference to technologies ranging from the fermentation of products to the selective breeding of plants. In recent years, the term modern "biotechnology" has been used interchangeably with biotechnology and has become almost synonymous with genetic modification and the targeted utilization of the methods of molecular biology. The "modern" in modern biotechnology presumably differentiates between the present applications of genetic engineering and cell fusion from the past conventional methods of biotechnology such as fermentation and selective breeding. The definition of biotechnology as given by the European Federation of Biotechnology is "Biotechnology is the integration of biochemistry, microbiology and engineering sciences to achieve technological (industrial) application of the capabilities of microorganisms, cultured tissue cells and parts thereof" [3]. The 2003 OECD definition of biotechnology is the "application of scientific and engineering principles to the processing of materials by biological agents to provide goods and services; new biotechnology involves the use of cellular and molecular processes to solve problems or make products." The Office of Technology Assessment [4] in the United States describes modern biotechnology as incorporating "a specific focus on industrial usage of recombinant deoxyribonucleic acid (rDNA), cell fusion, and novel bioprocessing techniques; industrial use of living organisms."

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Biotechnology is therefore a multidisciplinary and interdisciplinary field, rather than a single discipline, that has given rise to a range of products and processes in life sciences. The technology has been applied to the development of new medicines, improved crop plants and animals, as well as the more efficient manufacture of everyday products. A color code was created to distinguish between the various applications. Red, green, white, and blue biotechnology refer to applications to medicine, agriculture, industry, and marine and aquatic environments, respectively; these are further explained in later sections of the chapter.

28.2 BIOTECHNOLOGY, BIOENGINEERING, AND BIOMEDICAL ENGINEERING

Depending on the tools and applications, there is notable overlap with biomedical engineering or bioengineering and biotechnology [5,6]. Biomedical engineering or bioengineering is a rapidly growing transdisciplinary field that is regarded as the bridge between technology, medicine, and biology. In other words, the primary focus of bioengineering is the application of the principles of engineering and design concepts to medicine and biology. Harmon in 1975 defined bioengineering as the "field that uses the tools and concepts of the physical sciences to analyze biological systems" [7]. In 1997, the US National Institutes of Health (NIH) published its working definition: "Bioengineering integrates physical, chemical, or mathematical sciences and engineering principles for the study of biology, medicine, behavior, or health; it advances fundamental concepts, creates knowledge form the molecular to the organ systems levels, and develops innovative biologics, materials, processes, implants, devices, and informatics approaches for the prevention, diagnosis, and treatment of disease, for patient rehabilitation, and for improving health" [8]. Although NIH's definition of bioengineering is focused on human health, which is a key component of biological engineering, bioengineering also addresses the full spectrum of life sciences, including applications to agricultural, environmental, and ecological systems [9]. Industrial applications of bioengineering go beyond medicine to, e.g., the transformation of feedstocks into ethanol, hydrogen, biodiesel, nutraceuticals, and bioplastics.

While the scope and applications of biotechnology and biomedical engineering overlap to some extent, both possess their own characteristics. Bioengineering employs the concepts and principles of physical science to solve problems but biotechnology is more dependent on natural sciences. Much of biotechnology is associated with agricultural engineering and chemical engineering, rather than bioengineering. The interface with agricultural engineering arises because of applications, e.g., in the development of pesticides and genetic engineering of microorganisms, plants, and animals. As a result, the interface with chemical engineering is the use of industrial scale-up processes and various computational approaches.

28.3 HISTORY OF BIOTECHNOLOGY

The field biotechnology is considered a part of a continuum that began centuries ago. Three main stages, from a historical point of view, are recognized [10], namely, ancient biotechnology, classical biotechnology, and modern biotechnology.

The roots of biotechnology can be traced back to the prehistoric civilizations between 5000 and 10,000 BC, when Egyptian and Indus Valley Civilizations initiated the domestication and selection of plants and animals for better taste, high-yield, and disease resistance [11]. Various sexual and vegetative propagation methods were used to produce these plants [12]. There was also the use of microorganisms to produce cheese, bread, beer, and wine [13] and the use of herbal remedies for the treatment of wounds and ailments. This period in the history of biotechnology is described as ancient biotechnology. Early civilizations used traditional biotechnology techniques in a trial and error manner, apparently without understanding the underlying scientific principles. Their activities are typically referred to as discoveries rather than developments as they came about based on observations of nature.

The second phase in the history of biotechnology, referred to as classical biotechnology or traditional biotechnology [14], charts the development of fermentation technology between the mid-19th century and the 1970s. During this period, there was the widespread use of methods from ancient biotechnology, especially fermentation, and their adaptation to the industrial production of enzymes, antibiotics, and various types of organic acids such as vinegar, citric acid, amino acids, and vitamins [15]. Koch, Pasteur, and Lister founded institutes with the mandate of investigating fermentation along with other microbial processes [16]. By the end of the 19th century, Mendel's work on the basic principles of heredity revolutionized genetics and led to the beginning of controlled plant breeding experiments [17].

The discovery of the structure of DNA in the 1950s marks the era of modern biotechnology. Two techniques contributed greatly to the transition, namely rDNA technology and monoclonal antibody or hybridoma technology. rDNA technology is the field of molecular biology in which DNA from two or more sources is "edited" to form new synthetic molecules. Hybridomas are the hybrid cells of myeloma cells with antibody-producing cells. rDNA technology enables

the transfer of genetic material from one species to another, and thus the production of food crops and animals with traits that are different from those obtained using traditional breeding techniques [18]. The hybridoma technology, on the other hand, enabled the generation of unlimited quantities of monospecific antibodies directed against any antigen [19]. Both technologies rapidly found industrial applications and led to the rapid development of diagnostic procedures in the fields of parasitology, virology, and cancer [20] as well as biopharmaceuticals (e.g., the production of insulin in recombinant *Escherichia coli* and *Saccharomyces cerevisiae*) [21]. The recently developed, fast, and massive sequencing technologies have been used to decipher the genomes of hundreds of organisms and viruses: human [22], cultivated tomato (*Solanum esculentum*) [23], *Agrobacterium tumefaciens* [24], nematode (*Caenorhabditis elegans*) [25], domestic cattle (*Bos taurus*) [26], *E. coli* [27], and viruses such as human immunodeficiency virus [28] among numerous others. Together with improved computational approaches, sequencing has led to ongoing studies on the function of genes and the proteins they encode. The sequencing and evolution of human and microbial genomes, the understanding of the genetic basis of many diseases including the development of antibiotic resistance, have led to the development of novel diagnostic procedures, vaccines, treatments, cures, and management of many diseases [29]. Additionally these data will invariably provide opportunities for increased understanding of the genetics and history of domestication, and accelerate crop and animal improvement [30,31]. Modern biotechnology, the third generation of biotechnology, is explicitly based on underlying scientific progress, whereas the first and second generations (ancient and classical biotechnology) were more technological applications, lacking solid understanding of the underlying scientific principles.

Although the discovery of the structure of DNA and rDNA technology is regarded as a huge driver for the recent developments in modern biotechnology, two other influences have contributed to the growth and development of the field [32]. One, the need to replace fossil resources and move to renewable raw materials, and two, the need for green or clean processes, where efficient use is made of the energy during production. All three drivers, rDNA technology, renewable raw materials, and environmental impact, are shaping biotechnology growth. The history of biotechnology is still being written and will continue to be written as new uses are realized.

28.4 BIOTECHNOLOGY IN COLOR

The global society faces increasing challenges in health care, energy, and food production. The primary health care needs of people worldwide are growing steadily. The world has therefore responded with the development of various solutions to face these challenges through the development and advances made in biotechnology, which enhances the bioactive molecules discovered in various medicinal plants [33]. There are four main topics of biotechnology being explored to date. These include industrial biotechnology also called white biotechnology and blue biotechnology, which encompass the aquatic environment, red biotechnology and green biotechnology, which involve medical and agricultural biotechnology applications, respectively. These technologies are all based on the use of biological systems and molecules contributing to the development and advancement in therapeutic solutions for humanity and the environment.

28.5 WHITE BIOTECHNOLOGY (INDUSTRIAL)

White biotechnology is the application of biotechnology for industrial purposes in which the processing and production of end products and intermediates are from renewable sources. This new approach to sustainable resource management and conservation uses less and cleaner energy, thus reducing our environmental footprints and resulting in an overall reduction in production costs. Some of these areas of application include the practices of using microorganisms and enzymes to generate industrially useful products.

Industrial biotechnology started as far back as the 1800s when Louis Pasteur showed that fermentation was caused by microbial activity. Fleming later discovered antibacterial compound in molds and this was further developed as medicine (penicillin) in the early 1900s by Florey and Chaim [34]. Since then biotechnology has revolutionized human life with various modern technologies. One such technology involves human proteins, such as insulin and growth hormones, which are being produced in microorganisms such as *E. coli* and *S. cerevisiae*. Various other therapeutic proteins produced in bacteria and yeast include a clot-dissolving protein called tissue plasminogen activator as well as interferon, which is currently being used against certain types of cancers and for certain skin conditions [35–37]. There are several pharmaceuticals on the market that are very expensive because they were discovered in rare plants in small amounts. With the recent advances in biotechnological techniques, strategies are being developed to offer alternative and viable sources of these compounds in the future. This recombination synthesis (also called combinatorial biosynthesis) has already being employed by pharmaceutical companies such as Eli Lilly in natural products, such as alkaloids like vinblastine and vincristine for cancer chemotherapy [38].

The soil bacterium *A. tumefaciens* is largely used in plant transformation. This genetic transformation technology of inserting foreign genes into plant cells using the bacterium has been very successful and very popular. *A. tumefaciens* is a plant pathogen which causes tumor development in susceptible plants called crown gall disease. The bacterium contains a plasmid called Ti (tumor-inducing) which inserts its T-DNA into the cell which causes the tumor development. Modified Ti plasmids with the virulence genes removed have been used to introduce desired genes into the plant [39]. This method of genetic engineering is being examined and could possibly be used in medicinal plants to enhance secondary metabolite content. The production of secondary metabolites through plant cell culture became a leading area of research involving different scientific disciplines, including pharmacognosy [38]. Several biotechnology techniques are being investigated to produce transgenic medicinal plants; these use various biotechnological tools to transform plants such as the alkaloid-producing plant *Hyoscyamus muticus* and the chloroplast transformation of *Arabidopsis thaliana*. In these transformed plants various desired agronomic traits are conferred or high levels of vaccine antigens and other biopharmaceuticals are expressed in the plants [40].

It is anticipated that novel clinical drugs such as antibiotics and reserpine (the first tranquillizer), which were derived from natural sources, would eventually be produced synthetically [41]. One example Artemisinin is an antimalarial drug isolated from the plant *Artemisia annua* (Asteraceae). Alternative production is being investigated via transgenic plants or using other biosynthetic pathways in less complex host cells such as *E. coli*. Thus the natural products become templates for the synthetic version for various reasons; depletion of plants in the wild or the costs for collection, extraction, and isolation. Biotechnology is one of the main tools that are currently being engaged to drive the process.

28.6 BLUE BIOTECHNOLOGY (MARINE)

Blue biotechnology is using biotechnological tools to harness biomolecular processes from marine and freshwater resources to benefit society [42]. The products from blue biotechnology have markets in a wide range of fields in medicine, cosmetics, health care, and nutraceutical food supplements. The markets have also extended into the environmental and industrial applications such as biofuels and bioremediation systems. Marine biotechnology therefore includes any area that involves marine bioresources as the source as well as the target of biotechnology applications. Blue biotechnology is unique in that it is defined by its source rather than by its market [43].

The marine environment is a rich potential source of therapeutic agents with novel mechanisms of action. Drug discovery is one of the most promising outcomes of marine biotechnology especially with the growing need for novel compounds for the treatment of human diseases. The discovery of various marine-derived bioactive pharmaceuticals have led to the development of compounds with antioxidant, anticancer, antiviral, anticoagulant, antibacterial, and anti-inflammatory activities [44]. The potential for these marine natural products as pharmaceuticals, which started in the 1950s, led to several marine-derived pharmaceuticals that were later commercialized. There are several marine organisms such as marine-derived fungi, which are important sources of novel pharmaceutically active secondary metabolites. These included the antibiotic Cephalosporine from marine fungi, anthelmintic insecticides from kainic acid found in red algae, and analgesic Zincototide from mollusks. Other important pharmaceuticals include Ara-C which is an anticancer drug (effective against acute myelocytic leukemia and non-Hodgkin's lymphoma) and Ara-A which is used as an antiviral drug for treating herpes [45]. Both of these drugs were derived from natural compounds found in marine sponges found off the coast of Florida. Globally, the marine pharmaceutical industry has accepted the world's marine environment as a major active resource for medical research [45,46]. To date, the marine pharmaceutical industry's current successes consist of three Food and Drug Administration approved drugs, 1 EU registered drug, 13 other natural products (or derivatives thereof) in different phases of the clinical trials, and a large number of other marine chemicals in the preclinical stage of development [47].

The three main groups of marine raw material include seaweeds and their constituents, chitin and chitosans from crustacean shells, and the lipids, natural colorants, and vitamins from the microalgae [48]. Alginate, a constituent of seaweeds, is increasingly being used in bead encapsulation for processes such as drug delivery. The chitin and chitosans raw materials are widely used in health care especially in the area of wound-healing; there is an increased demand for molecules such as glucosamine, an acetylated derivative of chitosan. High levels of docosahexaenoic acid and ARA, long-chain polyunsaturated fatty acids found in abundance in breast milk have also been found in marine microalgae [48].

Besides the pharmaceutical uses, other useful compounds discovered and isolated from the marine environment include enzymes, such as DNA polymerase, which was isolated from deep sea hydrothermal vents (Vent DNA polymerase). These are used in foundational biotechnological techniques such as polymerase chain reaction (PCR), which is used in many applications, e.g., gene discovery and diagnosis [49]. Other important molecules used as tools in biotechnology include the hydrocolloids such as agar and agarose extracted from the Rhodophyacean seaweeds.

Biopolymers of marine origins have many applications in the food industry often because of their ability to remain stable at extreme temperature. Polysaccharides such as algin and carrageenan from several species of brown seaweed and of red algae, respectively, have been used as thickening and stabilizing agents in dairy products and processed meats. Fish collagen (a protein) has been used as a gelatin substitute. Omega-3-fatty acids have been used in fish oil capsules and many nutraceutical preparations. Many food coloring agents have been sourced from marine algae [50]. Biodegradable bioplastics such as polyhydroxyalkanoate (PHA) are synthesized by many microorganisms, some of marine origin, and have many industrial applications because of their thermal and elastic properties [51] and are often used to replace plastic of petroleum origin because of their environmentally friendly status.

28.7 RED BIOTECHNOLOGY (MEDICAL)

Medical biotechnology has contributed over the years to combating, diagnosing, and reducing the rates of diseases and other life-threatening conditions by using the genetic makeup of organisms and harnessing their vast natural resources available. It involves technologies that use the biological processes of microorganisms to combat debilitating and rare diseases in the medical sector. To date there are over 250 health care products and vaccines for previously untreatable diseases available to patients as a result of biotechnology [52]. Mapping the human genome has assisted researchers in making genetic associations with certain genetic conditions such as familial breast cancers and other diseases. Significant advances have been made with the Human Genome project which began in 1990 in identifying and sequencing the over 20,000 genes in human DNA. Through this project new insights and understanding into the causes of diseases has led to various therapies, diagnostic tools, and drugs that are being developed as solutions, e.g., the diagnosis of diseases such as cystic fibrosis where a gene mutation has occurred and is passed down from generation to generation. Additionally, for some cancers such as breast and ovarian cancers where individuals carry specific genetic mutations, individual's DNA can be tested and then the individual can be treated [53,54].

Biotechnology plays a crucial role in the elucidation of the molecular causes of diseases such as diabetes, cancer, and rheumatic diseases. Scientists have been able to not only diagnose with new methods and detect genetic predispositions to these diseases but also approach the treatment more accurately. The biopharmaceutical field has developed novel classes of drugs that target specific areas in the body resulting in a more effective treatment of patients. New techniques in biotechnology have fast-tracked early detection and diagnosis as an integral step, which has greatly enhanced treatment. Pharmacogenomics is the arm of medical biotechnology, which designs the most effective drug therapy and treatment strategy based on the specific genetic profile of a patient. Individuals have been known to react to the same drugs differently; it is therefore hoped that research will improve the efficacy of drugs by designing personalized drugs, which would be safer and more effective [52]. One of these personalized and tailored treatments designed and being used is the breast cancer drug Herceptin which is specific for patients who test positive for the human epithelium growth-factor receptor 2 gene [55].

Biotechnology has revolutionized the production of medically important biomolecules especially in the production of therapeutic proteins, which could be enzymes, chemical messengers, as well as monoclonal antibodies. New technologies that enabled the insertion of human genes into microorganisms, such as bacteria and yeast, opened the doors to large-scale production of medicine. One of the first drugs that was produced using these technologies was insulin. The human insulin gene was inserted into a plasmid (small circular DNA) and placed into bacterial cells. The bacterial cells multiplied and produced insulin in large quantities. The insulin was isolated and processed into its active form [56]. The resulting genetically engineered bacterium enabled much cheaper production of large quantities of human insulin when compared to the extraction of insulin from animals. Before rDNA technology insulin production was at a ratio of 2 tons of pigs pancreas required for 8 ounces of purified insulin. However, over time people were developing immune reaction to insulin from animals, thus making the drug less effective and unsustainable [48].

Biotechnological advances also feature prominently in both the diagnosis and the treatment of HIV/AIDS. The antibodies produced by the body in an HIV infection are used as biomarkers in the diagnosis of an infection and treatment. Various new techniques are revolutionizing the approach to detection and treatment of HIV. The newest technique in gene therapy is in the use of transcription activator-like effectors (TALEs), which are proteins secreted by the *Xanthamonas* spp. TALEs are being used to modulate gene expression in the hosts by binding to specific binding sites on a DNA molecule and modifying a specific target genome sequence, in this case the HIV provirus [57–59]. The successful increased production of vaccine is the result of the applications of rDNA technology. The hepatitis B vaccine that is currently being sold on the market was manufactured in recombinant yeast cells that had a vector DNA carrying viral genes. The vaccine is safe since it contains no complete viral particles [60]. Similarly, experimental vaccines against AIDS are being explored.

28.8 GREEN BIOTECHNOLOGY (AGRICULTURAL)

Green biotechnology is commonly considered as the next phase of the green revolution with the aim to challenge hunger. It uses technologies for the production of more fertile and resistant plants, ensuring application of environmentally friendly fertilizers, biopesticides, and soil ameliorants. Major technologies applied include various tissue culture techniques. These include methods for obtaining secondary metabolites (biofarming) and for conservation and multiplication of elite varieties (micropropagation, somatic embryogenesis, cryopreservation). Green technology also includes the application of genetic engineering for selection of crop plants and farm animals with designed traits. An important tool is the use of marker-assisted breeding, which includes the development of molecular markers or DNA fingerprinting to tag, map, and identify thousands of genes encoding desired proteins. Plant breeders can then screen this library of identified genes for the trait of interest. There is also the concept of reverse breeding and doubled haploids, a method for efficiently producing homozygous plants from a heterozygous starting plant that has all the desirable traits [61].

A transgenic technology of potential value to pharmacognosy is in the use of transgenic hairy root or shoot teratomas culture to overproduce secondary metabolites. Hairy roots have been induced by the Ri plasmid of *Agrobacterium rhizogenes* for over 63 plant species. Hairy roots grow rapidly and are genetically stable in vitro and thus can produce more secondary metabolites than the corresponding untransformed plant roots [62]; elicitors can further increase production [63]. For metabolites normally produced in leaves, a similar result is obtained from shoot teratomas induced using the Ti plasmid of *A. tumefaciens* [62]. Another method manipulates secondary pathways by transferring specifically modified genes to modify which secondary metabolites are produced [64].

The understanding of biosynthetic pathways brings with it the possibility to use genetic engineering to increase phytochemical yields. For example, *Atropa belladonna* is naturally rich in hyoscyamine (a drug that blocks the action of acetylcholine in the muscle and nervous system). However, there is a 10-fold greater demand for scopolamine (a drug fighting motion sickness) than for hyoscyamine. Therefore *A. belladonna* was transformed using *Agrobacterium* to include a gene from *Hyoscymus niger* that encodes an enzyme which catalyzes the reaction leading from hyoscyamine to scopolamine. While *A. belladonna* was naturally rich in hyoscyamine, the alkaloid content in the leaves and stem in the transgenic *A. belladonna*, were almost exclusively scopolamine [65].

Micropropagation is a method of choice for rapid multiplication of elite varieties including medicinal plants. Micropropagation is a biotechnology technique that can produce multiple copies of a single plant. To maintain genetic integrity, a part of the plant (explant) with a preformed meristem is used such as a shoot apex, axillary bud, or shoot meristem tip. The smaller the explant, the longer the time taken for the plant to become established in culture but the greater the chance the plant will be free from viruses. Serology tests are used to ensure the plantlets grown from meristem tips are indeed free from viruses. Micropropagation of such plants in the laboratory can ensure that each new crop is planted with virus-free materials, thus increasing yields [66]. In the Caribbean, many crops are produced this way including yam, sweet potato, cassava, banana, plantain, pineapple, ginger, turmeric, sarsaparilla, and other medicinal and economically important plants [67–69]. These cloned disease-free plants are ideal for pharmacognosy uses, whether directly from in vitro material or after growth by hydroponics or in the soil, preferably using organic methods. Somatic embryogenesis, another in vitro technique, is often used for slow-growing herbs such as ginseng and trees such as *Eucalyptus*, as a method for obtaining and maintaining elite cultivars and as part of breeding programs [69,70].

Parent plants of crops used for the production of hybrid seeds are also multiplied via micropropagation. Somaclonal variations may modify the phenotype of the plants; such plants need to be discarded or may be used to develop new traits.

Micropropagated plants can also be used as an important source of phytochemicals [13,70,71]. Biofarming is the production of phytochemicals in vitro. Elicitors are used to increase yield such as vincristine and vinblastine from *Catharanthus roseus* flowers [72]. Plants are not genetically modified. Biopharming is the term used when plants are genetically modified to produce valuable products such as plant-made pharmaceuticals or plant-made biologics (PMBs) such a vaccines and human proteins [73].

While transgenic technology have been used to alter biosynthetic pathways of target metabolites, and tissue culture can improve the quality of a herbal extract by ensuring clonal production, genetic integrity, and extract consistency while removing toxic components and contaminants, there are still issues to be overcome in using in vitro plant material for herbal and drug extracts for pharmacognosy. These include the difficulty in predicting which extracts will become or remain marketable and therefore worthy of developmental efforts and whether the market will still prefer naturally sourced extracts [74].

28.9 GENETIC ENGINEERING TECHNIQUES

Genetic engineering involves the manipulation or alteration of an organism's genes using biotechnology. rDNA technology is a major arm of genetic engineering which has been applied to the manufacturing of pharmaceuticals, particularly

therapeutic proteins such as insulin [21,56], human serum albumin, human papillomavirus vaccine, and hepatitis B vaccine [37,60]. rDNA technology essentially involves isolating a gene of interest, inserting the gene into a cloning vector, and allowing the gene product to be expressed within an appropriate host. Several foundational tools and protocols are integral to the field; they include nucleic acid isolation, gel electrophoresis, DNA hybridization, and DNA sequencing. These techniques focus on obtaining and confirming the identity of the desired gene. Other techniques such as PCR and DNA cloning aim to amplify genes or express proteins encoded by the desired gene. This section will explain in further detail the principles governing these methods and how they are performed.

28.10 NUCLEIC ACID ISOLATION

DNA is found within the nucleus while RNA is found either in the nucleus or cytoplasm. The aim of nucleic acids isolation is to remove all the organic components within the cell leaving behind the nucleic acid. The first step involves breaking the cell wall; this is usually achieved by mechanical methods such as grinding or bead beating. Enzymes such as lysozymes are also capable of hydrolyzing the glycosidic bonds present in the peptidoglycan cell wall of the bacteria and lysing the cell.

The phospholipid bilayer of the cell membrane can be disrupted by the addition of a detergent such as sodium dodecyl sulfate (SDS). Cell lysis results in the release of all organic components within the cell into solution. Such components include nucleic acids, proteins, carbohydrates, lipids: the most abundant of these being proteins [75].

A mixture of phenol and chloroform (1:1 ratio) may be used to separate proteins from nucleic acids. When the mixture is vortexed and centrifuged, the denser organic layer containing lipids settles at the bottom of the tube while aqueous phase containing nucleic acids remains at the top. Proteins may be found at the interface of the aqueous and organic phase (Fig. 28.1) [75,76].

Alternatively, proteases may be used to degrade proteins. The precipitation of the protein−SDS complex may be aided by salts such as ammonium or potassium acetate. The mixture is usually centrifuged to allow the protein complex to settle to the bottom of the tube. If DNA is being isolated RNAse is added to degrade any RNA present: alternatively DNAse is added when isolating RNA. Nucleic acid may be precipitated using ethanol (2 volumes) or isopropanol (0.5 volumes) and centrifuged for the retrieval of the nucleic acid pellet. [75].

28.11 GEL ELECTROPHORESIS

Gel electrophoresis is a method used to visualize and separate nucleic acids of different sizes. DNA separation is achieved by the application of an electric field. DNA, being negatively charged, will move from the cathode (−) to the anode (+) when voltage is applied. Separation occurs within different types of gels. These gels contain pores allowing DNA molecules to pass through depending on the size of the fragment. Larger fragments will encounter greater obstruction from the gel matrix and therefore tend to move the least distance along the gel. Smaller fragments are able to maneuver through gel pores more easily and therefore tend to move the furthest. Once electrophoresis is complete, the gel is stained with an intercalating dye such as ethidium bromide. Ethidium bromide binds to the bases of DNA and fluorescence under UV light to allow for viewing (Fig. 28.2). The relative size of the fragments produced on the gel is determined by comparing their position to that of a molecular weight marker [75,77].

Two types of gel are commonly used, agarose gels and polyacrylamide gels. Agarose is a carbohydrate extracted from seaweed and may be used to prepare gel containing 0.7−3% agarose (Fig. 28.3). Agarose is generally used to separate DNA fragments from 300 to 10,000 bp (base pairs). The higher concentrations of agarose produce a denser matrix

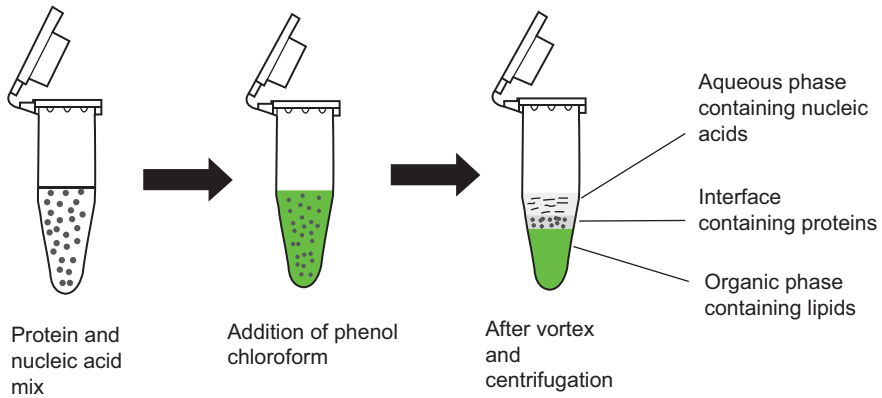

FIGURE 28.1 Separation of nucleic acids from organic cell components using phenol and chloroform.

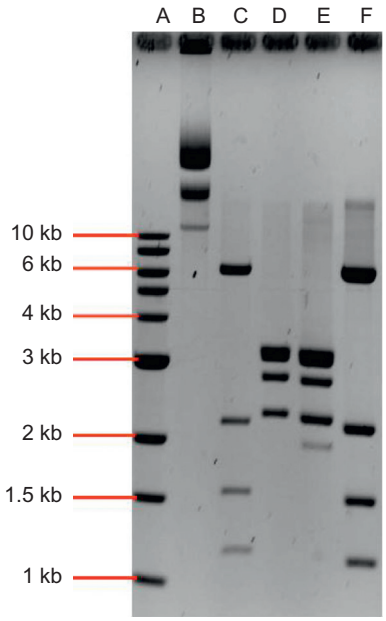

FIGURE 28.2 Agarose gel electrophoresis viewed under UV light. Lane A contains 1-kb molecular marker while lanes B–F contain DNA samples of varying fragment sizes. The size of the fragments is determined by comparing their position on the gel, relative to that of the molecular marker.

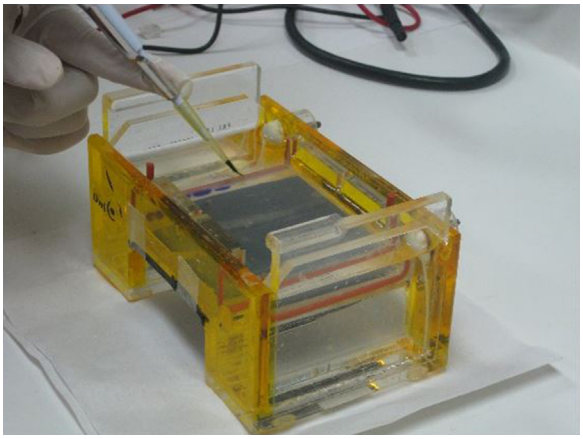

FIGURE 28.3 Loading DNA into the wells of an agarose gel.

and are therefore used to separate smaller fragments. Polyacryamide gels contain much smaller pores than agarose and are used to separate very small DNA fragments ranging from 10 to 500 bp. Fragments that differ by a single base pair may be separated using this gel matrix. Polyacryamide gels were also used in DNA sequencing [75,77].

Large fragments between 30 and 50 kb are separated using a form of gel electrophoresis called pulse-field electrophoresis. The principle of separation is the same; however the voltage is not applied across the gel. Instead an electric field is applied toward the center and at two 60 degree angles. The direction of the electric field is changed at equal time intervals. Time intervals can range from 0.1 to 1000 s depending on the size of the DNA fragment. Each time the direction of the electric field is changed the DNA fragment has to reorient itself. Overall the movement results in a net forward migration of the fragments [75,77]. Pulse-field electrophoresis is often used to separate complete genomes.

28.12 SOUTHERN BLOTTING

DNA fragments resolved on the electrophoresis gel may be transferred to a nitrocellulose or nylon membrane for further downstream manipulation, such as DNA hybridization. The membrane is placed above the gel; the two are layered with

filter paper and seated in a buffer solution. The DNA on the gel is transferred to the membrane by capillary action [75,78,79]. This process is referred to as Southern blotting; other similar procedures include northern blotting, which is the transfer of RNA, and western blotting, which is the transfer of proteins from a polyacrylamide gel.

28.13 DNA HYBRIDIZATION

DNA hybridization is the process of joining two single strands of DNA thus forming dsDNA. Joining of the two strands is facilitated by the formation of hydrogen bonds between the nucleotide bases. DNA hybridization may be used to assess the genetic similarity between two populations of DNA or to detect a specific sequence of DNA in a population of DNA molecules. This is achieved by using a nucleic acid probe. A probe is a piece of single-stranded DNA (ssDNA) or RNA with a marker attached for detection after hybridization. Probes may be labeled with radioactive atoms (such as ^{32}P, ^{35}S, and ^{3}H) or nonradioactive compounds (such as biotin-11-dUTP, dioxigenin-dUTP) or fluorescence molecules (such as Cy3 or Cy5).

During DNA hybridization the hydrogen bonds of the target DNA are disrupted by incubating at 95°C or exposure to an alkaline pH. This leads to the formation of ssDNA. The target ssDNA is incubated with the probe at approximately 65°C or below for a prolonged period. If the target DNA sequence is complementary to the probe sequence then hybridization will occur by the formation of hydrogen bonds. The greater the complementarity, the greater the stability of the bonds formed between the target and the probe. Radioactive probes may be detected by autoradiography—an X-ray image indicating the pattern of decay produced by beta or gamma particles. The detection of nonradioactive probes such as biotin is based on their reaction with a specific antibody. The chosen antibody is paired with a chromogenic substance or chemiluminescent substrate. Common chromogenic substances such as NBT (nitro-blue tetrazolium chloride) or BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) yield an intense purple spot on the membrane in the presence of the target DNA (Fig. 28.4). Chemiluminescent substances emit light, which produces etching on photographic film.

28.14 DNA SEQUENCING

DNA sequencing is a method used to determine the order of nucleotides (adenine, guanine, cytosine, and thymine) in a strand of DNA. Several methods have been developed since the 1970s. The chemical method was developed by Alan Maxam and Walter Gilbert and the dideoxy method by Fred Sanger [80,81]. The Sanger method was the most commonly used method in molecular biology until the advent of mass-sequencing technologies, also called next-generation DNA sequencing methods [82]. The latter include pyrosequencing, Illumina, and SOLiD platforms. These technologies allowed generating millions of reads of 50−1000 nucleotides long in a single run, which are assembled in large DNA molecule thanks to powerful bioinformatic programs. The costs involved are constantly decreasing; today a given eukaryotic genome can be entirely sequenced for less than US$1000. The comparison of the genomes of many individuals can help identify genes and loci involved in disease, resistance to biotic and abiotic stresses, yield, and phylogeny [83].

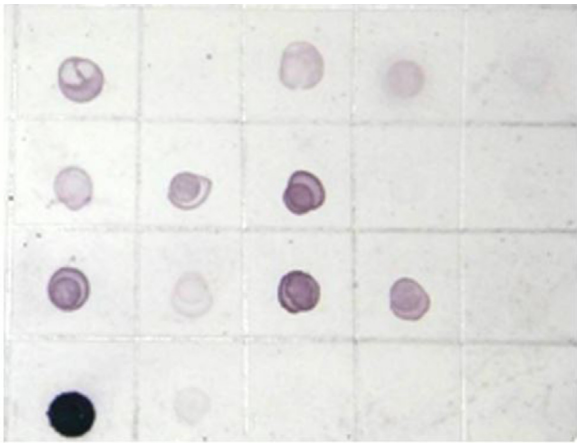

FIGURE 28.4 Hybridization membrane produced when using dioxigenin-labeled probe. Purple spot indicates positive hybridization of the probe to the target DNA.

28.15 POLYMERASE CHAIN REACTION

PCR is a technique used to amplify a segment of DNA, producing millions of copies. The principles of in vitro DNA amplification are based on the process of in vivo DNA replication. PCR, as with cellular DNA replication, relies on four main components: template DNA, primers, DNA polymerase, and nucleotides [84].

Template DNA describes the DNA, which is to be amplified. The DNA double helix is unwound to produce single strands of DNA with exposed bases. Each of the single strands of DNA will serve as a template for the construction of a new double helix. DNA copies produced will therefore be composed of one parent strand and one newly synthesized strand. This form of DNA replication is referred to as semiconservative [84,85].

A primer is a small piece of RNA or DNA of 15−30 bp. Primers are capable of binding to a specific region of ssDNA complementary to its sequence and serve as a starting point from which DNA polymerase constructs a new strand. Primers therefore target specific regions in the genome, which need to be amplified and may therefore be artificially synthesized for this purpose. Two primers are required for the amplification of both the 5′−3′ strand and the 3′−5′ strand on the template DNA.

DNA polymerase is an enzyme responsible for constructing a new DNA strand by adding deoxynucleotide triphosphates (dATP, dCTP, dGTP, dTTP) complementary to those found on the template strand. DNA polymerase will begin this process of extension from the site at which the primer anneals onto the template. The DNA polymerase used in PCR is heat stable *Taq* polymerase. This enzyme is obtained from a strain of bacteria called *Thermus aquaticus*, which is found in hot springs. This heat stable polymerase is able to withstand the high temperatures involved the PCR. *Taq* DNA polymerase requires Mg^{2+} and a buffer [76,83,86].

Appropriate volumes of each component are dispensed in a tube and placed in a thermal cycler. A thermal cycler alternates between cycles of heating and cooling which are necessary for amplification to occur. PCR involves three steps: template denaturation, primer annealing, and template extension. During template denaturation the double-stranded DNA (dsDNA) template is denatured or made single-stranded by heating at 95°C. After which the reaction is cooled to between 40°C and 60°C where the primers will anneal to complementary sequences on the template DNA. This is followed by template extension; the temperature is raised to 72−74°C and dNTPs are added to the 3′ end of the primer using the base sequence of the template. As outlined in Fig. 28.5, at the end of each cycle the amount of DNA would increase twofold. This temperature cycling is repeated many times; after 30 cycles the target DNA is increased a million-fold [84,85]. Due to the specific nature of primer annealing, PCR is used to identify bacteria and viruses (Fig. 28.6), diagnose diseases, and link criminals to a crime by identifying genetic fingerprints.

28.16 DNA CLONING AND RESTRICTION DIGESTION

DNA cloning is a technique used to make several copies of a piece of DNA within a cell. DNA cloning may be applied to the isolation and characterization of a specific gene or it may be used to yield useful proteins such as insulin. The overall process involves placing genes of interest into a host cell whose machinery is utilized for the replication of these genes or the synthesis of proteins encoded by the genes.

DNA cloning commonly requires placing the gene of interest within a vector. The most common vector used is a plasmid, which is circular, dsDNA, extra chromosomal DNA found in prokaryotes. For insertion to occur both the plasmid and genes of interest must be excised with the same (or compatible) restriction endonuclease. Restriction endonucleases are DNA cutting enzymes found naturally within bacterial cells; they protect bacterial cells from invading bacteriophages by binding to specific sequences or recognition sites within the viral DNA and cutting the phosphodiester backbone of dsDNA. Recognition sites are palindromic and result in the formation of sticky or blunt end fragments. Table 28.1 outlines examples of restriction enzymes and their recognition sites. The properties of these enzymes have been utilized to achieve DNA cloning.

Once the plasmid and gene of interest have been digested by the restriction endonuclease, they are capable of being joined together to form rDNA. This is achieved with enzyme DNA ligase. The rDNA is placed into a host cell, such as *E. coli*, in a process called transformation. Bacterial cells must first be made competent before transformation can occur. Competency describes the cell's ability to allow DNA to enter through its membrane. One common method for achieving competency is by exposing bacterial cells to cold calcium chloride followed by a brief heat shock at about 42°C. Another method is electroporation: cells are exposed to an electric field, which temporarily creates holes within the cell's lipid bilayer membrane.

Once bacterial cells are transformed with the recombinant plasmid, they are placed on nutrient agar containing an appropriate antibiotic. Bacterial cells that have been successfully transformed are expected to contain genes responsible for antibiotic resistance conferred by the recombinant plasmid and are thus able to replicate on the media [76,79]. Replication of bacteria may result in the production of millions of copies of rDNA, which may be isolated for further analysis (Fig. 28.7).

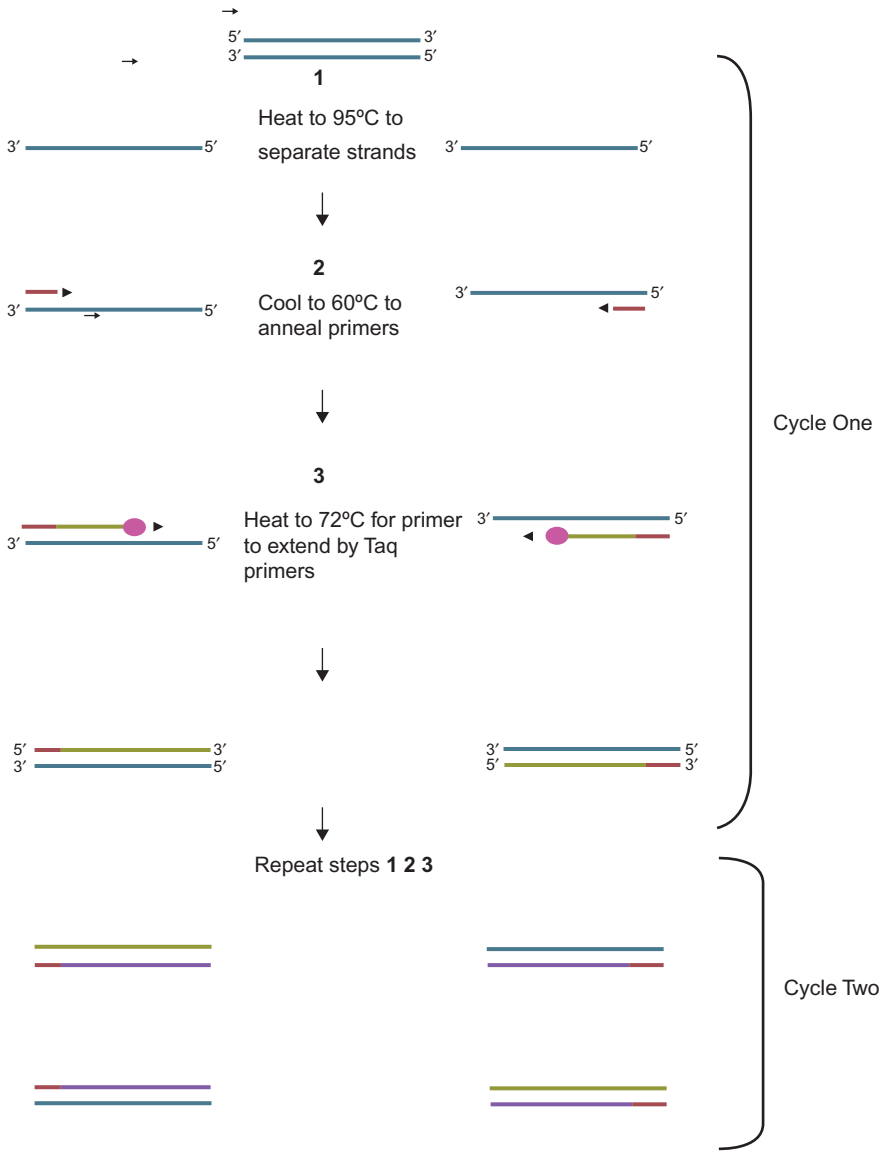

FIGURE 28.5 Amplification of DNA using PCR. Blue strands represent the original dsDNA which serves as a template for the construction of a new strand. At the end of the first cycle, two dsDNA molecules are produced; the newly synthesized strands are shown in green. The process is repeated in the second cycle, which yields four dsDNA molecules with the newly synthesized strands shown in purple.

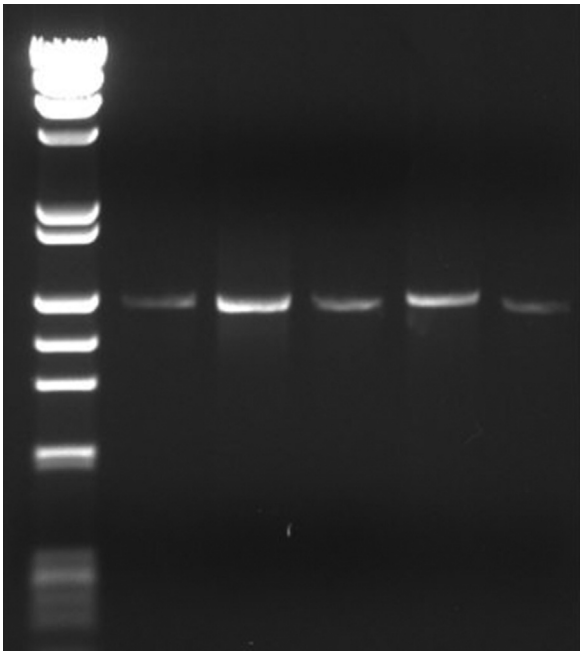

FIGURE 28.6 Polymerase chain reaction amplification of the reverse transcriptase and protease gene of HIV.

TABLE 28.1 Frequently Used Restriction Enzymes, the Microorganism They Are Obtained From, and the Recognition Sites at Which They Cut[a]

Microorganism	Restriction Enzyme	Recognition Site
Bacillus amyloliquefaciens	*Bam*HI	5′-GGATCC-3′
Escherichia coli	*Eco*RI	5′-GAATTC-3′
Haemophilus infleunzae	*Hind*III	5′-AAGCTT-3′
Providencia stuartii	*Pst*I	5′-CTGCAG-3′

[a]*Watson JD, Baker TA, Bell SP, Gann A, Levine M, Losick R. Molecular biology of the gene, 6th ed. New York: Cold Spring Harbor Laboratory Press; 2008.*

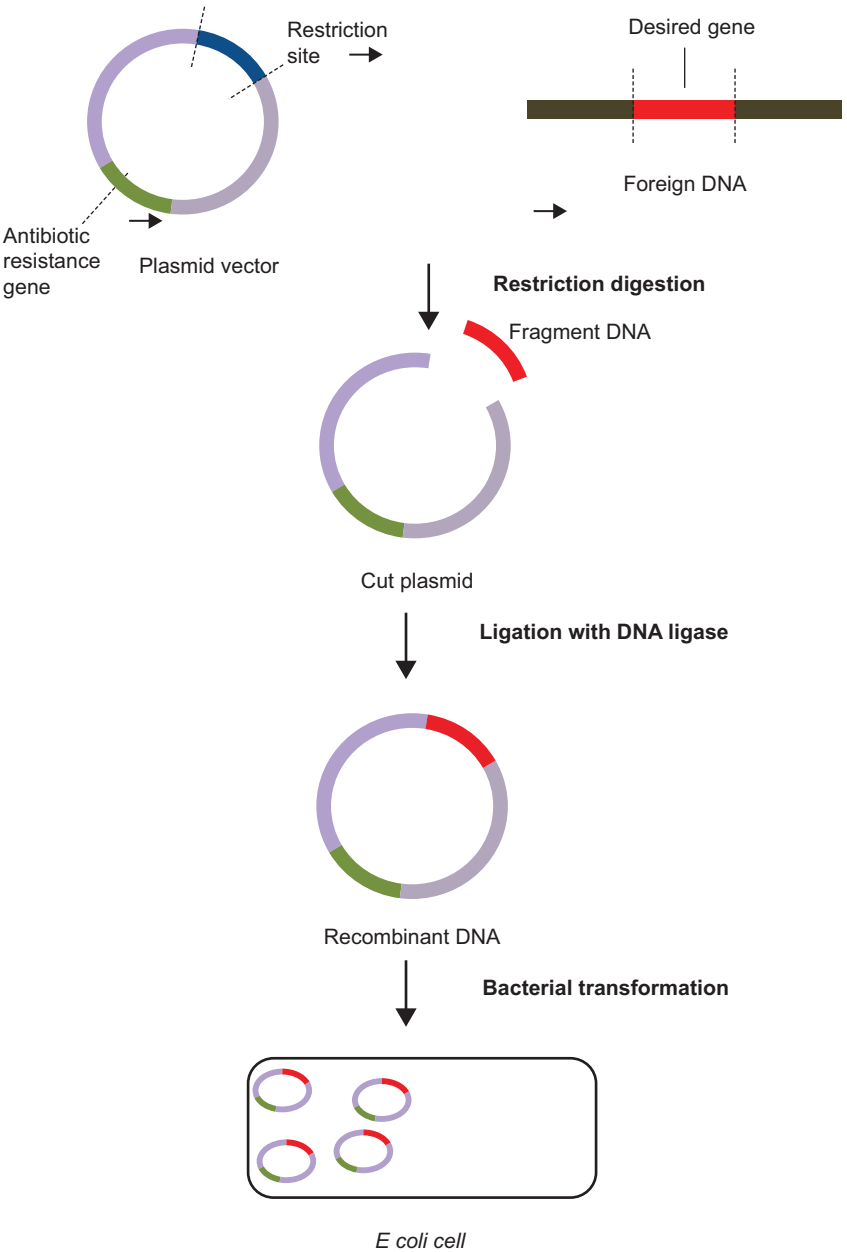

FIGURE 28.7 The steps in DNA cloning. The plasmid vector and desired DNA fragment are digested with the same restriction enzyme. The desired DNA is ligated into the plasmid by DNA ligase to form recombinant DNA. Bacterial cells are transformed by recombinant DNA. Several copies of the recombinant are produced during growth and multiplication of bacterial cells.

28.17 REVIEW QUESTIONS

1. Define biotechnology? Has your perception of biotechnology been changed by this definition.
2. What are some of the products of:
   a. Ancient biotechnology.
   b. Modern biotechnology.
3. Discuss the color code used to differentiate between the different areas of biotechnology.
4. Discuss some of the applications of biotechnology in medicine and agriculture.
5. Natural fresh water bodies and the ocean are large untapped sources of biotechnology products. Discuss using specific examples.
6. What do you understand by the term "recombinant DNA technology"? How is recombinant DNA technology different from hybridoma technology?
7. Differentiate between:
   a. Polymerase chain reaction (PCR) and DNA cloning.
   b. DNA hybridization and DNA sequencing.

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Chapter 29

Natural Product Structure Elucidation by NMR Spectroscopy

W.F. Reynolds
University of Toronto, Toronto, ON, Canada

Chapter Outline

Learning Objectives

- To provide a basic understanding of NMR spectroscopy as used in pharmacognosy research.
- To illustrate how different NMR methods can be used in combination to determine the structures of natural products.
- To provide practical advice on how to obtain the best quality NMR spectra and how to avoid making errors in structure elucidation.

29.1 INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy provides by far the highest resolution of any of the widely used spectroscopic methods. NMR spectra can often provide distinct peaks for each chemically different hydrogen or carbon atom in a molecule, thus providing a wealth of information for structure elucidation. In the past, the use of NMR spectroscopy in pharmacognosy has been hindered by its intrinsically low sensitivity. However, there have been dramatic improvements in sensitivity in recent years, due to higher field magnets and vastly improved probes and electronics. The first NMR spectrometer that I used as a graduate student (in 1960) had a signal/noise of ca. 10:1 for a 1.0% ethylbenzene sample. With the latest high-field NMR spectrometers, signal/noise is approaching 10,000:1 on 0.1% ethylbenzene. This, along with the development of two-dimensional (2D) NMR, has turned NMR spectroscopy into a powerful method for structure elucidation of natural products. These developments, along with parallel developments in X-ray crystallography, have revolutionized natural product research, in terms of both amount of sample required and time

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needed to determine an unknown structure. An example is provided by strychnine. Many leading synthetic organic chemists worked on the structure of strychnine in the first half of the 20th century, carrying out numerous synthetic and degradation reactions, until the structure was unambiguously confirmed in 1954. Now, it would be possible to determine its structure with as little as 1 mg of sample and 24 h of spectrometer time on a state-of-the-art NMR spectrometer, followed by data analysis with a computer-aided structure elucidation program (CASE).

The purpose of this chapter is to introduce nonexpert users to the use of NMR in pharmacognosy. Most organic chemists who use NMR only want to find whether their reaction went as anticipated and thus treat NMR as a "black box." However, to use NMR spectroscopy effectively in pharmacognosy research requires a better understanding of NMR. Unfortunately, space limitations force a relatively superficial treatment of this subject. However, there are a number of sources to which the reader can turn for more in-depth coverage. These include books on the application of NMR in organic chemistry [1,2], and similar books covering all major areas of spectroscopy [3,4]. In addition, I have coauthored two recent reviews on the use of NMR specifically in the natural product field [5,6].

29.2 BASICS OF NMR SPECTROSCOPY

29.2.1 Spin Angular Momentum and Magnetic Moments

Many nuclei possess spin angular momentum, P, and an associated spin quantum number I. The magnitude of the spin angular momentum vector is given by:

$$P = h(I(I+1)/2\pi) \tag{29.1}$$

where h = Planck's constant. If a nucleus is placed in an external magnetic field, B_0, only certain values of P along the field axis (defined as the z axis) can be observed:

$$P_z = hm_I/2\pi \tag{29.2}$$

where $m_I = I, I-1, \ldots -I$, i.e., a total of $2I+1$ possible values. Thus, I is quantized. Spin quantum numbers can either be zero, half-integral (1/2, 3/2, etc.) or integral (1, 2, 3, etc.). Which group that a particular nucleus is in can be predicted from its atomic and mass numbers. Nuclei with even atomic and mass numbers have $I = 0$. These nuclei give no NMR signals. Two important nuclei in this class are $^{12}C_6$ and $^{16}O_8$. Nuclei with odd atomic and mass numbers have half-integral quantum numbers while those with odd atomic numbers and even mass numbers have integral quantum numbers. The nuclei which are most important for high resolution NMR are those with $I = ½$ since these give sharp NMR signals while those with $I = 1$ or greater give broader (and sometimes very broad) signals (see Section 29.3.3). Table 29.1 lists the nuclei with $I = 1/2$ which are particularly important in pharmacognosy, along with their natural abundances and sensitivities relative to ^{1}H. ^{14}N, with almost 100% abundance would seem to be a better choice than ^{15}N for this type of research, but ^{14}N has $I = 1$ and gives very broad lines.

Along with angular momentum, a nucleus has a magnetic moment, $\boldsymbol{\mu}$, which is proportional to P and either parallel or antiparallel to P:

$$\boldsymbol{\mu} = \gamma P \tag{29.3}$$

TABLE 29.1 Importance NMR Nuclei for Organic Chemistry

Nucleus	% Abundance	NMR Frequency[a]	Sensitivity[b]	
^{1}H	99.9	100.00	1.000	
^{13}C	1.108	25.14	1.76×10^{-4}	(1.59×10^{-2})
^{15}N	0.37	10.14	3.805×10^{-6}	(1.04×10^{-3})
^{19}F	100.00	94.09	0.834	
^{29}Si	4.70	19.87	3.69×10^{-4}	
^{31}P	100.00	40.48	6.65×10^{-2}	

[a] *All frequencies are relative to hydrogen at 100 MHz*
[b] *All sensitivities are relative to hydrogen as 1.0000 The values in brackets refer to nuclei enriched to 100% abundance rather than natural abundance.*

The sign of γ, the magnetogyric ratio, is positive when the two vectors are parallel and negative when they are antiparallel. Although an oversimplification, the magnetic moment can be thought of as arising from the circulation of electric charge in the spinning, positively charged, nucleus. $\boldsymbol{\mu}$ is also quantized with observable values in a magnetic field, B_0, given by

$$\mu_z = \gamma h m_I / 2\pi \tag{29.4}$$

29.2.2 Magnetic Moments and Their Interactions With Magnetic Fields

There is an energy of interaction between a magnetic moment and a magnetic field given by:

$$E = -\mu\ B_0 = -\mu_z B_0 = -\gamma h m_I B_0 / 2\pi \tag{29.5}$$

Thus there are two energy levels for a spin ½ nucleus in a magnetic field for $M_I = +1/2$ and $-1/2$:

$$E_{+1/2} = -\gamma h B_0 / 4\pi$$

and

$$E_{-1/2} = +\gamma h B_0 / 4\pi \tag{29.6}$$

Thus the energy difference between the two levels is:

$$\Delta E = \gamma h B_0 / 2\pi \tag{29.7}$$

Since there is an energy difference between the $+1/2$ and $-1/2$ spin states in a magnetic field, with the $+1/2$ state being lower in energy for both ^{1}H and ^{13}C, the $+1/2$ spin state will have a higher population than the $-1/2$ spin state. However, with the magnitudes of currently available magnetic fields, this population difference is surprisingly small. For example, in a 500-MHz spectrometer, there are only about 80 more protons out of one million in $+1/2$ level than in $-1/2$ level. Since the signal intensity in an NMR experiment is determined by this population difference (see Section 29.2.3), this accounts for the intrinsically low sensitivity of NMR.

29.2.3 Two Simple Pictures of the NMR Experiment

Right from the first observation of NMR signals in 1945, two different descriptions of the NMR experiment were proposed, one based on quantum mechanics and the other on classical mechanics. A simplified quantum mechanics picture is given in Fig. 29.1.

As shown in Fig. 29.1 and indicated by Eq. (29.7), the energy difference between the two spin states increases linearly with magnetic field strength. According to Planck's relationship, the frequency, ν, corresponding to this energy separation is given by $\Delta E = h\nu$. Thus

$$\nu = \Delta E / h = \gamma h B_0 / 2\pi h = \gamma B_0 / 2\pi \mathrm{s}^{-1} \tag{29.8}$$

According to the simplified quantum mechanics model, irradiation with radiofrequency energy with the frequency defined by Eq. (29.8) will induce transitions between the two spin states, since there is a slight excess population in the

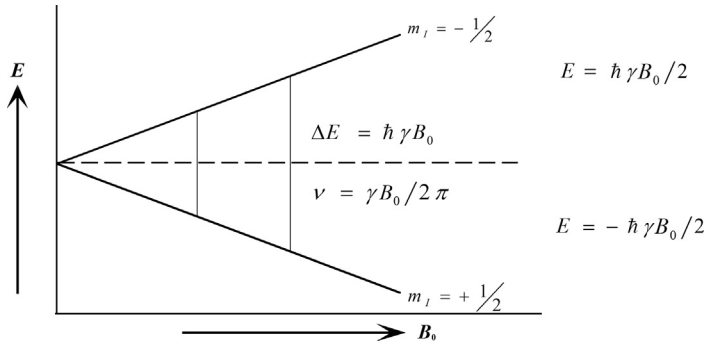


FIGURE 29.1 Energy levels for an $I = 1/2$ nucleus in a magnetic field B_0 and the frequencies for an NMR transition at a given value of B_0.

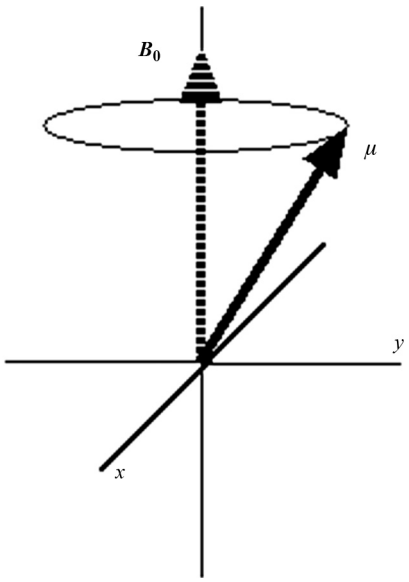


FIGURE 29.2 Precession of a magnetic moment, **u**, about a magnetic field, B_0.

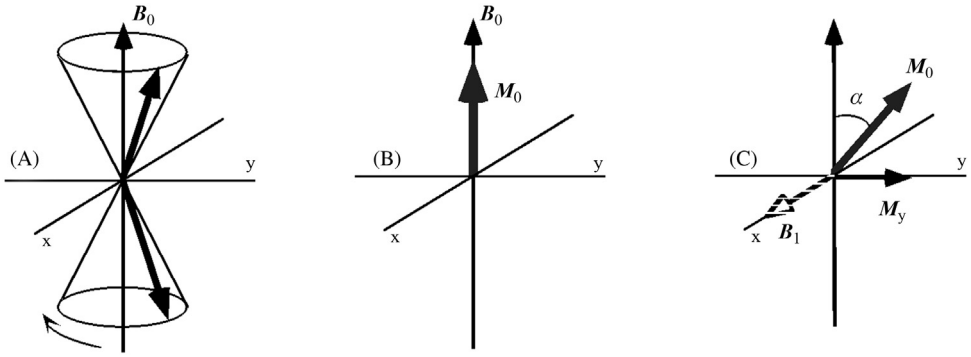


FIGURE 29.3 (A) Precession of individual magnetic moments for an $I = ½$ nucleus about B_0. (B) The resultant magnetization, $\boldsymbol{M}$, due to the sum of the individual magnetic moments. This arises from the small excess population of nuclei with $M_I = +1/2$. (C) The precession of $\boldsymbol{M}$ about the radiofrequency field, B_1, in the rotating frame coordinate system, i.e., with the coordinate system rotating at the same frequency as B_0.

lower energy state, this corresponds to energy absorption which can be detected by the receiver of an NMR spectrometer.

The classical model of the NMR experiment appears entirely different but, in the end, makes the same prediction. It also gives more useful insights into the nature of the NMR experiment, particularly when carried out as a pulsed Fourier transform (FT) experiment (see Section 29.4). According to classical physics, the interaction of a magnetic moment, $\boldsymbol{\mu}$, with a magnetic field, B_0, results a torque, L, which causes $\boldsymbol{\mu}$ to precess or rotate around B_0 (see Fig. 29.2), with an angular velocity, ω, given by:

$$\omega = \gamma B_0 \text{ rad } s^{-1} \tag{29.9}$$

This corresponds to a frequency, ν:

$$\nu = \gamma B_0 / 2\pi \text{ } s^{-1} \tag{29.10}$$

This is identical to the frequency predicted by the quantum mechanical model (see Eq. 29.8).

When, there are a large number of magnetic moments, $\boldsymbol{\mu}$, they will be rotating randomly about x, y with a slightly larger number pointing in the direction of B_0. The result will be a net magnetic moment, M_z, pointing along the $+z$ axis (see Fig. 29.3).

Now consider a second magnetic field, B_1, intermediate in strength between the other two and rotating in the x, y plane (see Fig. 29.3). When this frequency of rotation matches the frequency given by Eq. (29.9), the magnetic moment rotates around B_1, corresponding to a net absorption of energy which can be detected in a receiver.

29.3 BASIC NMR PARAMETERS

29.3.1 Chemical Shifts

If different protons in a molecule all gave NMR signals at the same frequency, there would no useful chemical information in an H NMR spectrum. Fortunately, this is not true. The external magnetic field induces circulation of electrons within a molecule, setting up induced magnetic fields which are different at each chemically distinct nucleus in the molecule. The induced field at a nucleus is proportional to the external magnetic field but opposite in sign:

$$B_{\text{induced}} = -\sigma B_0 \tag{29.11}$$

where σ is called the screening constant for that nucleus. Thus each nucleus "sees" a local magnetic field which is the sum of the external and induced fields:

$$B_{\text{local}} = B_0 + B_{\text{induced}} = B_0 - \sigma B_0 = B_0(1-\sigma) \tag{29.12}$$

Consider two chemically distinct nuclei with screening constants σ_A and σ_B, where $\sigma_B > \sigma_A$. In the early days of NMR spectroscopy, spectra were recorded by varying the magnetic field at constant frequency. Since the induced filed is then greater at nucleus B than at nucleus A, the local field will be smaller at nucleus B. Thus, a higher applied field, B_0, will be needed to produce a signal for B than for A (see Fig. 29.4). Conventionally, spectra were recorded with higher magnetic field on the right. However, for about the last 50 years, NMR spectra have been obtained by varying the frequency at constant magnetic field. For spectra obtained in this way, $\nu_A > \nu_B$ (see Fig. 29.4). To keep the same spectral appearance, spectra are now plotted with the lowest frequency on the right. Historically, one referred to high field and low field chemical shifts. While this terminology is still commonly used, it is more accurate to instead refer to low frequency and high frequency chemical shifts.

As can be seen from Eq. (29.12) and Fig. 29.4, the frequency difference for a pair of nuclei in a spectrum obtained at constant external magnetic field depends upon the difference in induced fields, with this difference being directly proportional to the eternal magnetic field strength, B_0. To facilitate comparisons of spectra obtained on different spectrometers with different magnetic fields and frequencies, the chemical shift difference is usually reported as a dimensionless ratio of frequency differences over spectrometer frequency, i.e., $\Delta\nu/\nu$. These values are typically in the range of ca. 10 parts per million (ppm) for ^{1}H and over 200 ppm for ^{13}C. By convention, both ^{1}H and ^{13}C chemical shifts are reported relative to the signal for tetramethylsilane (TMS) as an internal reference when using organic solvents. TMS was chosen as an inert substance which gives both ^{1}H and ^{13}C signals to low frequency of almost all common signals for organic compounds. With this reference, chemical shifts are reported on the δ scale where $\delta = 1$ corresponds to a 1 ppm chemical shift difference and TMS is defined as having $\delta = 0$ in both ^{1}H and ^{13}C spectra:

$$\delta = (\nu - \nu_{\text{TMS}})/\nu_{\text{TMS}} \times 10^6 \tag{29.13}$$

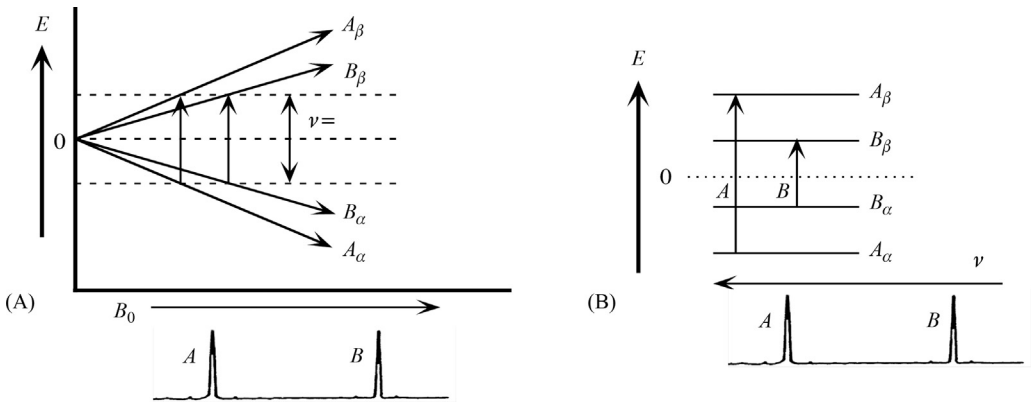


FIGURE 29.4 (A) Energy level for two nuclei, A and B, where nucleus B is more heavily screened than nucleus A. For a spectrum obtained by varying B_0, nucleus B occurs at higher field than nucleus A in an NMR spectrum. (B) If the experiment is carried out at constant B_0, nucleus B gives an NMR signal at lower frequency than nucleus A.

For aqueous solutions, either $(CH_3)_3SiCD_2CD_2CO_2^-$ Na^+ or $(CH_3)_3SiCD_2CD_2CD_2SO_3^-$ Na^+ can be used as an internal reference compound with the methyl signal assigned a chemical shift of zero.

Screening constants are actually quite complex parameters. There is some relationship with electron density at a nucleus, e.g., both ^1H and ^{13}C chemical shifts of CH_N groups with attached electronegative groups tend to occur at larger δ values. However, it is not a direct relationship. Thus the best approach is to rely on empirical correlation tables for help in assigning ^1H and ^{13}C signals. These can be found in Refs. 2, 3, and 4 and many standard Organic Chemistry texts. However, caution is needed in using these tables. They provide typical chemical shifts when the indicated substituent is attached to a CH_N group. However, if more than one substituent is near the CH_N group, the chemical shift may fall outside the indicated range.

29.3.2 Coupling Constants

A coupling constant between a pair of nuclei arises from the interaction of the magnetic dipoles for the nuclei. These can be of two forms. The first is the direct (or through-space) interaction between the two dipoles. This can be quite large and have a significant effect on spectra obtained in the solid state. However, due to rapid random tumbling of molecules in solution, these usually average to zero and are not observed in solution spectra. What remains in solution are smaller couplings, called scalar couplings, which are transmitted through bonding electrons. Because these depend on the orientations of bonds within the molecule, they do not average to zero with molecular tumbling. A coupling constant has a sign, with the sign being positive if the pair of nuclei prefer to be of opposite spin, i.e., paired. However, this normally has little effect on the appearance of the spectrum and thus is usually ignored. Because they involve interactions between nuclear dipoles, coupling constants are independent of magnetic fields. Thus they are NOT reported as δ values but rather in frequency units (Hz) and given the symbol J. The fact that chemical shift differences increase with increasing field while coupling constants do not, along with increased sensitivity due to larger population differences, has provided a powerful incentive to develop spectrometers with higher magnetic fields and operating frequencies. This benefit is most realized for ^1H spectra where one can more often observe a clean multiplet pattern for each separate proton signal at higher field. This not only provides information about the numbers of protons on adjacent carbons (see below) but also, from the size of coupling constants, information about the relative orientation of adjacent CH bonds (see Section 29.8.2).

The exact appearance of individual ^1H multiplets depends upon both the number of other protons coupled to that proton, the sizes of the different couplings, and also upon the ratio of the chemical shift difference between a pair of protons and the coupling between them, both measured in Hz. If that ratio is ca. $>10:1$, this is called a first-order case and there is little or no distortion of the relative intensities of the different components of each multiplet. However, the relative intensities change as this ratio gets smaller. For a spectrum for two coupled protons, the inner pairs of peaks get more intense while the outer peaks get less intense as the ratio decreases. This is called a second-order (or strong coupling) case. At the limit when the chemical shift difference goes to zero, no coupling can be observed between the pair of protons. This is a general rule, i.e., no coupling is observed between protons with the same chemical shift, even if they are actually coupled to one another. The first-order spectrum for two protons is called an AX spectrum, while the intermediate, second-order case, is called an AB spectrum (the limit where the chemical shift difference is zero is called an A_2 spectrum). A further advantage of higher magnetic fields is that multiplets are more likely to be first order and easier to interpret.

There are other simple multiplet patterns that one may observe in natural products. Perhaps the most common is that for an ethyl group which will either give an A_2X_3 an A_2B_3 spectral pattern, depending upon the chemical shift difference. In the former case, the CH_2 group will appear as 1:3:3:1 quartet of relative area two while the CH_3 group will appear as a 1:2:1 triplet of relative area three. The multiplicities reflect the number of different spin orientations from the adjacent group that is "seen" by the group being observed. The A_2B_3 case will be similar, except that it shows the characteristic "sloping roof" pattern, similar to AB spin system, with the inner lines for the two multiplets being more intense than the outer lines.

However, it is important to realize that many proton multiplets do not show simple patterns. First, it is very common for the proton(s) if a CH_n group has to be coupled to protons on two or more adjacent carbons, often with different couplings. Second, in natural products, it is uncommon for the two protons of a methylene group to have the same chemical shifts and the same coupling constants to adjacent proton(s). One reason for inequivalent methylene protons is the presence of an adjacent chiral center in the molecule. The chemical shift difference of the pair of protons (called diastereotopic protons) is usually largest when the chiral carbon is directly bonded to the methylene carbon but some inequivalence may still be observed when the chiral center is further away. A second, common, situation when CH_2 protons are nonequivalent is when the methylene group forms part of a nonplanar ring system which is either locked

rigidly in one conformation or where the ring has one strongly preferred conformation. It is also important to recognize that the observed splittings in multiplet patterns may not exactly correspond to coupling constants. This difference can be significantly larger than the experimental error when measuring the splitting in a strong coupling case.

Finally, most observed $^{1}H-^{1}H$ couplings in natural products and other organic compounds are either two-bond couplings between nonequivalent methylene protons (called geminal coupling constants) or three-bond couplings between protons on directly bonded carbons (called vicinal coupling constants). As will be discussed in Section 29.8.2, the latter couplings often yield important stereochemical information. However, small (<3 Hz) couplings can sometimes be observed between protons that are four or even five bonds apart.

29.3.3 Relaxation Times

After the net nuclear magnetic moment, **M**, is disturbed from its equilibrium position by B_1 (see Fig. 29.3), it gradually returns to its initial position by what is called relaxation. In solution, the main relaxation mechanism for spin ½ nuclei is provided by the random thermal motions of the magnetic moments of the nuclei, inducing fluctuating magnetic fields. Relaxation is most effective when the frequency of these motions most closely matches the acquisition frequency. However, for typical organic molecules in solution, the frequency of thermal motions is generally higher than the spectrometer frequency, so relaxation is relatively inefficient. This is both good and bad. It is good because the line width of an NMR signal is inversely proportional to the relaxation time and longer relaxation times lead to sharper NMR signals. It is bad because, if one is collecting repeated scans to improve signal/noise (see Section 29.4), one may have to wait longer for the system to reestablish equilibrium before the next scan. This becomes an important consideration in choosing acquisition parameters, particularly when one wants quantitative data (see Section 29.6.4).

There are two types of relaxation times that one can measure. The first is the spin-lattice relaxation time, T_1. This measures the return of **M** to equilibrium along the z-axis. The second, the spin−spin relaxation time, T_2, measures the decay of magnetization that had been generated in the x, y plane. While these relaxation times can be quite different for spectra obtained on solid samples, they are almost the same in solution for typical natural products, at least in nonviscous solvents. The only difference is that there is an additional contribution to T_2 arising from any inhomogeneity in the external magnetic field. Spectral line widths are inversely proportional to T_2.

These comments apply specifically to spin ½ nuclei. However, nuclei with $I = 1$ or greater have electric quadrupole moments which interact with any asymmetry in the electric field at the nucleus due to the surrounding distribution of electrons. This provides an extremely effective T_2 relaxation mechanism and consequently very broad lines.

29.4 PULSED FOURIER TRANSFORM NMR

Prior to 1966, NMR spectra were obtained by either slowly varying the magnetic field or the frequency to obtain a spectrum. It typically took a few minutes to acquire a high-resolution spectrum. This was clearly inefficient since only one part of the spectral region was excited at any given time. However, in 1966, Richard Ernst and Weston Anderson demonstrated a revolutionary new approach that allowed one to excite the entire spectral region at once, measure the time response of the nuclear spin system to this excitation in the form of a signal, and convert this time response to a frequency spectrum by Fourier transformation [7]. The actual acquisition of a single set of experimental data took at most a few seconds and often as little as 1 s. The whole process could be repeated multiple times, with the time responses added together in computer memory, Since the signal was coherent (i.e., the same each time) while any noise was random, the signal increased relative to the noise with each successive acquisition of data (at a rate equal to the square root of the number of acquisitions [7]). Since one could typically collect data at least one hundred times in the time previously required to collect a spectrum by the older method, this corresponds to a 10:1 increase in signal/noise in the same time. By 1969, the first commercial spectrometers which used the pulsed FT method became available. This new approach, combined with other improvements in spectrometer hardware, made routine acquisition of ^{13}C spectra possible for the first time, greatly improving the usefulness of NMR spectroscopy for organic chemists.

Fig. 29.5 shows that the application of a rotating field B_1 of the correct frequency will cause **M** to rotate away from its equilibrium position along the z-axis toward the x, y plane. The angle of rotation in radians, α, away from the z-axis, is given by:

$$\alpha(\text{rad}) = \gamma B_1 \tau \tag{29.14}$$

where τ is the duration for which B_1 is applied. If the duration of the pulse is just long enough to rotate **M** through $\pi/2$ rad (90 degrees), this is called a 90-degree pulse.

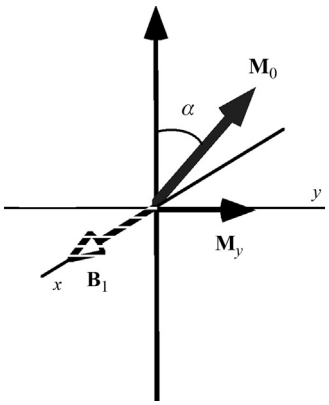


FIGURE 29.5 Application of a rotating magnetic field, B_0, of the correct frequency will cause **M** to rotate away from the z axis toward the x, y plane.

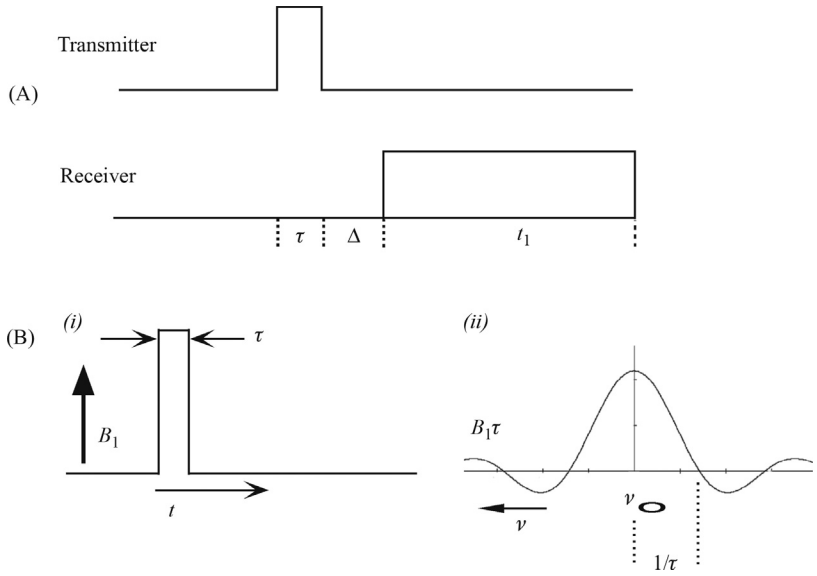


FIGURE 29.6 (A) The basic FT NMR experiment. Tau represents the duration of the RF pulse (typically ca. 10 μs) while delta is a short delay (of similar magnitude) after the pulse, before the receiver is gated on. The signal is then collected in the receiver during t_1. (B) The frequency excitation profile due to the pulse. The frequency excitation profile is the FT of the time profile.

One of the keys to pulsed FT NMR is that an RF pulse will excite not just a single frequency but a band of frequencies which is inversely proportional to the duration of the pulse. This potentially allows one to excite a band of frequencies wide enough to cover the full range of chemical shifts for ^{1}H or ^{13}C. A simple pulsed FT NMR experiment is illustrated in Fig. 29.6. The transmitter is on long enough to generate a 90-degree pulse, typically ca. 10 μs. As shown in the Figure, this generates a complex excitation profile. Although excitation is not uniform, a 10-μs pulse will produce a positive excitation range of 200 KHz. Since the range of frequencies to be excited is far smaller than this, there is essentially uniform excitement over the entire chemical shift range, particularly if the frequency of the transmitter is chosen to be at the midpoint of the expected frequency range. After the transmitter pulse is turned off, there is a short delay of similar magnitude, followed by turning on of the receiver to start acquiring the desired data. This is in the form of a signal generated in the receiver coil by the rotating x, y magnetization generated by the pulse. The period of data acquisition, t_1, is typically 1−4 s. The receiver is then turned off and the whole process can be repeated again.

After the pulse, magnetic moments associated with chemically distinct nuclei will be precessing in the x, y plane at slightly different rates. Here, the idea of the rotating coordinate system is extremely useful for visualization. With the x, y coordinate system assumed to be rotating at the transmitter frequency, individual magnetic moment vectors will be rotating relatively slowly, either in a positive or negative sense, at the difference between their rotation frequencies and that of the transmitter. This, in practice, is how the receiver actually works, i.e., instead of measuring absolute frequencies (of many MHz), it measures frequency differences (of a few KHz), something that is far easier to do accurately (see Fig. 29.7).

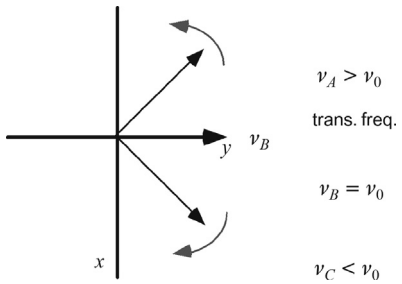


FIGURE 29.7 Individual magnetization vectors rotate slowly in the rotating frame as frequency differences, relative to the transmitter frequency. The receiver measures frequency differences which are in the audiofrequency range, rather than actual frequencies which are in the radiofrequency range.

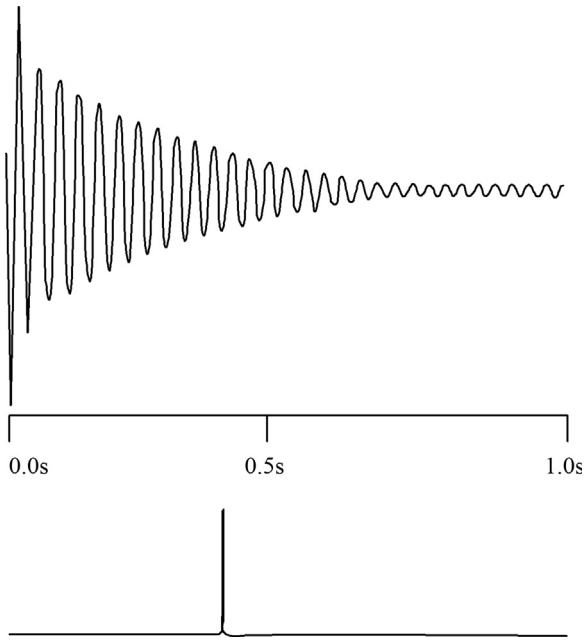


FIGURE 29.8 The FID time signal and the Fourier-transformed NMR signal for a single, off-resonance, peak.

The signal detected in the receiver is called the free induction decay (FID) signal. An example of a FID for a single NMR peak is shown in Fig. 29.8. As the magnetic moment precesses, it alternatively produces positive and negative signals in the receiver as it goes in and out of phase with transmitter frequency (at a rate determined by the frequency difference). The intensity of the peak is determined by the strength of the initial signal while the decay rate of the signal (T_2 relaxation) reflects the line width of the signal. Thus, the time response of the signal encodes all of the information required to produce a frequency spectrum. This can be generated by Fourier transformation of the FID signal (see Fig. 29.8). A molecule with many different types of protons will generate a much more complex FID but this can still be Fourier transformed to produce a spectrum (see Fig. 29.9).

There is still one problem. The FID signal collected in a receiver along the *y*-axis is identical, regardless of whether the frequency difference is positive or negative (see Fig. 29.10) and thus FT cannot distinguish between positive and negative frequencies. This is solved by using a second receiver at right angles to the first. In this case, the signal starts as zero but either increases or decreases, depending on the sign of the frequency difference (see Fig. 29.10). By separately Fourier transforming the two FID signals and coadding them in the computer, a correct spectrum is obtained, as shown in Fig. 29.9. This method, called quadrature detection, is incorporated in all modern NMR spectrometers.

Finally, the signal is analogue (i.e., continuous) and must be converted to digital form for acquisition and processing in the computer. This is done with an analogue to digital convertor (ADC). There are two key characteristics of an ADC. The first is the sampling rate. Information theory tells us that to adequately define a spectrum of width F Hz, we must

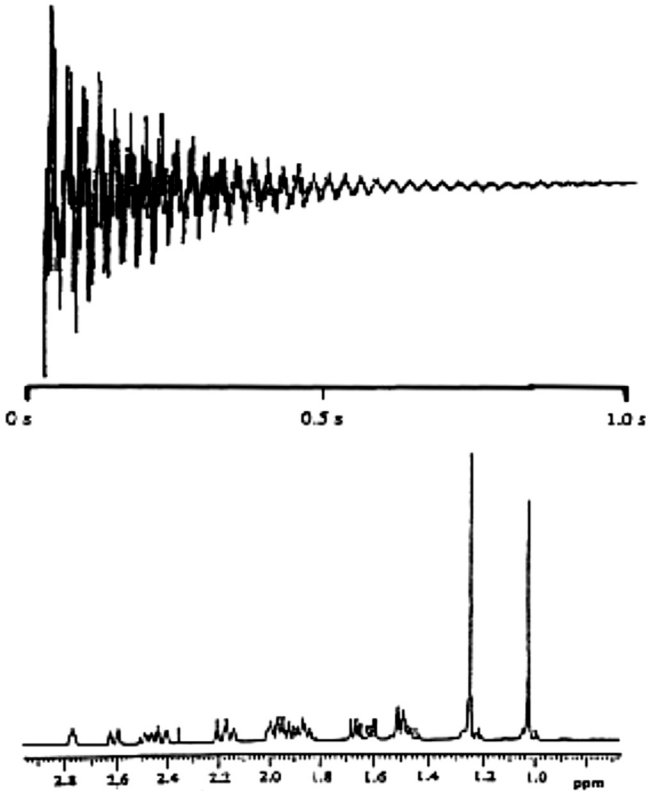


FIGURE 29.9 Similar to Fig. 4.4 except that it is for a multipeak spectrum.

sample at a rate 2 F Hz. However, many recent spectrometers have ADCs with sampling rates far in excess of that requirement (up to 100 MHz). The second requirement is the bit length. Each bit stores data as 0 or 1. One bit determines sign while the remaining $n - 1$ bits determine peak intensities. The ratio of strong to weak peaks that can be detected is given by 2^{n-1}:1. Typical ADC bit lengths are 14 or 16, respectively, corresponding to ratios of 8192:1 and 32,768:1.

However, since it really takes at least three bits to adequately define the intensity of a weak peak, the effective ratios are correspondingly smaller. However, the high speed of ADCs in modern spectrometers allows one to use a method called digital oversampling which effectively produces a bit length of about 20 bits (see Ref. 5 if you are interested in a description of this method).

29.5 KEY FEATURES OF NMR SPECTROMETERS

All current high-resolution spectrometers use magnets which are superconducting solenoids with coils made of alloys which have near-zero resistance at liquid He temperatures. The outer jacket contains liquid N_2 while the inner Dewar contains liquid He. The bore of the solenoid which holds the probe also is fitted with a series of shim coils which are used to produce as homogeneous a magnetic field as possible. Typical refill times for cryogens are 14 days for N_2 and 5−6 months for He. Recently, some solenoids have been equipped with He refrigerators which prevent He boil-off. These reduce operating costs at the expense of a significant increase in purchase price.

Most probes used in pharmacognosy research will have one coil tunable to ^{1}H and a second to other nuclei, including ^{13}C. A third coil will be provided for the deuterium lock signal which is used to keep the field/frequency ratio stable during acquisition. The relative sensitivity of the probe for ^{1}H and ^{13}C is determined by the geometry of the coils. If the inner coil is for ^{1}H (what is called an inverse detection probe), it will have relatively higher sensitivity for ^{1}H and relatively lower sensitivity for ^{13}C. The reverse is true if the inner coil is for heteronuclei (called a direct detection probe). This is a dilemma since one wants both high ^{13}C sensitivity for obtaining ^{13}C spectra and high ^{1}H sensitivity for obtaining most 2D spectra. Probes will usually also have a *z*-axis gradient coil (to aid in shimming and 2D data acquisition). Most probes are designed to take 5 mm tubes, although probes for smaller diameter (e.g., 3 or 1.7 mm) are

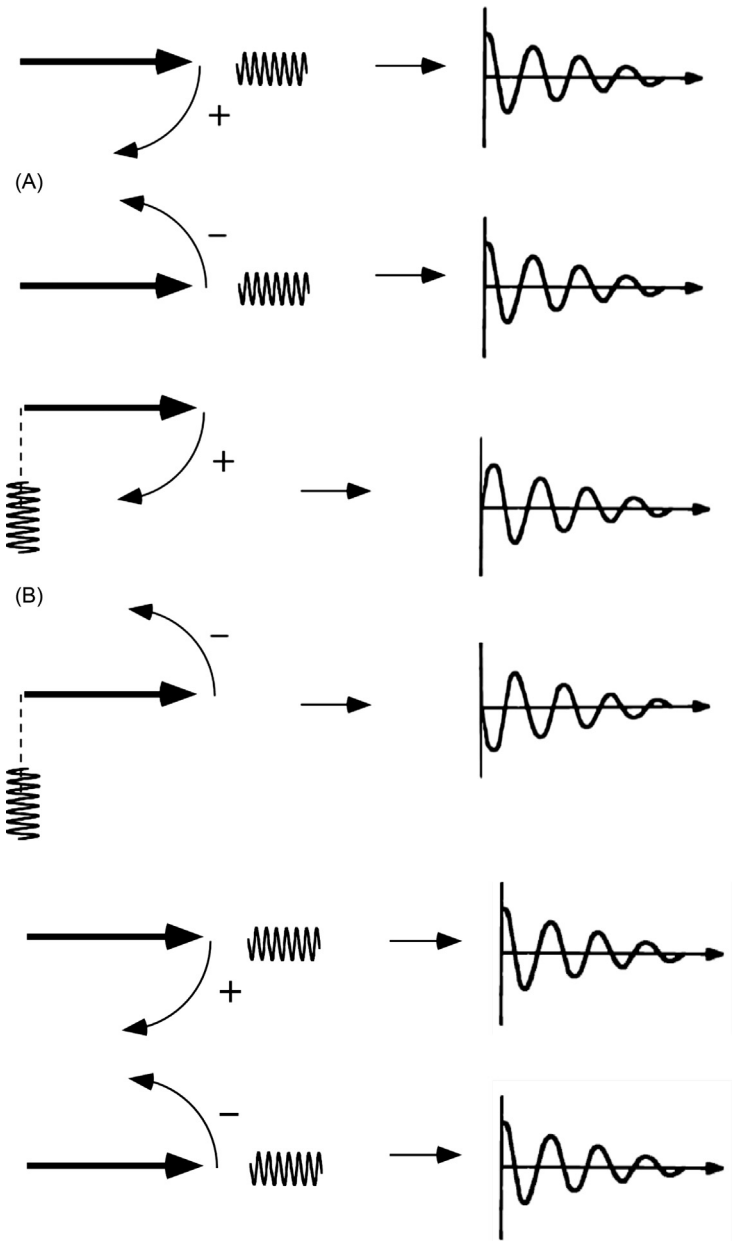


FIGURE 29.10 (A) Illustration of how a single receiver along the *y* axis cannot distinguish between positive and negative frequencies. (B) Illustration of how a second receiver set up to detect signals shifted by 90 degrees can make this distinction.

also available for sample-limited cases. While most probes operate at near room temperature, probes are available in which the coils and preamplifiers are cooled with liquid He (cryoprobes). Reduction of random noise in the coils at this low temperature results in a ca. fourfold increase in signal/noise. However, cryoprobes are significantly more expensive to purchase and operate. Finally, probes are also available with liquid N_2 cooling. These are intermediate in both cost and sensitivity and thus may be a good choice for pharmacognosy research.

The console will have at least two channels, one for detecting ^{1}H spectra and the other for ^{13}C and other nuclei plus a deuterium lock channel. It will also have the ADC, wave form generators for producing shaped RF pulses, a temperature control unit, preamplifiers for the 1 H and heteronuclear channels, a control unit for the shim coils and computers for spectrometer control and data processing. Other useful accessories for a spectrometer include a sample changer and units for automatic probe tuning.

29.6 ACQUIRING AND PROCESSING ^{1}H AND ^{13}C SPECTRA

29.6.1 Sample Preservation

The quality of sample tubes used can have a major impact on the quality of your spectra. NMR tube manufacturers produce tubes of different grades for spectrometers of different frequencies. You should always use at least the minimum grade for your spectrometer. NEVER dry NMR tubes in an oven for glassware. This may warp the tubes, risking probe damage. If you wish to reuse an NMR tube, the sample should be removed immediately after use. Cleaning solution should NEVER be used to clean NMR tubes since paramagnetic ions will be absorbed on the glass, degrading resolution. A suggested procedure is to first use a detergent solution, followed successively by distilled water, acetone, and then ether, followed by air drying. New tubes should also be cleaned before use since they may contain grease or other impurities from the manufacturing process.

Since one needs a source of deuterium to act as a lock, all common NMR solvents are deuterated. These include $CDCl_3$, CD_3OD, acetone-d_6, and DMSO-d_6. Of these $CDCl_3$ is often the solvent of choice. However, some caution is needed since opened bottles of $CDCl_3$ may, over time, produce traces of acid which could induce a chemical reaction in your sample. DMSO-d_6 is close to a universal solvent for organic molecules but, due to its very high boiling point, it is hard to recover samples dissolved in that solvent and is also highly prone to picking up water during sample preparation. The other two solvents are often useful for relatively polar compounds such as saponins. Finally, there should be instructions near the spectrometer, often in the form of a depth gauge, concerning optimum sample depth in your NMR tube. Too little sample will cause trouble in shimming.

29.6.2 Starting the Experiment

If you are lucky, this will be a trivial exercise. If your spectrometer has a sample changer, you place the sample in a spinner in a vacant slot in the sample changer, go to the computer keyboard, indicate the slot used, choose your desired experiment, and type "GO" or whatever the appropriate parameter is for your spectrometer. When your turn in the queue comes up, the sample is picked by the robot and inserted into the magnet. The spectrometer then searches for the deuterium lock signal, locks on to it, tunes the probe for your sample, performs a quick gradient shim to improve resolution, and then runs your chosen experiment(s). It may even send you a message to your email address to inform you that your experiment is finished and that the sample and data can be collected. The acquisition parameters used will be the default parameters in the spectrometer program for that experiment. Fortunately, newer spectrometers provide most, if not all, of these features. If not, manual probe tuning is not recommended for simple one-dimensional (1D) spectra. Minor mismatches in probe tuning have a minimal effect on spectral quality and there is a significant risk of probe damage when tuning is done by an inexperienced user. However, shimming is essential if it is not done automatically by the spectrometer. If there is a relatively up-to-date default shim set stored in the spectrometer computer, AND if you have followed instructions concerning sample depth, this should not be difficult. Sample spinning averages out field gradients in the x, y plane. Consequently, the resolution during sample spinning is mainly controlled by the Z shims ($Z1$, $Z2$, etc.) while resolution without spinning depends on X, Y, and on higher order shims involving combinations of X, Y, and Z shims. Normally, a nonexpert user should only adjust Z shims. If the other shims are off, they will produce spinning sidebands at frequencies equal to the spinning speed (see Fig. 29.11). If these are observed, the facility manager should be informed so that a full reshimming can be carried out. Otherwise, it is usually only necessary to adjust $Z1$ and $Z2$ shims. $Z1$ determines the overall width of the signal while $Z2$ corrects for asymmetry in the signal (see Fig. 29.11). $Z1$ and $Z2$ may have to be adjusted iteratively since changing one has some impact on the other, particularly when they are initially far from optimum settings. The goal is to provide as sharp a signal as possible which is also symmetric. Higher order Z shims only have an impact near the bases of signals ($Z3$ similar to $Z1$ in function and $Z4$ similar to $Z2$). Finally, it should be noted that, with recent model spectrometers with modern shimming algorithms based upon gradient shimming, sample spinning is often unnecessary.

29.6.3 Data Processing and Reporting of Data

For reasons too complex to explain at this level, the collected FID signal contains information for two types of spectra, a real one and an imaginary one. Thus only half of the collected points contribute to the real spectrum. However, this problem can be overcome by a clever trick called zero filling [8]. Adding an equal number of zeros to the end of the FID signal stored in the computer effectively allows all of the collected data points to be used to generate the real

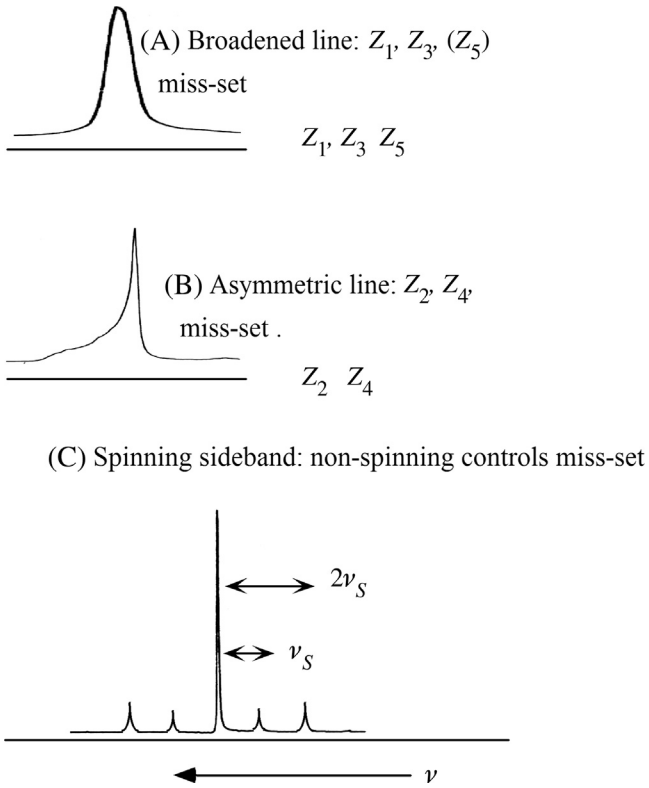


FIGURE 29.11 Determination of shimming problems from the appearance of an NMR peak (A) A broadened line indicates *Z*1 and/or *Z*3 are miss-set. (B) An asymmetric line indicates *Z*2 and/or *Z*4 are miss-set. (C) Spinning sidebands indicate that nonspinning shims are miss-set.

spectrum, improving resolution by a factor of two. Even more zero filling can be used, if desired. This will more accurately define peak positions but will not further improve resolution. Spectra may also need phasing so that all peaks are exactly upright. The required procedure is different for different spectrometers, so the manual should be consulted for instructions.

Because of their much lower sensitivity, ^{13}C spectra often have poor signal/noise. Processing the FID with an exponential line broadening factor of 1 or 2 Hz will improve signal/noise at only a slight loss in resolution. However, ^{1}H spectra typically have excellent signal/noise and no line broadening should be applied.

Standard practice is to report ^{1}H chemical shifts to the nearest 0.01and 0.1 ppm for ^{13}C chemical shifts. While greater accuracy is possible, changes in sample concentrations or temperature can cause shifts of these magnitudes. In my opinion, coupling constants measured from multiplets should only be reported to the nearest 0.5 Hz since it is difficult to measure splittings more accurately than that.

29.6.4 Quantitative NMR and High-Quality Spectra

If obtained under optimum conditions, ^{1}H spectra are quantitative and the integrated areas of different peaks provide accurate measurements of the relative numbers of protons. In recent years, there has an increased interest in taking advantage of this in natural product investigations [9]. Unfortunately, the default values provided are often not ideal for this purpose. If one uses a 90-degree pulse in combination with a short acquisition time, **M** will not have time to return to equilibrium along the *z*-axis before the next pulse. This will get worse with each successive pulse, a problem called saturation. The solution is to use a less than 90-degree pulse [7]. Consider Fig. 29.12. If one applies a 90-degree pulse, there is no magnetization along the *z*-axis immediately after the pulse. However, if one uses a 45-degree pulse, this produces ca. 71% of the *y* magnetization that would be generated by a 90-degree pulse while still leaving 71% of the *z* magnetization. Thus, if one uses this in combination with a longer acquisition time, e.g., 4 s, this will usually be sufficient for complete recovery of *z* magnetization before the next pulse. Alternatively, if relaxation times are unusually long, a 30 degree pulse will produce 50% *y* magnetization and 87% *z* magnetization. The same approach is

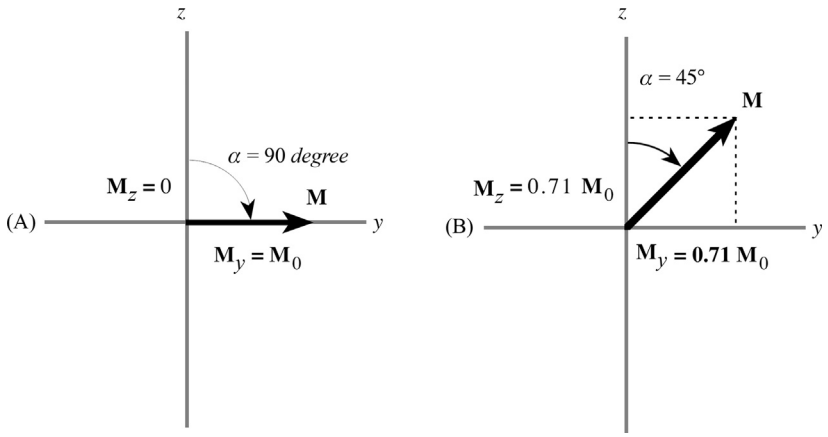


FIGURE 29.12 Comparison of the resultant magnetization in the *x*, *y* plane and along the *z*-axis immediately after (A) a 90-degree pulse (B) a 45-degree pulse.

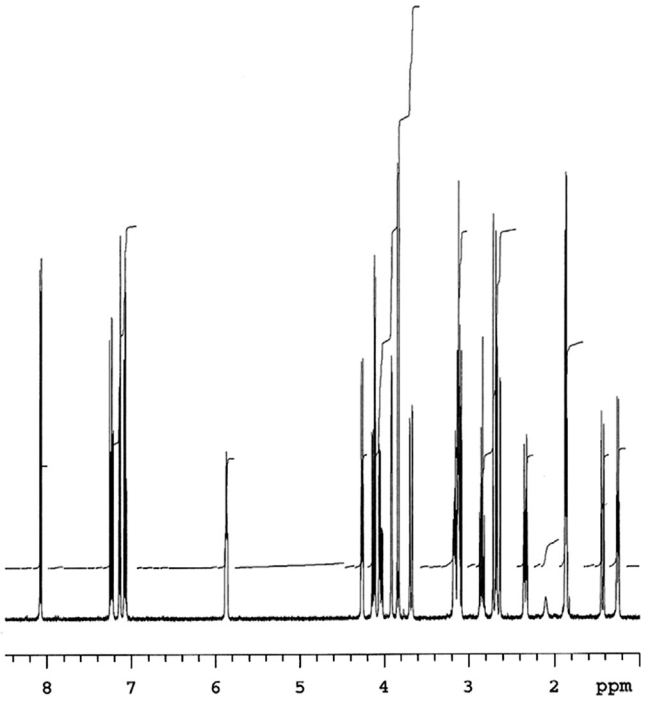


FIGURE 29.13 A 500 MHz ^{1}H spectrum of strychnine, **1**, in $CDCl_3$. This and all subsequent spectra were obtained on an Agilent DD2 NMR spectrometer.

recommended if one wants high-quality spectra for publications or theses, where the improved resolution, due to the longer acquisition time, is helpful.

Unlike ^{1}H NMR, it is extremely difficult to get quantitative ^{13}C NMR spectra, at least in any reasonable time. There are two problems. First, while ^{1}H decoupling during acquisition collapses $^{1}H-^{13}C$ multiplets to singlets, improving signal/noise, it also perturbs spin populations. This, results in a further enhancement of signal intensity, called a nuclear Overhauser enhancement (nOe), of up to a factor of three for protonated carbons. However, nOe values are usually much smaller for nonprotonated carbons. At the same time, relaxation times, which are dominated by $^{1}H-^{13}C$ dipolar interactions, are much longer for the latter carbons, resulting in greater saturation. Together, these result in weaker peaks for nonprotonated carbons. The use of a 45-degree or even shorter excitation pulse is particularly important in acquiring ^{13}C spectra in order to minimize saturation. Figs. 29.13 and 29.14, respectively, show high-resolution ^{1}H and ^{13}C spectra for strychnine, **1**, as representative examples of these types of spectra (Structure 29.1).

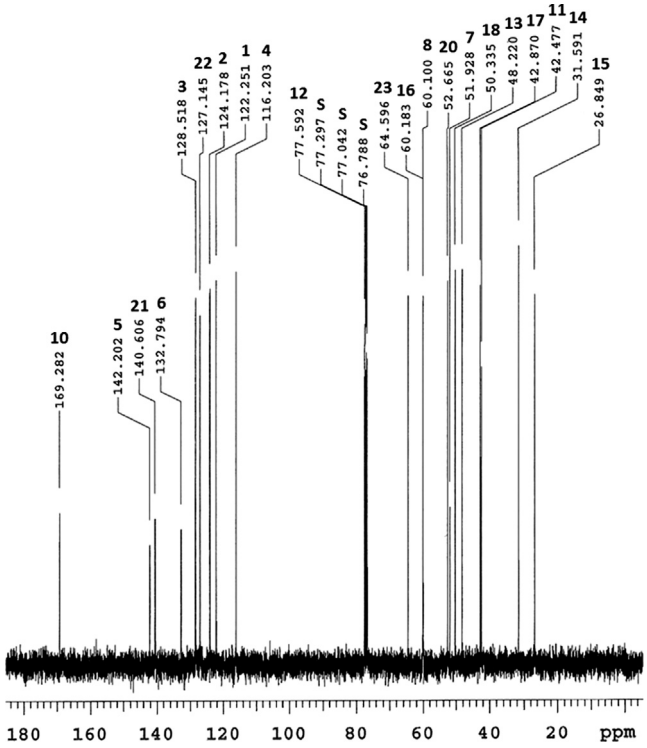
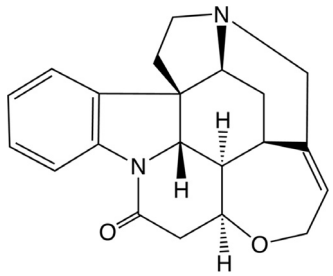


FIGURE 29.14 A ^{13}C spectrum of Strychnine, **1**, in $CDCl_3$. ^{13}C chemical shifts for the different carbons are listed above the peaks, along with carbon assignments.



1

STRUCTURE 29.1 Strychnine.

29.6.5 ^{13}C Spectral Editing

In addition to the sensitivity gains from pulsed FT NMR, another advantage is the ability to carry out acquisitions involving a series of pulses, called pulse sequences. These include spectral editing sequences which provide ^{13}C spectra where peaks are either upright or inverted, depending on the number of attached protons, helping in interpreting ^{13}C spectra. While there are a number of these sequences, they fall into two basic groups. The first are called polarization transfer sequences in which magnetization is transferred from ^{1}H to ^{13}C via $^{1}H-^{13}C$ coupling constants [10]. Of these, the most widely used is the DEPT sequence, most commonly in the form called DEPT135 [11]. The latter sequence gives CH and CH_3 signals upright and CH_2 signals inverted. Polarization transfer gives a greater enhancement than nOe and DEPT is also insensitive to variations in $^{1}J_{CH}$. However, it gives no peaks for nonprotonated carbons. A modified version, called DEPTQ [12], does give all peaks but, at least in our experience, the nonprotonated carbon peaks are quite weak. A spectrum of strychnine, **1**, obtained with DEPT135 is shown in Fig. 29.15.

The second class of sequences are called spin-echo sequences since they use ^{13}C 180-degree pulses to refocus ^{13}C magnetization. Of these, the first and most widely used version is the APT sequence [13]. This produces upright peaks

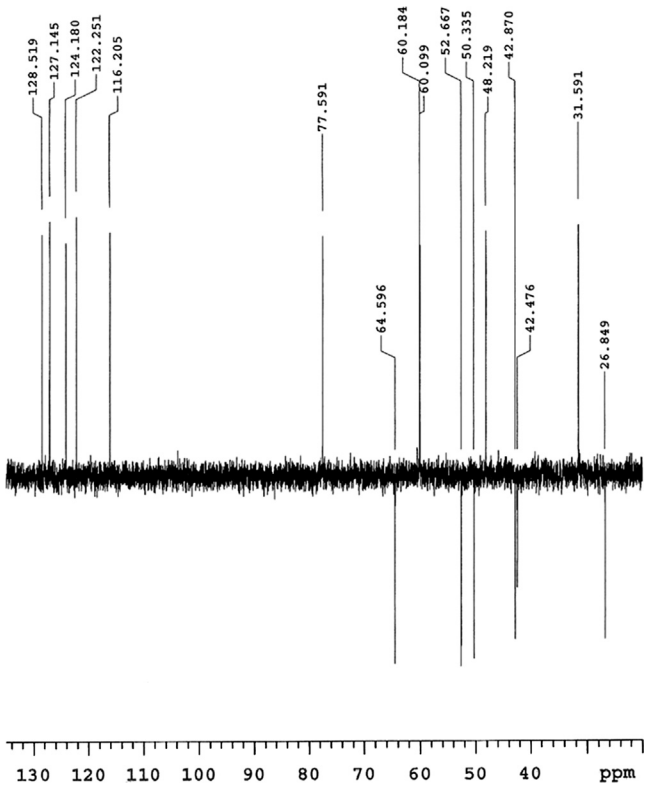


FIGURE 29.15 An edited DEPT135 spectrum of strychnine, **1**. CH signals are upright and CH_2 signals are inverted.

for CH_2 and nonprotonated carbons and inverted peaks for CH and CH_3 carbons. While the ability to detect quaternary carbons is valuable, the main problem is that APT is far more sensitive than DEPT to variations in $^1J_{CH}$, sometimes providing unreliable results for protonated carbons. This has discouraged its use. However, we have recently developed a modified version of APT, called CRAPT [14]. This replaces the ^{13}C 180-degree pulses of APT with CRISIS pulses which are far less sensitive to variations in $^1J_{CH}$ [15]. Fig. 29.16 shows CRAPT, APT, and ^{13}C spectra for **1**, each obtained in the same total acquisition time. As can be seen, CRAPT is more sensitive than APT and almost as sensitive as the ^{13}C spectrum, while providing edited spectra. This suggests that CRAPT is a viable alternative to the common practice in pharmacognosy research of acquiring a ^{13}C spectrum plus a DEPT135 spectrum.

29.7 DEREPLICATION OF NATURAL PRODUCTS

In isolating natural products as possible new drug candidates, one does not want to spend too much time characterizing already known compounds. Thus it is important to have a rapid and reliable method for distinguishing between known and unknown compounds, a process called dereplication. Unfortunately, at least as far as I am aware, there is no simple way to do this by NMR alone. ^1H NMR would seem to be the obvious choice since ^1H spectra can be acquired very quickly. However, the appearance of a ^1H spectrum is quite complex and changes with spectrometer frequency and solvent. Thus, even if a spectral data base includes ^1H spectral data, it may not be clear whether a compound is known or unknown. Due to their simplicity, ^{13}C spectra, in either normal or edited mode, are more likely to give a definitive answer. However, unless one has access to a ^{13}C-optimized cryoprobe, this may take an unacceptably long time.

At the moment, it seems to me that the best starting point for dereplication is a high-resolution mass spectrum (MS). This can be obtained with a small fraction of an isolated compound and will hopefully give its molecular formula. This can be used to prepare a list of possible compounds of this formula by searching online. Then a ^1H spectrum, or perhaps an edited HSQC spectrum (see Section 29.8.2), can be used to identify a specific compound. The latter gives chemical shifts for all protonated carbons and their attached protons and can usually be obtained more quickly than a regular ^{13}C spectrum and in no more time than for a DEPT135 spectrum.

It might seem that HPLC-NMR would be useful for this purpose. However, there are problems, particularly when used in flow mode. It is usually only possible to get a ^1H spectrum in the limited time that the sample is in the flow

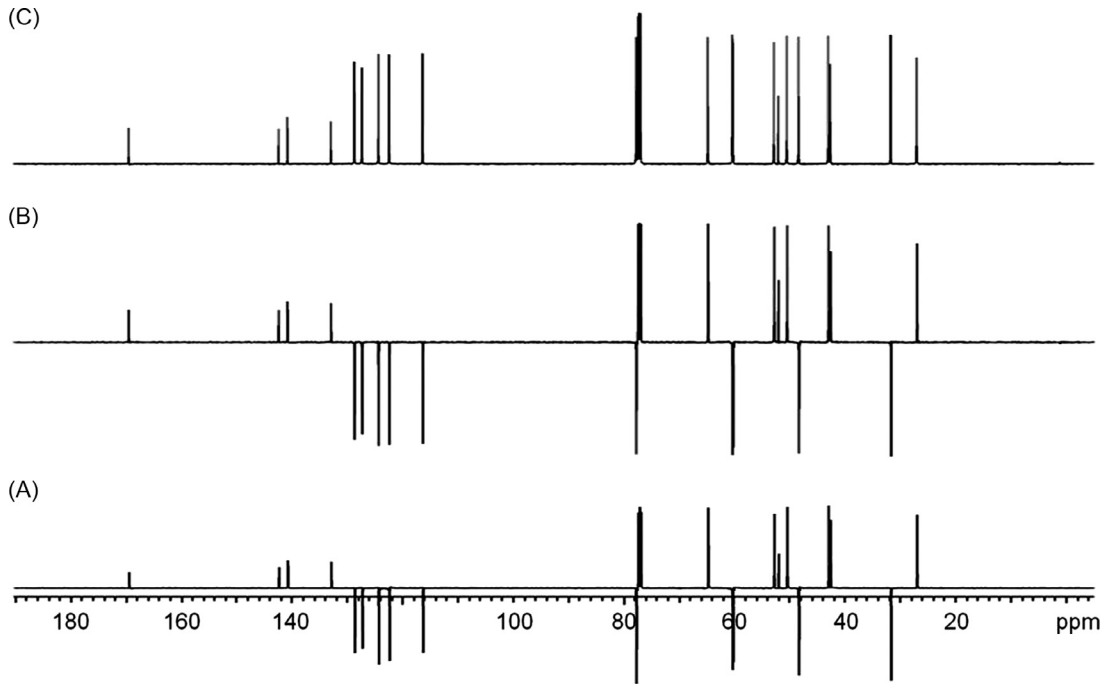


FIGURE 29.16 (A) A ^{13}C APT spectrum. (B) A ^{13}C CRAPT spectrum. (C) A regular ^{13}C spectrum. All spectra are for strychnine, **1**, in $CDCl_3$ and were obtained in the same total experiment time. CH_2 signals are upright and CH signals inverted in the APT and CRAPT spectra. This figure was taken from Ref. 14 with permission of the publishers.

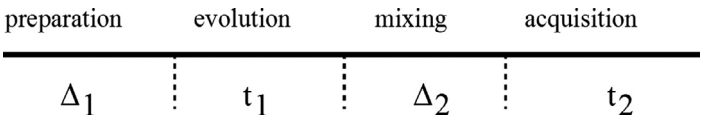


FIGURE 29.17 A generic 2D pulse sequence. The preparation period is usually just a relaxation delay. The time-incremented evolution period is t_1. There may be additional RF pulses during t_1. The mixing period is usually either a single 90 degree pulse or a simultaneous pair of 90 degree pulses on different channels. The acquisition time is t_2.

cell. Also, use of deuterated solvents is expensive while mixtures of protonated solvents require multiple peak suppression of solvent peaks, often obscuring sample peaks. A better approach is to use protonated solvents and solid-phase extraction (SPE) cartridges to trap separate compounds. These can then be washed into sample tubes with a deuterated solvent. One approach that looks particularly promising to me uses an HPLC/MS/SPE/NMR combination [16]. After an HPLC peak is detected, a small fraction is sent for MS while the remainder is trapped on an SPE cartridge. Depending on the MS results, this then may or may not be used to obtain NMR data. A modification of this approach might be to send a second fraction for bioassay screening, allowing one to only concentrate on fractions with promising activity.

29.8 TWO-DIMENSIONAL NMR

29.8.1 Basis of 2D NMR

This represents the second of Richard Ernst's key contributions to NMR for which he received the 1986 Nobel Prize in Chemistry [17]. The basic approach used in 2D NMR is illustrated in Fig. 29.17. The preparation time is usually just a relaxation delay to ensure that magnetization, **M**, is fully recovered before the next cycle of the pulse sequence. The evolution time, t_1, is regularly incremented from zero up to some maximum value, with a separate FID collected for each t_1 value. Double FT, first with respect to t_2 and then with respect to t_1, yields a 2D spectrum with frequency axes f_1 and f_2 containing information respectively generated during t_1 and t_2. There are usually pulses at the beginning and the of end of t_1, most often 90-degree pulses, and there may be additional pulses during t_1.

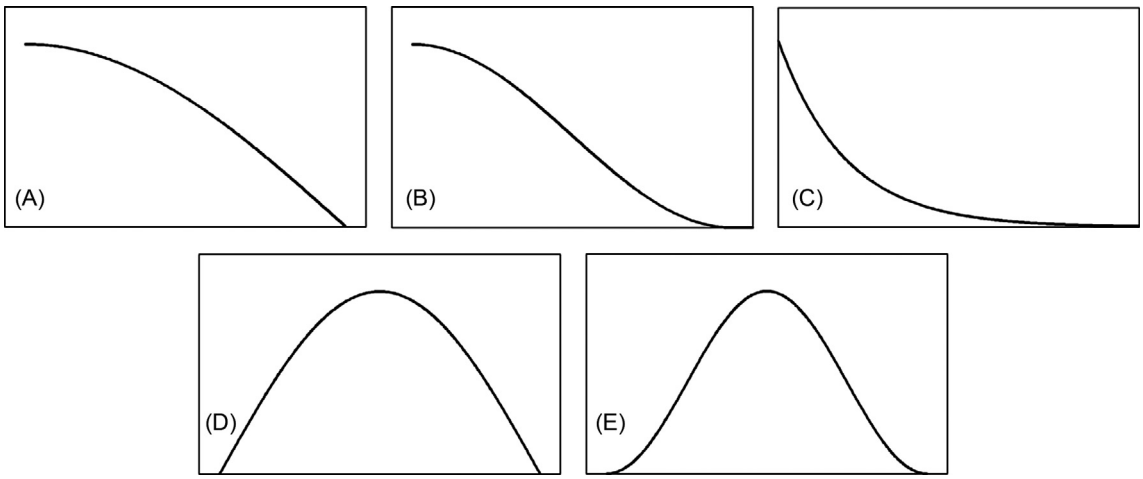


FIGURE 29.18 Different weighting functions that can be used for processing NMR FID signals. In each case, the weighting function results in zero signal intensity at the end of t_1 or t_2. (A) A 90 degree-shifted sine bell function. (B) A Gaussian function. (C) An exponential (line-broadening) function. (D) A sine bell function. (E) A squared sine bell function.

There are two basic types of 2D spectra. The first are homonuclear experiments (almost always ^{1}H), where the 1D spectrum appears as a diagonal from bottom left to upper right of the spectrum, with oft-diagonal peaks between interacting protons spaced symmetrically about the diagonal. The second, heteronuclear, 2D experiments have a proton spectrum along one axis and a heteronucleus (usually ^{13}C) along an orthogonal axis, with peaks at the frequencies of coupled pairs of ^{1}H and ^{13}C nuclei. In recent years, these are almost entirely obtained by ^{1}H detection during t_2, to take advantage of the higher sensitivity of this approach. Conventionally, f_2 frequencies are displayed horizontally and f_1 vertically.

Earlier versions of 2D experiments relied on phase cycling to obtain the desired information. The relative phases of one or more pulses and the receiver were systematically varied in what was known as a phase cycle, designed to collect the desired signals while eliminating other unwanted signals. A typical phase cycle would either consist of four steps or some multiple of four. However, almost all current pulse sequences for obtaining 2D spectra use what is known as gradient selection [18]. Here, a pair of gradient pulses (usually of opposite sign) are applied along the z-axis during the pulse sequence. One purpose is to choose between alternate pathways that magnetization can follow during the course of the pulse sequence. However, other important practical advantages include more effective elimination of unwanted peaks and, in the case of COSY spectra, the ability to acquire a spectrum with only one scan per time increment spectrum, instead of at least four with phase cycling.

Some 2D sequences (COSY in particular) generate peaks which cannot be phased. This problem is avoided by plotting the spectrum in what is known as absolute value (or magnitude) mode. While the peaks are all upright in this mode, they have broad "tails" which hinder resolution. However this can be minimized by using a sine bell or squared sine bell weighting function when processing the data (see Fig. 29.18). The former gives better signal/noise while the latter gives better resolution. However, most of the widely used 2D sequences yield phase-sensitive spectra in which all peaks can be phased, often with some peaks upright and others inverted. For phase-sensitive spectra, processing can be done with an exponential line broadening function, a Gaussian function, or a 90 degree-shifted sine bell function (see Fig. 29.18). The first gives best signal/noise, while the third gives best resolution. I personally favor the Gaussian function as a compromise choice.

29.8.2 2D NMR Pulse Sequences Commonly Used in Natural Product Research and the Kinds of Information Which They Provide

29.8.2.1 COSY and TOCSY

The first of the homonuclear sequences is called COSY [19]. With this sequence, the equivalent of a 1D spectrum appears along the diagonal of the 2D plot from lower left to upper right. Peaks indicating coupled pairs of protons appear symmetrically on either side of the diagonal. The pairs of protons responsible for the off-diagonal peaks can be confirmed by tracing horizontal and vertical lines from the off-diagonal peaks to the diagonal. This provides a powerful

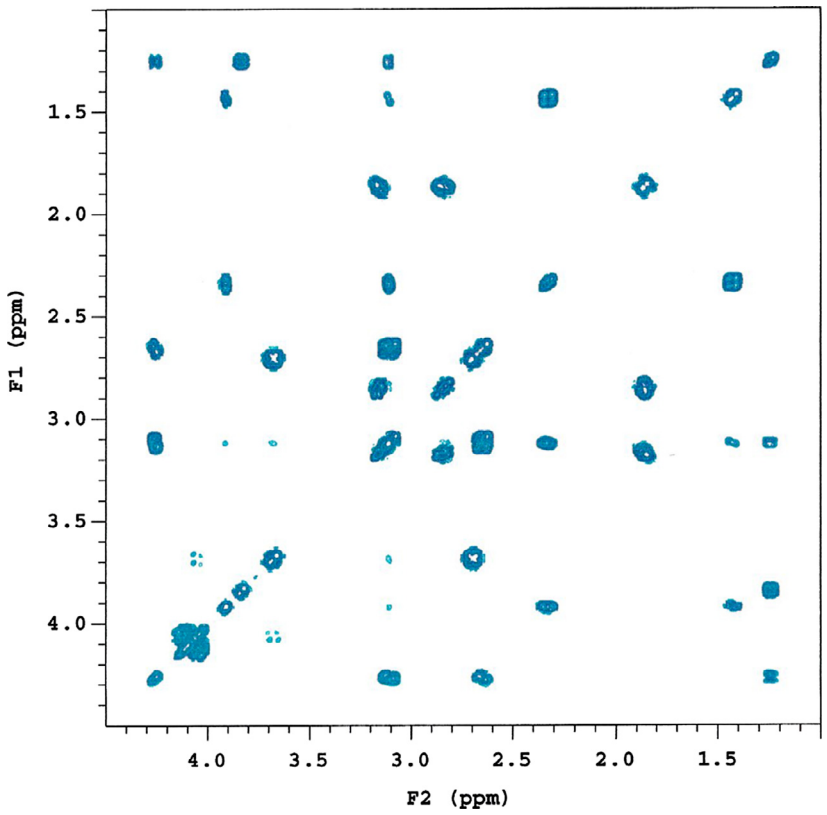


FIGURE 29.19 Expansion of the aliphatic region of a COSY spectrum of strychnine, **1**.

tool for working out networks of coupled protons within a molecule. It is also the most sensitive of the 2D experiments and a useable spectrum can often be obtained with as little as one scan per time-incremented spectrum. Fig. 29.19 shows a COSY spectrum for **1**.

TOCSY is essentially a COSY sequence with an additional series of pulses, called an isotropic mixing sequence, added before acquisition [20]. This effectively spreads COSY-like information throughout an entire coupled spin system. Thus if H(A) is coupled to H(B) and H(B) is coupled to H(C) but H(A) is not coupled to H(C), a COSY spectrum will show H(A)/H(B) and H(B)/H(C) correlations but not an H(A)/H(C) correlation. However, TOCSY will show all three correlations, observed by drawing a horizontal line through the chemical shift of any of these protons along the diagonal. A TOCSY spectrum for **1** is shown in Fig. 29.20. The extent to which the magnetization is transferred throughout a coupled spin network depends on both the duration of the mixing time and the size of coupling constants, with larger couplings being more effective in transferring magnetization along a coupled network. A mixing time of 60 ms will typically information to the third and sometimes the fourth of a sequence of coupled protons. The maximum recommended spin lock duration is usually ca. 100 ms. TOCSY is particularly valuable is in investigations of polysaccharides which typically have very crowded ^{1}H spectra. Provided that at least one proton in each sugar unit is resolved, it may be possible to get "edited" subspectra for each unit. Unlike COSY, TOCSY is acquired in phase-sensitive mode.

29.8.2.2 NOESY and ROESY

These spectra appear similar in appearance to a COSY spectrum. However, instead of providing information about coupled protons, the NOESY [21] and ROESY [22] spectra show off-diagonal peaks between pairs of protons that are close in space, even if they are several bonds apart. Since nOe effects are, to a first approximation, proportional to r^{-6}, they are very sensitive probes of distance between pairs of protons. Both spectra are obtained in phase sensitive mode, with the off-diagonal nOe peaks always opposite in phase to diagonal peaks for ROESY and usually for NOESY. NOESY uses a mixing time to build up the nOe while ROESY uses a series of pulses called a spin lock. A typical NOESY mixing time would be 500 ms but can be as large as 1 s. For ROESY, the spin lock duration is typically about 250 ms and it is not generally recommended to exceed 500 ms.

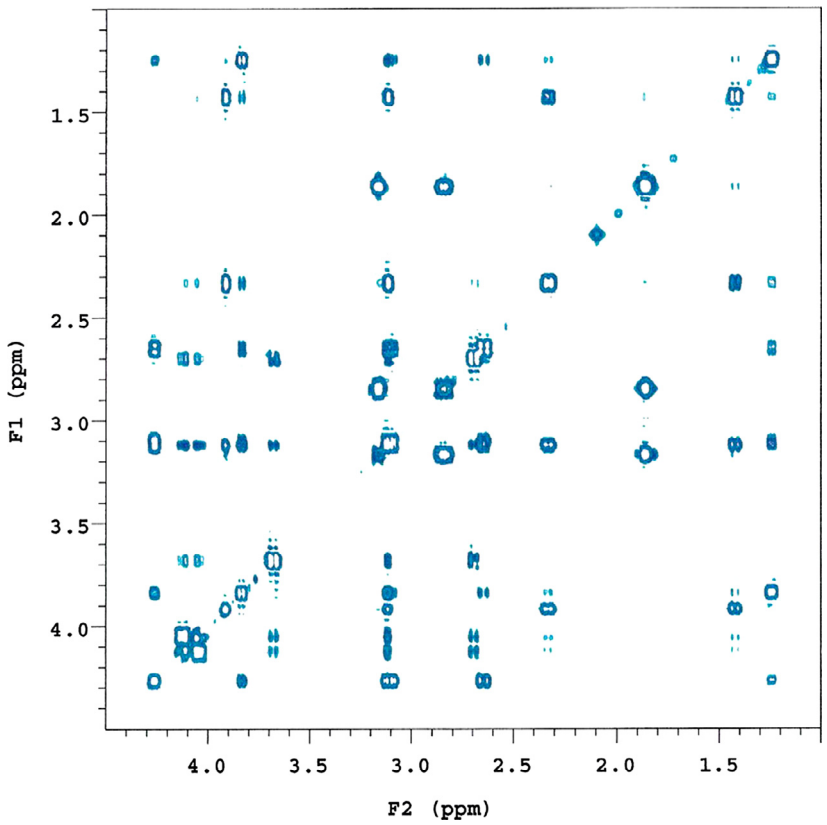


FIGURE 29.20 Expansion of the aliphatic region of a TOCSY spectrum (mixing time = 60 ms) of strychnine, **1**.

The key difference between the two sequences is that ROESY peaks are always positive while NOESY peaks switch from positive to negative with increasing molecular weight and solvent viscosity. The cross-over point seems typically to be in the 750−1000 molecular weight range but lower in DMSO-d6. Since nOe peaks are quite weak near the cross-over region, NOESY is recommended to compounds up to ca. 600 molecular weight but ROESY for compounds above that molecular weight. A NOESY spectrum for **1** is shown in Fig. 29.21.

29.8.2.3 Heteronuclear Single Quantum Coherence

This sequence provides correlations between directly bonded protons and carbons [23]. The ^{1}H spectrum is along f_2, conventionally the horizontal axis, while the ^{13}C spectrum appears along the vertical axis, f_1. Correlations between pairs of bonded nuclei are established by a drawing horizontal and vertical lines from a correlation peak to the two axes, the former establishing the ^{13}C chemical shift and the latter the ^{1}H chemical shift of the coupled pairs. In the case of diastereotopic CH_2 groups there will be two proton chemical shifts for each ^{13}C chemical shift.

Heteronuclear single quantum coherence (HSQC) spectra are normally obtained in phase-sensitive mode. They can also be obtained in edited mode with CH_2 peaks upright and CH and CH_3 peaks inverted [24]. While this involves some sensitivity loss (ca. 15%), the extra information provided by editing often outweighs any sensitivity losses. An edited HSQC spectrum provides the same ^{13}C information as a DEPT135 spectrum in similar time, with the added advantage of determining the chemical shifts of the bonded protons. An edited HSQC spectrum of **1** is shown in Fig. 29.22.

There are two additional sequences that have been used to provide similar information in the past. The first is the heteronuclear multiple quantum correlation (HMQC) sequence [25]. However, this gives ^{1}H multiplet structures along both f_1 and f_2. Thus, the ^{13}C resolution is poorer than for HSQC. In addition, due to the complex phase shapes of these multiplets, the sequence is usually run in absolute value mode, eliminating the possibility of spectral editing. For these reasons, it is now much less commonly used that HSQC. An even older sequence is HETCOR [26]. This is a ^{13}C-detected sequence and is much less sensitive than HSQC (by ca. a factor of 10) and thus is rarely used unless very high ^{13}C resolution is needed.

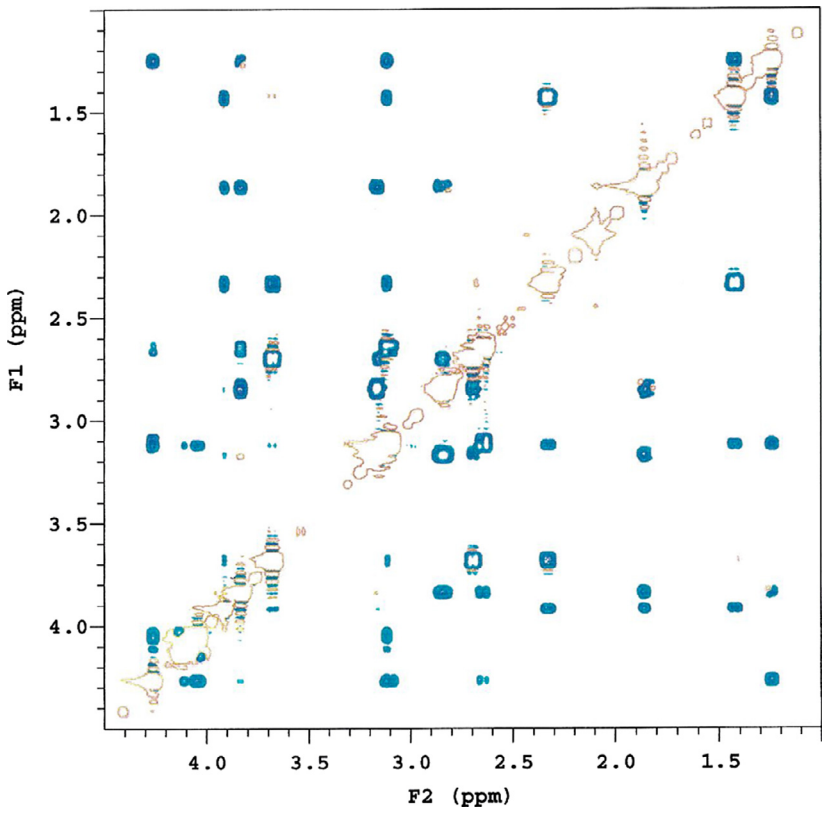


FIGURE 29.21 Expansion of the aliphatic region of a NOESY spectrum (mixing time = 0.5 s) of strychnine, **1**.

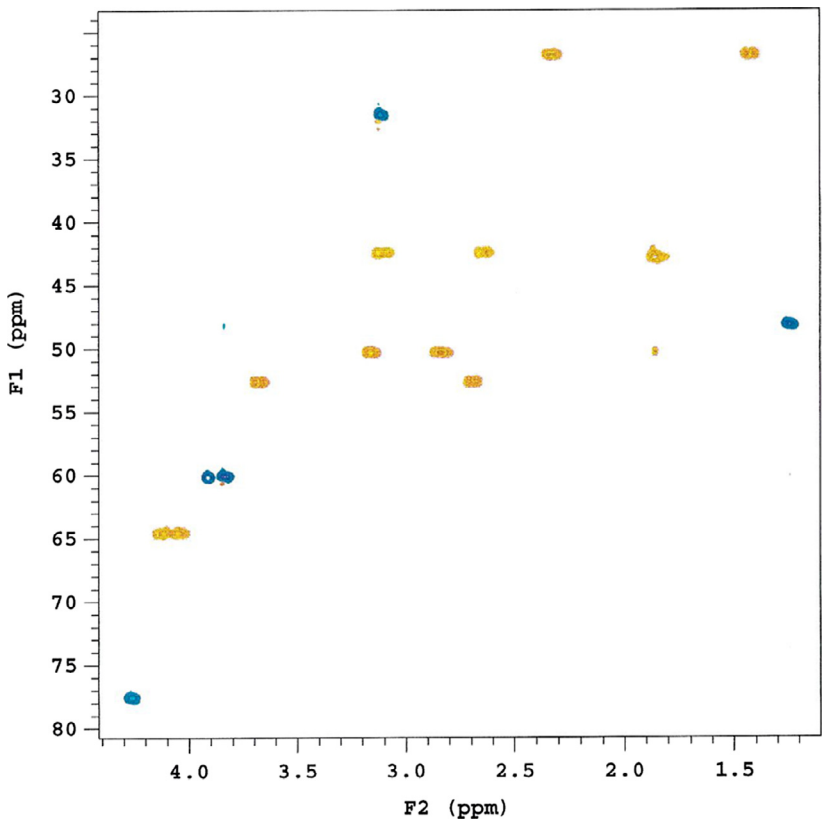


FIGURE 29.22 Expansion of the aliphatic region of an edited HSQC spectrum for strychnine, **1.** CH peaks (black) are upright while CH_2 peaks (gray) are inverted.

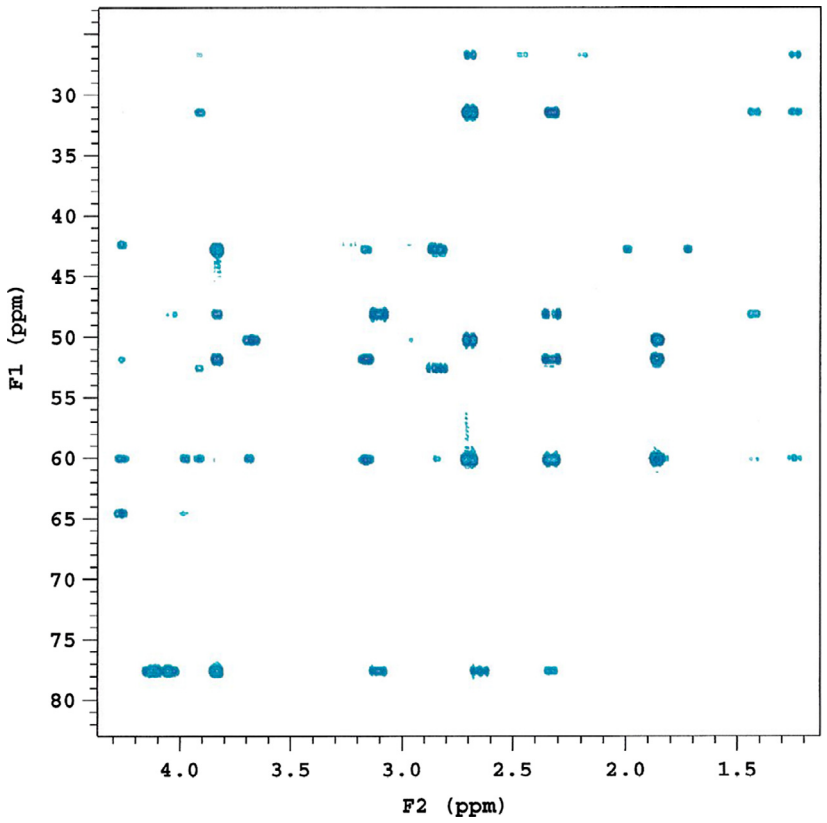


FIGURE 29.23 Expansion of the aliphatic region of an HMBC spectrum of strychnine, **1**.

29.8.2.4 Heteronuclear Multiple Bond Correlation

This sequence also detects correlations between carbons and protons but, in this case, they are not directly bonded [27]. Instead, HMBC relies on longer range (typically two-bond or three-bond but occasionally four-bond) $^{13}C-^{1}H$ couplings to generate correlation peaks. These coupling are much smaller (ca. 2−10 Hz) than one-bond coupling constants (which range between 125 and 210 Hz) and thus require longer delays during the pulse sequence. Typically, a compromise value of $^{n}J_{CH} = 8$ Hz is used to calculate the delay for the small couplings. HMBC spectra require significantly longer total acquisition times than HSQC spectra to acquire adequate quality spectra. Nevertheless, they are critical for structure elucidation (see Section 29.9). HMBC spectra are often obtained in absolute value mode. However, there are advantages, in both resolution and sensitivity, in processing them in mixed mode, i.e., absolute value mode along f_3 but phase-sensitive along f_1. An HMBC spectrum of **1**, obtained using mixed mode processing, is shown in Fig. 29.23.

29.8.2.5 Alternatives to HMBC

HMBC provides correlations between carbons that are mainly separated by two or three bonds. However, it does not distinguish between these two possibilities. This can lead to ambiguities in structure elucidation. The H2BC sequence does distinguish between two-bond and three-bond correlations, but only in cases where both carbons are protonated [28]. An alternative sequence which makes this distinction for both protonated and nonprotonated carbons is the 1,1-ADEQUATE sequence [29]. However, this relies on $^{1}H-^{13}C-^{13}C$ couplings. Since the natural abundance of ^{13}C is just 1.1%, the probability of adjacent ^{13}C carbons is only ca. 0.01%. Thus, a 1,1-ADEQUATE spectrum can often only be obtained in reasonable time if one has access to a cryogenically cooled probe.

29.8.2.6 $^{1}H-^{15}N$ HMBC spectra

While it is somewhat more difficult to obtain $^{1}H-^{15}N$ correlation spectra, the extra time needed is often justified by the extra information provided for nitrogen-containing natural products [30]. An HMBC spectrum will also often detect the presence of an N−H group which will appear as a doublet with a characteristic splitting (due to $^{1}H-^{15}N$ coupling) of ca. 90 Hz. This eliminates the need to acquire a separate HSQC or HMQC spectrum.

29.8.2.7 Selective 1D Versions of 2D Sequences

Modern spectrometers have wave form generators which can produce RF pulses of any desired shape. This can allow one to selectively excite a very narrow spectral region. This is essentially the reverse of the situation in Fig. 29.6 where a short, square pulse excited a broad spectral region. Here, a shaped pulse of longer duration and complex shape can cleanly excite a narrow spectral window. In the case of NOESY or ROESY measurements, one may only need a limited number of key correlations, results that can be obtained more quickly with selective 1D experiments. 1D TOCSY is even more valuable. By performing a series of experiments with increasing mixing time, one can sequentially assign a network of coupled protons. This is particularly useful for saponins and polysaccharides where one can sometimes produce separate ^{1}H spectra for each monosaccharide unit.

29.8.3 Forward Linear Prediction as a Time-Saving Method for Acquiring and Processing 2D Spectra

A FID signal can be regarded as a series of exponentially decaying cosine functions (see Figs. 29.8 and 29.9). When a sufficient number of points have been collected, further points can be mathematically predicted without the need to actually acquire them, a method called forward linear prediction (LP) [31]. This is particularly important for f_1 since doubling the number of time-incremented spectra will double the total experiment time. For HSQC and HMBC spectra, we have consistently found that one can get accurate, well-resolved, spectra by collecting 128 time-incremented spectra and predicting them out to 512 points (or 256 out to 1024 if even higher resolution is desired) [32]. This allows one to acquire spectra of identical quality in one quarter of the time needed to collect the full set of points. Alternatively, one can obtain a spectrum in the same time with twice the signal/noise by increasing the number of scans per spectrum by a factor of four. I always use LP when acquiring 2D spectra and I recommend that you do the same. Software for LP is available on all spectrometer programs.

29.9 USING COMBINATIONS OF SPECTRA TO DETERMINE STRUCTURES AND FULLY ASSIGN SPECTRA

29.9.1 Establishing the Molecular Skeleton

After standard ^{1}H and ^{13}C spectra are obtained, a combination of COSY (and/or TOCSY), HSQC, and HMBC spectra can often be used to determine the basic molecular framework [33]. First, the COSY and HSQC spectra can be used together to determine the different fragments of the molecule involving sequences of protonated carbons. The molecule can then be assembled into a full structure by using HMBC data to tie the fragments together into the full carbon framework (see Fig. 29.24). An example of this process as applied to kauradienoic acid, **2**, is shown in Figs. 29.25–29.28. In Figs. 29.25 and 29.26, a molecular fragment of protonated carbons corresponding to $C(5)H{-}C(6)H_2{-}C(7)H_2$. is labeled. A second fragment also involving only protonated aliphatic carbons ($C(1)H_2{-}C(2)H_2{-}C(3)H_2$, not shown) can also be deduced from the same two spectra. Expansions of an HMBC spectrum (Fig. 29.27) illustrate key correlations of the two methyl proton singlets (H(18) and H(20)) to various carbons. H(18) shows correlations to a quaternary aliphatic carbon, C(4), aliphatic carbons, C(3) and C(5), and to a nonprotonated carbon, C(19), with a chemical shift characteristic of a CO_2H group. These data can be used to work out the first part structure of **2** as shown in the top row of Fig. 29.28. Similarly, H(20) show correlations to C(1), C(5) and to nonprotonated aliphatic, C(10), and olefinic, C(9), carbons. This allows one to expand the part structure to almost complete the A and B rings of **2**, as shown in the second row of Fig. 29.28. Further COSY, HSQC, and HMBC data involving both aliphatic and olefinic carbons can be used to complete the skeletal structure, as shown in the rest of Fig. 29.28, while NOESY data can be used to determine the three dimensional (3D) structure. If a molecule contains one or more nitrogens, $1H{-}^{15}N$ HMBC spectra can be very useful in working out the structure [30]. There is no high resolution NMR technique that can be used for oxygen. However, the presence of bonded N or O atoms can often be deduced from the characteristic chemical shifts of carbons bonded to these atoms. Ultimately, a high-resolution MS will usually be needed to unambiguously confirm the molecular formula (Structure 29.2).

To elucidate the structure of a molecule from this type of 2D data, it is important to work in a systematic manner. I suggest preparing a spreadsheet or similar table of correlation data. The first column will contain ^{13}C shifts in increasing or decreasing order. The second column will contain the chemical shifts of attached protons (obtained from an HSQC spectrum) and their multiplicity patterns and coupling constants, if measurable. If there are diastereotopic

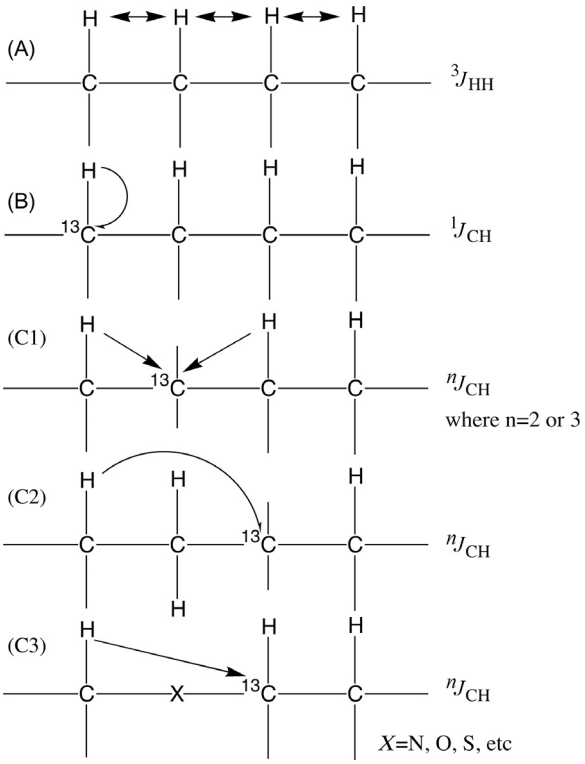


FIGURE 29.24 Using COSY, $^1J_{CH}$ and $^nJ_{CH}$ ($n = 2$ or 3) correlations to establish molecular skeletons of natural products. COSY (A) and $^1J_{CH}$ (B) correlations establish fragments of protonated carbons. $^2J_{CH}$ (C1) and $^3J_{CH}$ (C2) correlations to quaternary carbons tie these fragments together. Similarly $^3J_{CH}$ correlations through heteroatoms (C3) can tie two fragments together.

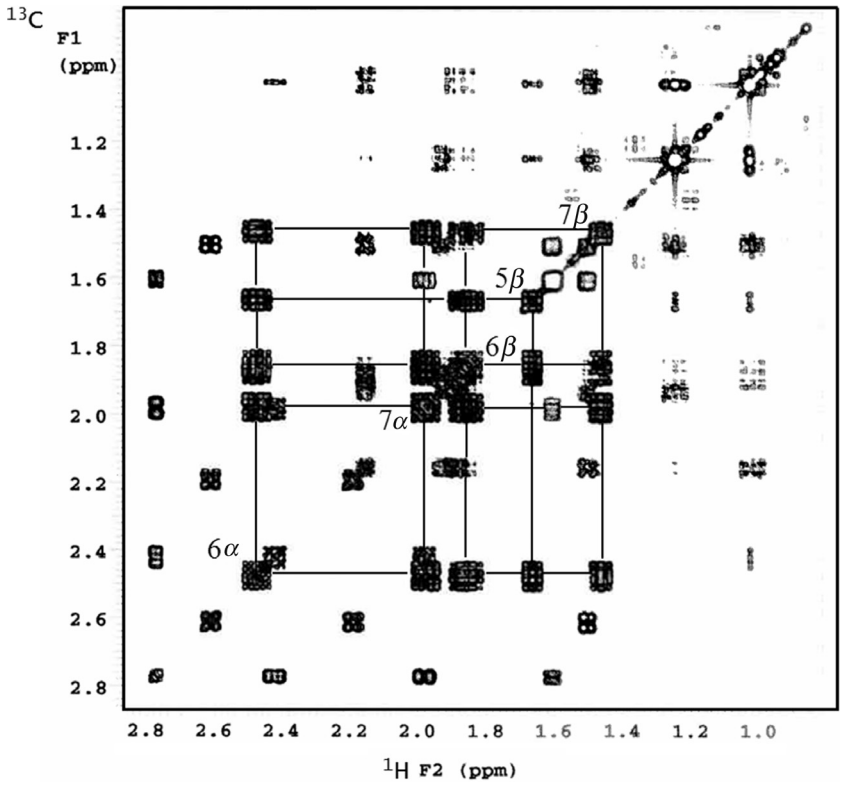


FIGURE 29.25 Expansion of the aliphatic region of the COSY spectrum of kauradienoic acid, **2**. COSY correlations used to deduce a series of coupled protons, corresponding to the molecular fragment C(5)H−C(6)H_2−C97)H_2, are outlined.

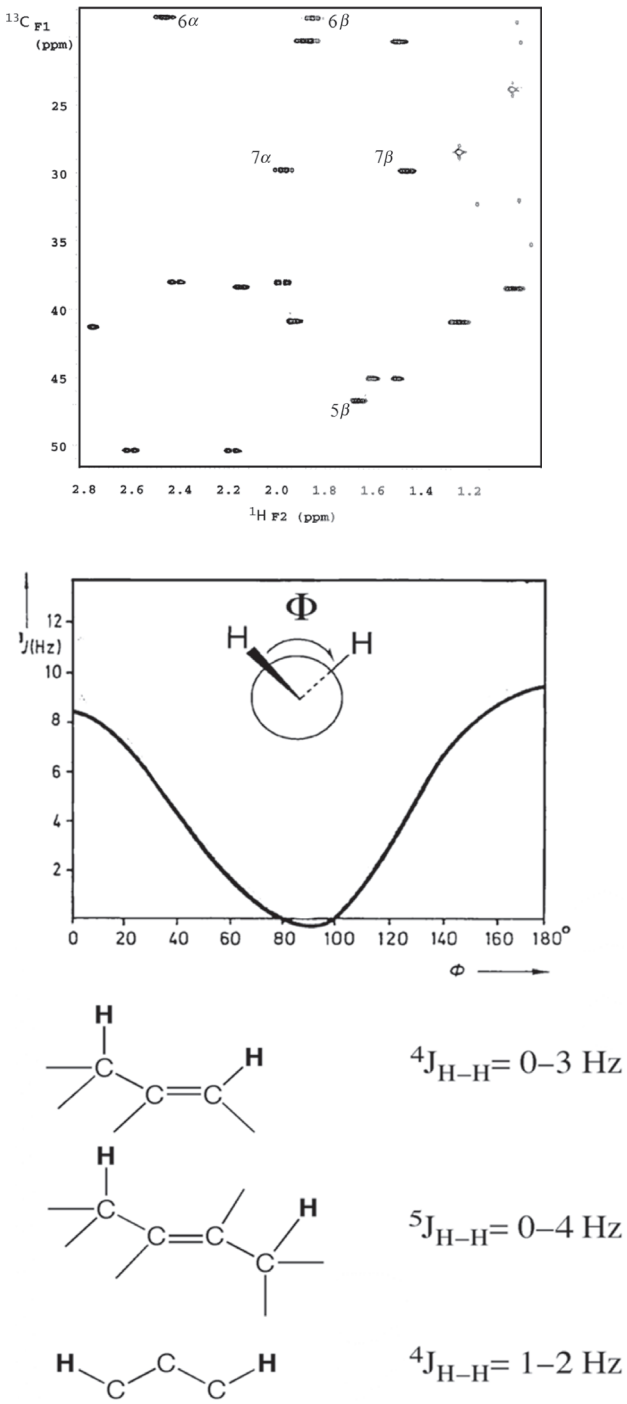


FIGURE 29.26 Expansion of the aliphatic region of the HSQC spectrum of **2** with peaks corresponding to C(5), C(6), and C(7) correlations labeled.

methylene protons, there should be a separate row for each of these protons. A third column will contain chemical shifts of other protons showing COSY correlations with the protons in column 2. Similarly, a fourth column will show the chemical shifts of carbons with HMBC correlations to the protons in column 2. Finally, a fifth column could list key NOESY or ROESY correlations to these protons. As an exercise, I suggest starting with a listing of the ^{13}C chemical shifts of the aliphatic carbons of **1**, taken from Fig. 29.14. Then use the HSQC, COSY, HMBC, and NOESY spectra from the figures in Section 29.8 to complete a table of the kind described above. Then check to see if the assignments are consistent with the actual structure of **1**.

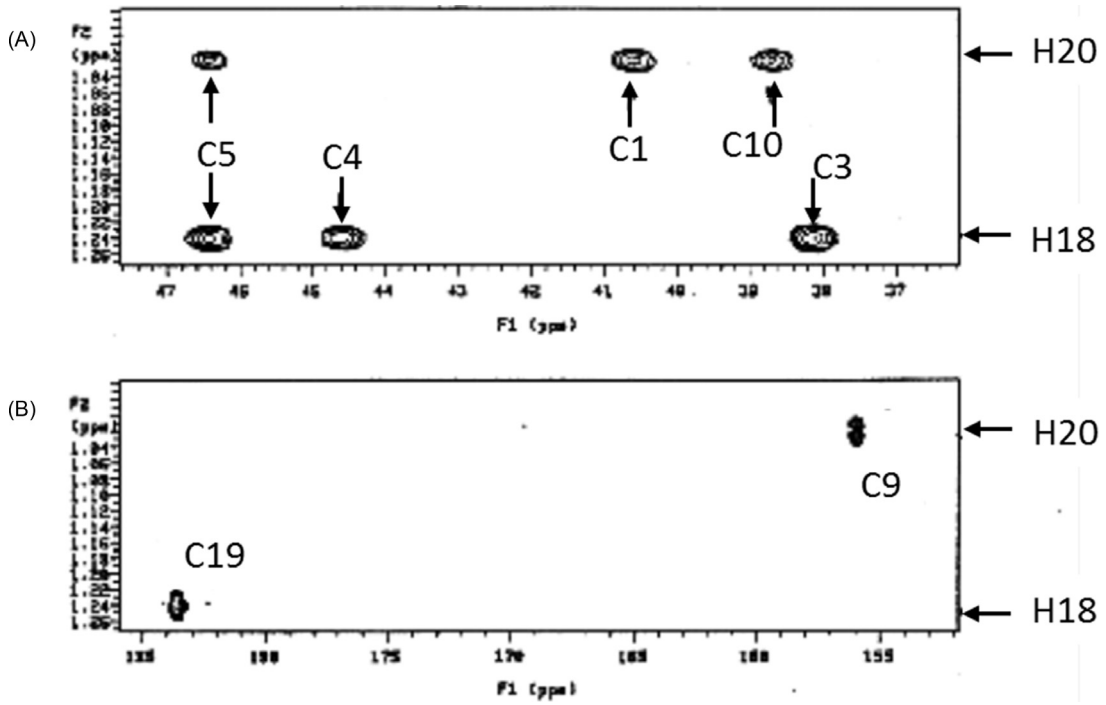


FIGURE 29.27 Expansion of an HMBC spectrum of **2**, illustrating key correlations involving the methyl proton singlets, H9(18) and H(20).

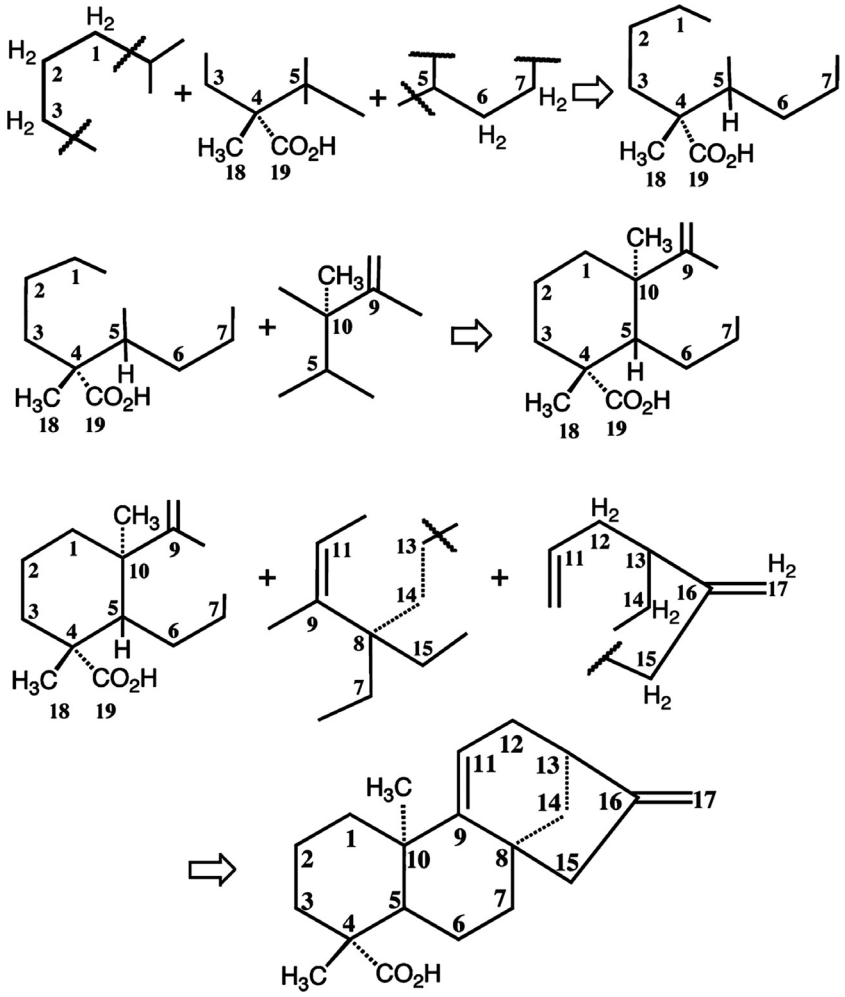
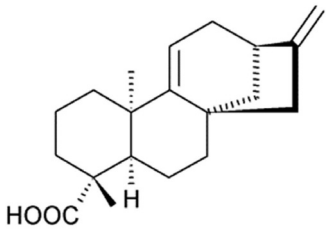


FIGURE 29.28 Illustration of How 2D data can be used to establish the skeletal structure of a natural product (kauradienoic acid, **2**). Fragments C(1)–C(2)–C(3) and C(5)–C(6)–C(7) were tied together via *n*-bond HMBC correlations from a methyl singlet to the quaternary carbon, C(4), and to protonated carbons C(3) and C(5). Similar HMBC correlations off a second methyl singlet to the quaternary carbon C(10) and to C(1) and C(5) allowed completion of ring A and most of ring B. Further correlations allowed completion of the structure.



2

STRUCTURE 29.2 Kauradienoic acid.

29.9.2 Determining the 3D Structure of the Molecule

Determining the 3D structure of a molecule is often difficult, particularly if the molecule is nonrigid since, in that case, the observed spectra will often be averaged values for two or more rapidly interconverting conformations. One often has to rely on information from several sources in an attempt to determine relative stereochemistry (note that absolute stereochemistry cannot usually be determined from NMR data alone). A much more detailed discussion of the types of information that can be used for this purpose is given in Ref. 5. Here, I will just focus on three possible sources of information.

The first is the size of vicinal $^{1}H-^{1}H$ coupling constants (i.e., those between protons on adjacent carbons). Vicinal coupling constants can be quite useful for deducing the relative orientations of groups attached to either end of an aliphatic C−C bond. Typical values, based on the dihedral angle for a pair of protons, are shown in Fig. 29.29. The same Figure also contains information concerning four-bond and five-bond $^{1}H-^{1}H$ coupling constants which may be observed in ^{1}H spectra of natural products. An equation, relating vicinal coupling constants to dihedral angles is available [34] but this is probably not accurate to better than ca. 10 degrees.

More key information can be provided by homonuclear nOe measurements, obtained with either 2D or selective 1D NOESY or ROESY measurements. The ability to detect spatially close protons which may be several bonds apart can be particularly helpful for determining molecular conformation and/or stereochemistry. By carrying out a series of careful nOe measurements for different pairs of protons in a molecule, it may even be possible to accurately determine interproton differences and consequently the complete 3D structure of a molecule of known molecular skeleton [35].

Finally, the rapid increase in computer power now makes it possible to accurately estimate not only energies but also ^{1}H and ^{13}C chemical shifts for different possible molecular geometries. This information can be used, either alone or in combination with vicinal coupling and/or nOe data, to determine 3D structures [36].

29.9.3 Avoiding Getting the Wrong Structure

There still are a surprisingly large numbers of incorrect NMR structures reported in the literature [37]. Obviously, one wants to avoid this and there are ways to at least minimize this risk. The first, as discussed in Section 29.9.1, is to tabulate and evaluate the data systematically. In doing so, it is important to let the data lead you to a structure, rather than try to fit the data to a preconceived structure. It is also important to obtain 2D spectra with sufficient resolution on both axes to provide unambiguous data. With HMBC in particular, an expected peak may sometimes not appear, because the $^{1}H-^{13}C$ coupling constant is quite small. A common case where this may occur is for two-bond couplings in olefinic or aromatic groups. Thus the absence of an expected peak is not enough to rule out a structure. However, the presence of a strong unexpected peak is usually a warning sign that you are on the wrong track. Finally, just because one structure appears to fit the available data, there may still be other structure(s) that fit as well or better. The best way to avoid a mistake is to use a CASE program which will systematically search for all possible structures that fit your data and predict chemical shifts for the different structures [38]. This is discussed in Ref. 5 with specific examples of systematic structure elucidation with the aid of CASE shown in Ref. 6.

29.10 REVIEW QUESTIONS

The following questions are designed to test how fully you have grasped the material covered in this chapter. The answers can be found within the body of the chapter.

Question 1: What are the two main advantages gained from acquiring NMR spectra at higher magnetic fields?

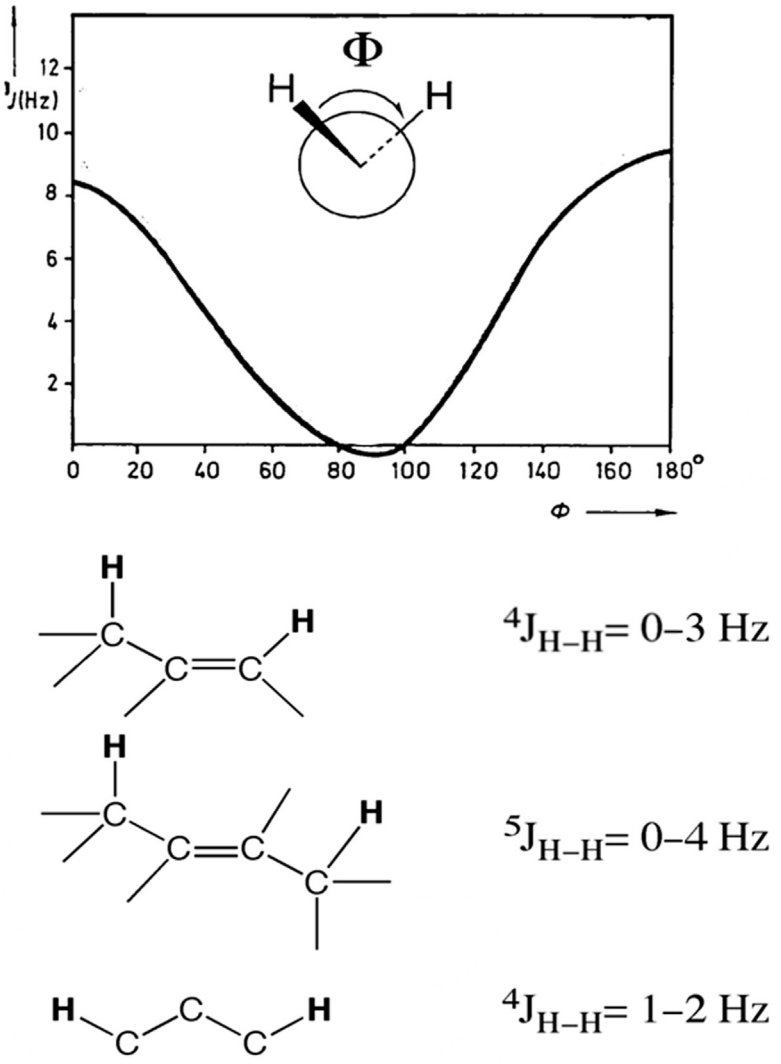


FIGURE 29.29 The graph at the top illustrates the approximate dependence of vicinal $^1H-^1H$ coupling constants on the dihedral angle between the two CH bonds. Typical four-bond and five-bond couplings that might be observed in natural products are shown at the bottom of the figure. The four-bond "*W*" coupling at the bottom is often observed in both aliphatic and aromatic systems.

Question 2: Why are ^{15}N spectra acquired instead of ^{14}N spectra in pharmacognosy research, even though ^{14}N has almost 100% natural abundance while ^{15}N is well under 1%?
Question 3: Why is it preferable to express chemical shifts in ppm rather than Hz while coupling constants should always be expressed in Hz?
Question 4: Which is more correct: to talk of low field/high field chemical shifts or high frequency/low frequency chemical shifts?
Question 5: Why are the relatively long relaxation times of NMR signals both an advantage and a disadvantage?
Question 6: What are two important advantages of Fourier transform NMR over the earlier method of sweeping through the spectrum?
Question 7: What is the main advantage and disadvantage of a liquid He-cooled probe as opposed to one operating at room temperature?
Question 8: Which shim controls are most important to optimize before acquiring a ^1H spectrum?
Question 9: Why is it often advantageous to acquire spectra using less than a 90-degree pulse when acquiring an NMR spectrum requiring multiple scans?
Question 10: Why do nonprotonated carbons usually give weaker peaks than protonated carbons in ^{13}C spectra?
Question 11: The carbon *alpha* to oxygen in a furan ring has an unusually large (ca. 205 Hz) $^1J_{CH}$ coupling constant. Which of APT or DEPT135 is more likely to yield a misleading result for this carbon in an edited ^{13}C spectrum?

Question 12: What are the advantages and disadvantages of ^{1}H spectra for dereplication?
Question 13: Under what circumstances is it better to acquire a ROESY spectrum in place of the more common NOESY spectrum?
Question 14: HMBC spectra cannot distinguish between two-bond and three-bond ^{13}C peaks. Which sequences discussed here can make this distinction and what are their advantages and disadvantages?
Question 15: While using 2D data to attempt to assign a structure of a natural product, you observe a very strong HMBC peak which would correspond to a four-bond peak in your proposed structure. Is this a serious concern?
Question 16: You find a structure which is consistent with all of your NMR correlation data. Is this enough to conclude that you have the correct structure?

29.11 CONCLUSION

This chapter provides an overview of NMR spectroscopy with particular emphasis of how it can be used to determine structures of new natural products.

ACKNOWLEDGMENTS

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Chapter 30

Metabolomics Approach in Pharmacognosy

N. Lajis, M. Maulidiani, F. Abas and I.S. Ismail
University Putra Malaysia, Serdang, Selangor, Malaysia

Chapter Outline

Learning Objectives

- To introduce metabolomics as the new unconventional approach in solving problems related to pharmacognosy.
- To explain the basic concepts of metabolomics-based research.
- To expose the students to the types of instrument platforms that can be used in metabolomics-based investigation.
- To expose to the students the types of research closely related to pharmacognosy.

30.1 INTRODUCTION

Pharmacognosy is the study about the physical, chemical, biochemical, and biological implications of natural products for medicinal or health benefit purposes. Continuous interest in this field has led to the emergence of many allied fields of study such as natural product, pharmacology, biomedicine, spectrometry, and biotechnology. Today, prospecting for bioactive constituents from natural resources (plants, animals, and microbes) with interesting and novel action mechanisms has become one of the most actively pursued endeavors in drug discovery programs conducted by many institutions. These constituents are "lead compounds" that are used as templates for more potent, selective, and safe drugs. Resulting from these efforts, a huge progress has been made in methodologies and instrumentation for isolation, purification, and molecular characterization, thus an enormous number of naturally occurring molecular entities have been identified.

Development in electronics and computation has also contributed to the rapid advancement in technologies associated with bioactive lead discovery and development activities. Currently, a large number of natural substances from various sources have been identified. These, along with their spectral and physicochemical data, as well as biological/pharmacological properties for most of them, are available in several databases, such as PubChem, NAPRALERT, and ChemBank [1]. Conventionally, the aim of a drug discovery program is to find new agents for a single or few predetermined therapeutic targets, such as hormones, enzymes, ion channels, specific proteins, and nucleic acid. Unfortunately,

Pharmacognosy. DOI: http://dx.doi.org/10.1016/B978-0-12-802104-0.00030-5

the methods employed have been based on reductionistic approach, where most of the parameters commonly present in the real living system are controlled or eliminated, thus many other potential action mechanisms such as synergism and multivalent pharmacology are missed [2].

The rising cost of medication has become a serious concern to the public. The reason for this is partly contributed by the escalating costs for the discovery of new drugs and their development. The current rate of discovery and development for a new drug is estimated at US$0.8−1.8 billion [3]. One of the major causes of this is due to the high rate of attrition of drug candidates, especially at the tail end of the program, the clinical trial [4]. New approaches are required to maximize the chance of success in this effort. Besides this, a new paradigm on disease treatment and management may now be required. Recent studies indicated that metabolomics may offer some potential solutions to these issues.

30.2 IMPORTANT ASPECTS IN METABOLOMICS

Metabolomics is the beneficiary from the advancement of knowledge in biological sciences, modern instrumentation, enhancement of computational capability, large chemical/biochemical/biological databases on biological molecules, and improved statistical capability. It is complementary to the other "omics" methods (genomics, transcriptomics, and proteomics). Being the most downstream products of gene functions on metabolism, metabolites mapping using metabolomics could become the ultimate tool in understanding chemically or biochemically implicated biological systems.

Metabolomics can be simply defined as "an unbiased study of all metabolites (usually involves the small molecules; MW < kDa) that are present in a specific cell, tissue, or organism." The term "metabolome," from which "metabolomics" was derived, refers to the collection of all these metabolites, which may include metabolic intermediates, hormones, signaling molecules, and secondary metabolites. Metabolomics is a new unconventional tool, which concerns the intermediate or end products of all regulatory activities of living organisms. The change in metabolite profile (qualitative and quantitative) represents the closest reflection of the phenotypes, which could help in the interpretation of the functional status of organisms. "Unbiased approach" is an important element in metabolomics study, because no metabolite is predetermined prior to analysis, and the metabolites that contribute to the biological conditions are unknown. Metabolomics is considered to be more robust as compared to other "omics" technologies for its ability to collect extremely large data sets, from which hypotheses on "biological consequences" may be developed.

The main and important components in metabolomics research are metabolite fingerprinting and metabolite profiling. Metabolite fingerprinting involves detection of all metabolites in the sample without the need for their identification. Metabolite fingerprints can, in principle, be generated from any data that describe the metabolites that are present in the sample. The earlier metabolite detection includes the use of paper and thin-layer chromatography. Currently, several more refined techniques such as Fourier-transform infrared (FTIR), Raman, nuclear magnetic resonance (NMR), and mass spectrometry (MS) are more commonly used. Comparison between fingerprints of different samples may lead to the discrimination of samples having differential metabolites, or clustering of samples with similar profiles. This approach has become the most commonly applied as the first stage of analysis in order to establish whether differences in metabolite profiles are present among the samples. The common processes involved in metabolomics studies may be represented as in Fig. 30.1.

NMR and MS are the most commonly used platforms in metabolite detection and identification due to their signal specificity and uniqueness. Hyphenated MS methods, such as gas chromatography-coupled MS (GC-MS) and liquid chromatography-coupled MS (LC-MS), have become more important and robust in metabolomics due to their high sensitivity of detection and quantification capability. Comparisons of the advantages and weaknesses between these platforms are listed in Table 30.1. Other separation methods such as capillary electrophoresis and ultrahigh pressure liquid chromatography have also been used in combination with MS.

The potential application of metabolomics in medicines, particularly in disease diagnosis, toxicology, and identification of novel biomarkers for diseases, has been demonstrated [5]. In more recent years, metabolomics approach has been adopted or suggested to be used in various research areas including drug discovery, neurosciences, agriculture, food and nutrition, and environmental sciences. Aspects of pharmacognosy could become the essential part in most of these areas.

30.3 IMPORTANT ISSUES IN METABOLOMICS-BASED RESEARCH

Identification and clear definition research problems are the starting point in any research project. In metabolomics the core questions revolve around the assessment of the effect of treatments on a biological system, or group discrimination, based on the data generated. Proper identification of the problem will help establish the scope of the research and in the

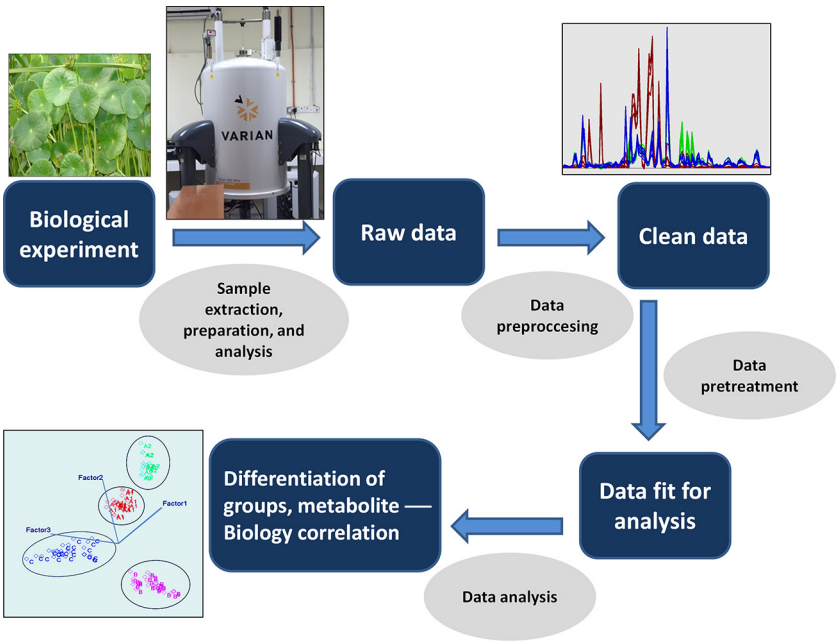

FIGURE 30.1 Schematic representation of process involved in metabolomics studies.

TABLE 30.1 Comparison of the Major Strengths and Weaknesses of NMR and MS Techniques in Metabolomics

Key points	NMR Techniques	MS Techniques
Detected metabolites	Universal detector	Universal detector for ionizable molecule
Reproducibility	High reproducibility	Lower reproducibility than NMR (especially when combined with LC)
Sensitivity	Low sensitivity (mg)	High sensitivity (ng)
Resolution	Low resolution but could be improved by 2D methods	High resolution
Quantitativity	Quantitation possible (no standard required)	Quantitation possible (standard required)
Sample preparation	Minimal	Sample preparation required
Structural information	Gives structural information to identify metabolites	Gives structural information to identify metabolites
Other	Nondestructive method	Destructive method

design of the experiment. A good design in metabolomics experiments can help minimize the influence of bias resulting from different sources (such as instrumental, biological, or human factors), and allow researchers to derive maximum information from the effort. Typical steps in metabolomics investigation include experimental design, data acquisition, data processing, data pretreatment, exploratory analysis/unsupervised modeling, supervised modeling, metabolites characterization and quantification, and finally, the interpretation.

A thorough experimental design including careful sample collection and storage are vital to ensure the quality of the data generated in the subsequent analysis. Factors that should be considered in the study design include, e.g., the variations due to age, parts of the organism, growth condition, diurnal variation, gender, animal strain, diet, and stress. The time gap between sampling and freezing or extraction may also alter the metabolite profile due to protraction of residual biochemical reactions. All of these issues can significantly modify the metabolite profile obtained from the experimental subjects. A number of publications on the protocols for sample handling and preparation have been published [6–10].

In a holistic analytical approach like metabolomics, sample preparation should ideally be minimal to avoid variation in the sample composition, which may arise from unintentional metabolite losses or enrichment. For example, in the case of urine sample, typical centrifugation and dilution are sufficient. However, for blood-derived samples (e.g., plasma and serum), proteins are usually removed by precipitation using organic solvents. Preliminary studies to estimate the efficiency of the extraction process by comparing the number of features detected may be useful (higher number means extraction efficiency) in order to optimize the protocol. In our work on the discrimination of *Nigella sativa* seed samples from different geographical origins, a preliminary PCA performance of the extracts obtained using different solvents was compared in order to select the most appropriate solvent system for extraction [11].

The issues on sample size and number of replicates required for the experiment are also fundamental in metabolomics studies. The general concept of "noise reduction" by increasing the sample size may be applied in metabolomics experiments. In designing experiments, it may be worthwhile to refer to experimental parameters (such as sample size, models, and statistical methods) implemented in other related fields, as well as in numerous publications on metabolomics studies available in the literature.

In the analytical stage, the ideal optimum analytical method is one that would provide direct and fast analysis, without the need for anything beyond the minimal sample preparation. The technique should also provide unbiased results for the myriad of metabolites present, despite their varying concentrations and molecular properties. In addition, the availability of high structural information content of the detection system used could facilitate the identification of potential marker molecules. Due consideration in all the practices, including the identification of possible source of variation, is essential to minimize unwanted variation arising from inconsistent procedures or instrumental factors. The variability introduced by the analytical measurement procedure and its respective sources should be identified and kept under control.

The two major analytical techniques currently used in metabolomics are the ^{1}H NMR spectroscopy and MS, which are mainly due to their effectiveness in both the quantitative and quantitative aspects. In the following discussion the strategies used in these two instrumental platforms will be discussed in more detail, although the same strategies are also applied in others.

30.4 NMR AND MS PLATFORMS IN METABOLOMICS-BASED ANALYSIS

The versatility of NMR spectroscopy in metabolomics studies has been shown in many recent studies, despite its inferiority in respect to sensitivity in comparison to the GC- and LC-MS techniques. The advantages in terms of rapid and simpler sample preparation perhaps has compensated for the lack of its sensitivity. The abundance and ubiquitous presence of protons in biological molecules makes the proton NMR spectrometry the most commonly used platform in metabolomics studies. Furthermore, the occurrence of nuclear spin in most of the elements constituting the metabolome allows the use of more convenient and robust NMR analyses. In most metabolomics-based research one-dimensional (1D) NMR technique is sufficient to provide the primary data for the fingerprinting and metabolite profiling tasks. However, numerous multidimensional experiments, such as correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY), have also been developed to enhance the sensitivity of the technique, and assist in metabolite identification.

The data generated by NMR spectroscopy are highly reproducible, thus allowing the application of library searches based on comparison of spectral data from different sources, such as those listed in Table 30.2. The use of statistical methods in analysis of constituents from a mixture further enhances the versatility of NMR in metabolomics analysis [3].

GC-MS has been applied in metabolomics studies much earlier than LC-MS and NMR spectrometry. It has been the preferred method for the analysis of volatile metabolites, especially in plant metabolomics. It offers high sensitivity and resolution, in addition to providing excellent and highly reproducible mass fragmentation data. The mass data generated are therefore very well suited for metabolite identification through spectral matching with those in the established MS databases (such as NIST, Wiley, and Fiehn Metabolomics libraries).

The disadvantage of GC-MS-based metabolomics is in the sample preparation, which is rather tedious, time-consuming, and can be error-prone. Involatile metabolites need to be derivatized in order to make them volatile. During this process, the derivatization efficiency may differ between different metabolites, and consequently, the true representation of their concentrations may be lost. Furthermore, different forms of derivatives may be produced during the derivatization reaction, adding new constituents to the sample. There are also other issues related to this process, such as degradation and by-products formation [12].

LC-MS has been the most widely used MS-based technique in metabolomics. Its ability to separate and detect a wide range of metabolites has been most attractive, especially in the bioanalytical field. The method can achieve

TABLE 30.2 List of Representative Nuclear Magnetic Resonance (NMR) Databases and Software Tools

Name of database, tools and address.	Description
NMRShiftDB2: http://nmrshiftdb.nmr.uni-koeln.de/	An open-source NMR database for organic structures and their NMR spectra including prediction (^{13}C, ^{1}H, and other nuclei); also spectral and structural search.
NMRDB: http://www.nmrdb.org/	A freely available database comprising three services: NMR resurrector; NMR assigner; and NMR predictor.
BMRB (Biological Magnetic Resonance Bank): http://www.bmrb.wisc.edu/	An open access database which collects, annotates, archives, and disseminates spectral and quantitative data derived from NMR spectroscopy.
MDL (NMR metabolomics database of Linkoping): http://www.liu.se/hu/mdl/main/	An online database and publically accessible depository dedicated to the omics of small biomolecules, to facilitate access to NMR parameters.
MMCD (Madison Metabolomics Consortium Database): http://mmcd.nmrfam.wisc.edu/	A freely available database hosting a large collection of raw ^{1}H, ^{13}C, and 2D NMR spectra.
Name of NMR tools.	**Description**
Chenomx NMR Suite: http://www.chenomx.com/software	An integrated suite of tools allowing researchers to identify and quantify in NMR spectra.
AMIX: http://www.bruker.com/products/mr/nmr/nmr-software/software/amix/overview.html	A software developed by waters for analysis of NMR and MS data.
Mestrelab: http://mestrelab.com/software/mnova/sma/	An NMR data processing, analysis, simulation, and reporting as well as prediction of NMR spectra from molecular structure.
ACD/NMR Predictor and ACD/Structure Elucidator Suite: http://www.acdlabs.com/products/	A commercially available tool for prediction and structure elucidation of NMR data developed by ACD/Labs.

collection of both quantitative and qualitative information at picogram per milliliter sensitivity. Mass accuracy is highly important in molecular identification, and thus most of the LC-MS-based metabolomics are conducted on the Time of Flight machines due to its sensitivity, rapid data acquisition, and accurate mass capability.

30.5 DATA ACQUISITION AND PROCESSING

The data generated from spectrometric instruments are in the form of spectra, which are denoted as absorption (ultraviolet, Raman, or infrared), chemical shifts (NMR), and mass over charge ratio (m/z) for MS. The data from chromatographic methods, such as GC-MS and LC-MS, are called chromatogram, and contain additional useful information, the retention time or retention factor of each metabolite. All these data are in their raw form and need to be converted into extracted data, such as peak tables, so that they can be processed by statistical, metabolite identification, and quantification tools.

There are a number of issues inherently present in metabolomics data sets, including the baseline drift, retention time shift, noise, and artifacts present in the raw data, especially in the GC-MS or LC-MS. The terms "preprocessing" and "pretreatment" have been used to refer to the preliminary steps before the real data processing can be done. Preprocessing in the case of NMR data can refer to the phasing of Fourier transform, while in the LC- or GC-MS it can refer to deconvolution of overlapping peaks [13]. Other tasks such normalization, scaling, baseline correction, and other methods performed on the data set are classified as pretreatment. The goal of data pretreatment is to eliminate or minimize variability unrelated to the property of interest. This step is necessary so that the data become more analyzable and comparable, hence pertinent changes can be effectively modeled.

A number of preprocessing tools are available commercially, as well as in open sources or open access domains. They are also often available or incorporated in the machine vendor software. In NMR spectroscopy, software tools such as AMIX and CHENOMX include methods, such as baseline correction, elimination of water or urea signals, spectral binning (also called bucketing), scaling, and alignment. The problem of pH-induced peak shift in NMR measurement can be addressed by the use of buffer.

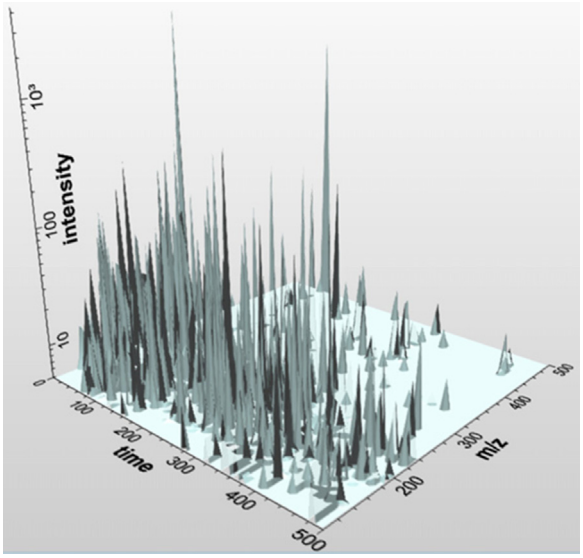

FIGURE 30.2 Representation of a 3D ion chromatogram obtained from the analysis of rat urine on UPLC−TOF-MS. *Reproduced with permission from: Theodoridis GA, Gika HG, Want EJ, Wilson ID. Liquid chromatography-mass spectrometry based global metabolite profiling: a review. Anal Chim Acta 2012;711:7−16.*

The data acquired from LC-MS analysis is very complex, and can be organized in a three-dimensional (3D) representation consisting of retention time, m/z value, and signal intensity (see Fig. 30.2). It represents a series of full scan mass spectra acquired at consecutive time points (typical scan rates are 2−20 ms). Preprocessing and treatment of this raw data may include noise filtering, baseline correction, centering, normalization, peak picking, peak integration, and retention time alignment. The 3D data were collapsed into a two-dimensional (2D) report containing data matrix in the form of a peak table, generally comprising m/z value, retention time, and intensity of the detected peaks.

Some important pretreatment and preprocessing procedures are briefly explained below.

30.5.1 Alignment

Metabolomics studies involve comparison between samples on the basis of their data sets, which represent the spectrum or chromatogram of each individual sample. Alignment is performed to achieve consistent data configuration and allows proper comparison between samples.

30.5.2 Binning

Continuous data, such as NMR spectrum or chromatogram, comprises thousands of measurements (variables). Binning is conducted to reduce the number of variables so that they can be handled by ordinary computer. It is achieved by dividing the spectrum into a desired number of bins, the same as for histograms, and summing all the measurements inside each bin to form new spectra with fewer variables (see Fig. 30.3 for reference). The most commonly used binning is an equidistant sectioning of 0.04 ppm each throughout the entire spectrum.

30.5.3 Normalization

Normalization involves a mathematical operation which attempts to justify the overall concentrations of the samples. This pretreatment strategy removes the “unwanted variation” between data of the different samples in order to focus only on the biological variation of interest.

30.5.4 Scaling

The concentration of different metabolites within a sample may differ greatly in orders of magnitude. However, highly abundant metabolites (such as glucose) may not be necessarily important from the biological aspect of the study.

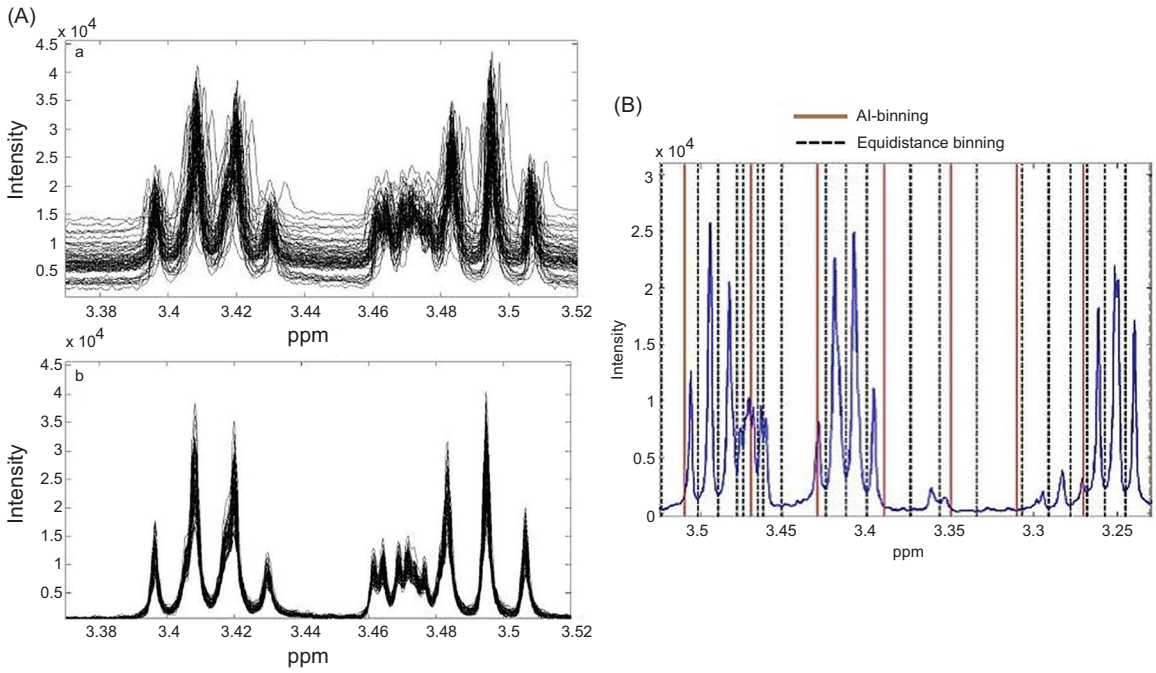

FIGURE 30.3 Examples of: (A) Alignment—before (a), and after (b); and (B) Binning—Adaptive Intelligent (AI) and equidistance binning. *Adapted from: Smolinska A, Blanchet L, Buydens LMC, Wijmenga SS. NMR and pattern recognition methods in metabolomics: rom data acquisition to biomarker discovery: a review. Anal Chim Acta 2012;750:82–97.*

Scaling takes account of the differences in concentration levels between metabolites that originate from differences in average abundance. Different scaling methods have been used for this purpose including auto-, univariate-, pareto-, range-, and vast- scalings, each of which uses different parameters as its scaling factor.

30.6 STATISTICAL DATA PROCESSING

The first step in data analysis is to explore the overall structure and find the pattern (trends and groupings) in the data. Precise inspection of the data and integration of individual peaks can provide valuable information on biochemical changes in an organism. A number of statistical methods are available to help achieve these goals, while maximizing information recovery from the data. Multivariate data analysis (MDA) has been the most commonly used among other developed methods. The most commonly practiced approach in pattern discovery is to carry out a blind unsupervised (i.e., those that do not assume any prior knowledge) data analysis, which allows an unbiased view of the data collected.

30.6.1 Unsupervised Multivariate Data Analysis

Principal Component Analysis (PCA) is one of the most commonly used methods for exploring the metabolite differences in a set of samples. This technique essentially allows the reduction of a large number of variables into a smaller number of principal components (PCs). Each PC is a linear combination of the original variables, whereby each successive PC explains the maximum amount of variance that was not accounted for by the previous PCs. Furthermore, each PC is orthogonal to the other PCs and therefore exhibits different information. The variation in spectral data is described by only a few PCs as compared to the huge number of the original variables. In addition, PCA can also be used to identify outliers in the data analysis [14].

Conversion of the original data set by PC results in two matrices known as scores and loadings, which graphically are represented as the scores and loadings plots, respectively. In the scores plot, each point represents a single spectrum. The plot summarizes all spectra and shows how they are related to each other. The points (data sets) that are close to each other would have similar profiles, while those lying far away are characterized by different profiles. The PC loadings indicate which variables have the greatest contribution in transforming them to the new variables, and show the relation among the measured variables. An important feature in the scores and loadings plots is that the directions in the

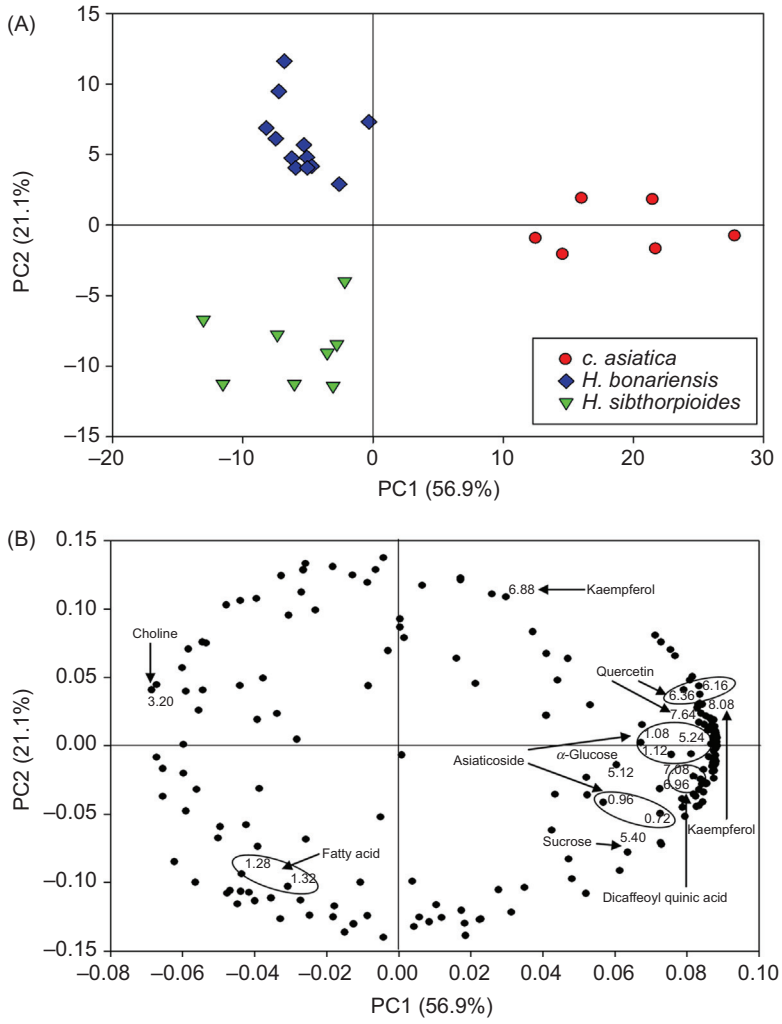

FIGURE 30.4 Two-dimensional scores (A) and loadings (B) plots of the principal component analysis (PCA) separated by PC1 and PC2 of the methanolic extracts of *C. asiatica, H. bonariensis*, and *H. sibthorpioides*. Score plot (A) shows the discrimination of three *Pegaga* varieties. Loading plots (B) indicate the ^{1}H NMR signals of compounds that are responsible for the separation of the three *Pegaga* varieties including sucrose at δ_H 5.40 (d, $J = 4.0$ Hz); asiaticoside at 5.24 (t, $J = 3.5$ Hz), 1.12 (s), 1.08 (s), 0.72 (s); dicaffeoyl quinic acid derivatives at 7.08 (d, $J = 2.0$ Hz), 6.96 (dd, $J = 8.0$, 2.0 Hz); kaempferol derivatives at 8.08 (d, $J = 8.0$ Hz), 6.88 (d, $J = 8.0$ Hz); quercetin derivatives at 7.64 (dd, $J = 8.5$, 2.0 Hz), 6.36 (d, $J = 1.5$ Hz), 6.16 (d, $J = 2.0$ Hz). *Reproduced from: Maulidiani H, Khatib A, Shaari K, Abas F, Shitan M, Kneer R, et al. Discrimination of three pegaga (*Centella*) varieties and determination of growth-lighting effects on metabolites content based on the chemometry of ^{1}H nuclear magnetic resonance spectroscopy. J Agric Food Chem 2012;60:410–417.*

former correspond to the direction in the latter. Therefore, any spectral clustering observed on the scores plot may be interpreted by examining the loadings. Graphical representation of these plots is shown in Fig. 30.4.

Hierarchical cluster analysis is another unsupervised technique widely used in metabolomics [15]. Using this method, samples are grouped into clusters according to their similarity to form a tree called a dendrogram (see Fig. 30.5). For classification purpose, the researcher has to decide on the similarity cut off, which divides the dendrogram into clusters. Unlike the PCA method, HCA does not carry information about the basis for the clustering. Thus, information on which metabolites that are responsible for the difference between clusters is missed. There are also several other unsupervised methods available, such as self-organizing map (SOM), robust-PCA, and K-means.

30.6.2 Supervised Multivariate Data Analysis

The supervised techniques make use of *a priori* knowledge of the class membership (e.g., age, bioactivity, origin) for the identification of spectral signals (or chromatographic peaks) that are different between groups or classes. The knowledge is also used to learn patterns and rules, and applies them for predicting new data. In these methods the relation between a matrix of predictors (NMR spectra or ion chromatograms) and matrix of vector or responses (e.g., class membership of biological activity) is learnt. A number of supervised methods have been developed, such as Partial Least Square Discriminant Analysis or Projection of Latent Structure Discriminant Analysis (PLS-DA), orthogonal PLS-DA (OPLS-DA), Random Forests (RF), support vector machine (SVM), and artificial neural network (ANN).

In metabolomics analyses PLS-DA and OPLS-DA are the most popularly used classification methods. In PLS-DA method, the approach is to maximize the covariance between the independent variables X (the metabolomics data) and

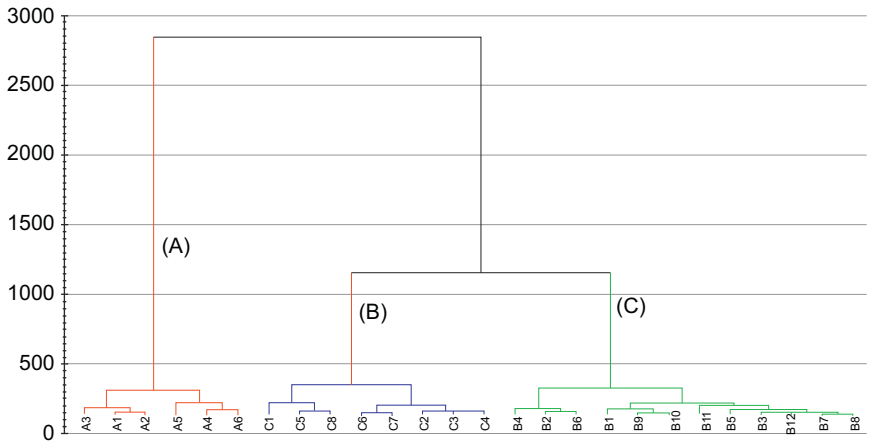

FIGURE 30.5 Dendogram of hierarchical cluster analysis (HCA) using Ward's minimum variance method of *C. asiatica* (A), *H. bonariensis* (B), and *H. sibthorpioides* (C). In HCA, clustering of samples are based on their similarity. *Reprinted from: Maulidiani H, Khatib A, Shaari K, Abas F, Shitan M, Kneer R, et al. Discrimination of three pegaga (*Centella*) varieties and determination of growth-lighting effects on metabolites content based on the chemometry of ^{1}H nuclear magnetic resonance spectroscopy. J Agric Food Chem 2012;60:410–417.*

the corresponding dependent variables Y (classes or groups, i.e., the targets of prediction, such as bioactivity) of a highly multidimensional data by finding linear subspace of the explanatory variables. The new subspace permits the prediction of Y variable based on a reduced number of factors (PLS components, also known as latent variables). The factors describe the behavior of dependent variables Y, and they span the subspace onto which the independent variables X are projected.

The main advantage of PLS-DA approach is its availability and ability in handling the highly colinear and noisy data, which are very common in metabolomics studies. It also provides several statistical parameters, such as loadings weight, variable importance on projection, and regression coefficient that can be used to identify the most important variables. The technique provides a visual interpretation of a complex data set through a low-dimensional and easily interpretable scores plot that illustrates the separation between different groups. Comparison of loadings and scores plot can support investigation in terms of identification of relationship between important variables that can be specific to the group of interest. A drawback of using PLS-DA model is the risk of constructing a model which fits the data perfectly, giving rise to 100% correct classification even if there is no real relation in the data (overfitting of the data). This is mainly caused by the presence of significantly large number of variables over the smaller number of samples. It is therefore imperative that the models constructed using this method be validated. Cross-validation and permutation testing are the means of validating the model [16,17].

OPLS-DA is an extension of PLS-DA. It is based on splitting the variations of the variables into two parts, namely, the response-correlated and the response-uncorrelated variations. The response-uncorrelated variation is often called structured noise that is caused by differences between subjects (e.g., gender, diet, age). This manner of variation separation helps the interpretation of the model and identification of the important variables. The fine structures of the original peaks are maintained and this type of plots (such as S-line plot and S- plot, see Figs. 30.6 and 30.7, respectively) usually aids metabolite identification.

The predictive power of OPLS-DA and PLS-DA are comparable. However, interpretability of OPLS-DA is superior to PLS-DA, since irrelevant variations are filtered out [18]. Just like PLS-DA, validation of OPLS-DA is extremely important when using these models, and the results of cross validation should be reported for each model.

SVM is a nonparametric machine learning technique applicable for solving classification and regression problems. This method is based on the mapping of data into a high-dimensional space that allows the separation of two groups of samples into distinct regions separated by "support vector." SVM is primarily developed to solve binary problems, such as "one against one" or "one against all" types of analysis [19]. RF is also used for either classification or regression purposes based on construction of a large number of decision trees (forest). For prediction, a majority tree-vote is taken for classification, while an average of output from different trees is taken for regression [20]. For nonlinear model, the ANN is one of the most popularly used to develop relationship between variables [21]. It is a processing tool made up of a number of simple, highly interconnected processing elements, which process information by their dynamic state response to external inputs. ANN is built by input, hidden, and output layers having several neurons. Every neuron in different layers is connected by a weight value, and it also has a nonlinear "activation function." A review on the recent development in statistical methods in metabolomics has recently been published [22].

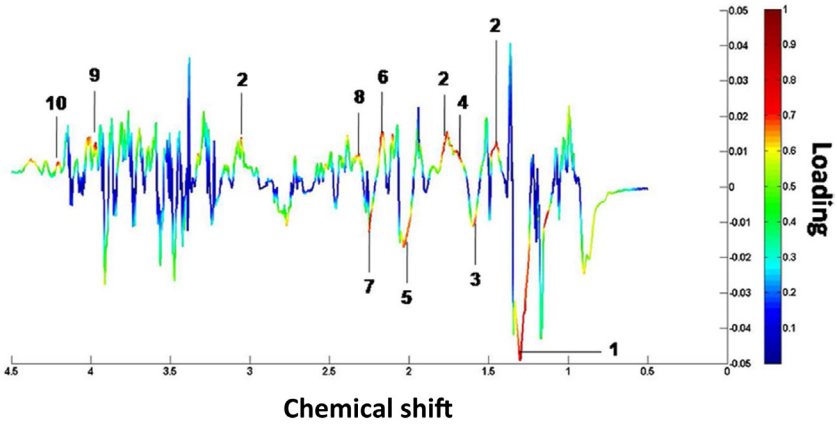

FIGURE 30.6 S-line plot. Significance of variation in metabolites between idiopathic recurrent spontaneous miscarriage (IRSM) and controls is represented by the color map plot. The blue color represents least correlation, whereas red color represents highest correlation. Peaks with positive loading signify increased metabolites in IRSM (2, L-lysine; 4, L-arginine; 6, L-glutamine; 8, L-valine; 9, L-histidine; 10, L-threonine), and negative represent decreased metabolites (1, lipoproteins; 3, adipic acid; 5, proline; 7, acetone). *Reprinted with permission from: Banerjee P, Dutta M, Srivastava S, Joshi M, Chakravarty B, Chaudhury K. ^{1}H NMR serum metabonomics for understanding metabolic dysregulation in women with idiopathic recurrent spontaneous miscarriage during implantation window. J Proteome Res 2014;13:3100−3106.*

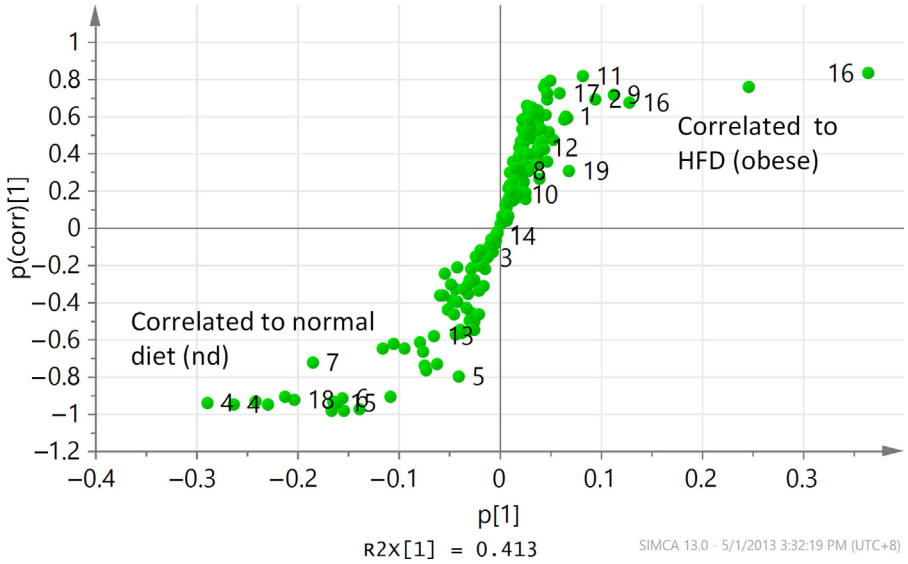

FIGURE 30.7 Example of the S-plot to identify putative biomarkers in obese and normal conditions (in rat model). In this plot the metabolites located in the upper-right region correspond (e.g., 16, 9, 11, 12) to the treated condition, in this case obese, while those in the lower-left region (e.g., 4, 18, 7, 6, 15) correspond to the normal condition.

30.7 METABOLITE IDENTIFICATION

Metabolite identification is usually carried out after the quantification and statistical data analysis. It is also one of the major tasks in metabolomics. Proper identification of metabolites is essential for accurate biological interpretation, particularly in multiple metabolomics studies. Currently, only the NMR- and MS-based, either alone or in combination, are the commonly used techniques. The approaches in metabolites assignment from NMR data are different from those used for the LC-MS or GC-MS data. Nevertheless, spectral libraries for both NMR- and MS-based metabolomics profiling are available as reference for the task. In some cases, the identification process has to be done manually and is time-consuming. Consequently, metabolites assignment is usually carried out after statistical data analysis, and only done on the most relevant compounds (also known as biomarkers).

30.7.1 NMR-Based Identification

1D ^{1}H NMR spectroscopy has become the primary source of NMR-based metabolomics in a diverse research areas ranging from plant to human diseases. The high reproducibility of this type of spectra made it possible to carry out

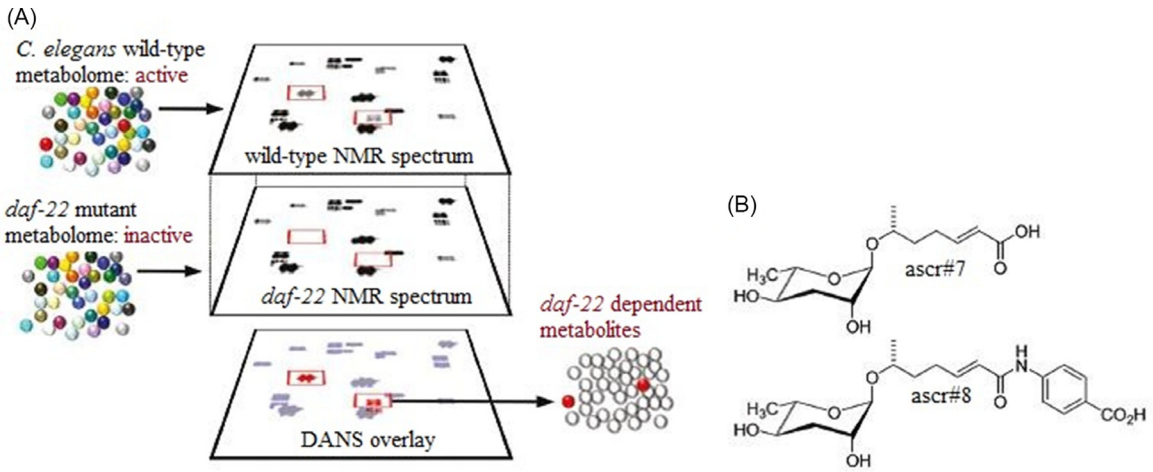

FIGURE 30.8 Schematic representation of differential 2D NMR spectroscopy (DANS) applied in the identification of male-attracting pheromones in *Caenorhabditis elegans* (CA) via differential analysis of 2D NMR spectra (DANS): (**A**) overlay of COSY spectra from CA wild-type and daf-22 mutant metabolomes reveals daf-22-dependent signals; (**B**) structures of two new metabolites, ascr#7 and ascr#8, that were identified from additional analysis of the daf-22-dependent signals. *Reproduced with modification from: Robinette SL, Bruschweiler R, Schroeder FC, Edison AS. NMR in metabolomics and natural products research: two sides of the same coin. Acc Chem Res 2012;45:288–297.*

metabolite identification based on comparison with those in the literature or databases. Over the years multiple tools have been developed to assist metabolite identification, and a sample of these are listed in Table 30.2.

Aside from the common 1D NMR, the use of 2D NMR is also popular [23]. Using these techniques, which basically utilize specific pulsing sequence during the measurement, direct evidence of the connectivity and spatial features of the molecule in question can be deduced. Among the 2D techniques, the 2D J-resolved spectroscopy has been the most popular technique used in metabolite identification. Using this, simpler visualization of spectra is achieved by presenting the chemical shift along one axis (x) and the coupling along the other (y) axis. COSY is another 2D method, which provides spectral information on couplings between a pair of nuclei represented as cross-peaks. The application of high-resolution double quantum filter-COSY has been proven successful in identification of unknown constituents from a complex mixture [24]. Differential analyses of 2D NMR spectra (DANS), a method for graphic comparison of 2D NMR spectra representing different biological states, were also applied to metabolite extracts (e.g., see Fig. 30.8). Differential comparison of 2D spectra using DANS may constitute a simple method for selecting signals in complex spectra that warrant more detail analysis [25].

The NMR spectra of mixtures are essentially a representation of superimposed spectra of the individual components making up the mixtures. On this basis, the spectra of a mixture can, in principle, be deconvoluted into individual spectra of the components. TOCSY was used to help simplify structural determination by dividing the proton signals into groups or coupling networks present in the entire molecule. Furthermore, a method named DemixC has been developed for deconvolution of (^{1}H, ^{1}H)-TOCSY spectra of mixtures into 1D traces, which represent the individual proton-spin systems of the components [26,27]. The 1D subspectra generated by this method closely resemble the normal ^{1}H-NMR spectra, which can then be applied for spectral matching with available in NMR spectral databases. Example of the application of DemixC in covariance spectroscopy is shown in Fig. 30.9. Statistical TOCSY (STOCSY) method was developed to generate a pseudo-2D NMR spectrum that displays the correlation among the intensities of the various peaks across the whole sample. Using this approach, two or more molecules involved in the same pathway can also display high intermolecular correlations, or can even be anti-correlation, because of biological covariance. A range of other statistical spectroscopy techniques have also emerged with varying degrees of usage in metabolomics [28].

30.7.2 MS-Based Identification

In GC-MS technique, the identification has mainly been resolved through library search, using appropriate matching criteria that map the fragmentation pattern, often in combination with retention time information. Pure GC-MS spectra are quite easily obtained by applying peak-picking or deconvolution. Unfortunately, the usefulness of library-search strategy in GC-MS for metabolites identification is limited to only those available in the libraries of known metabolites.

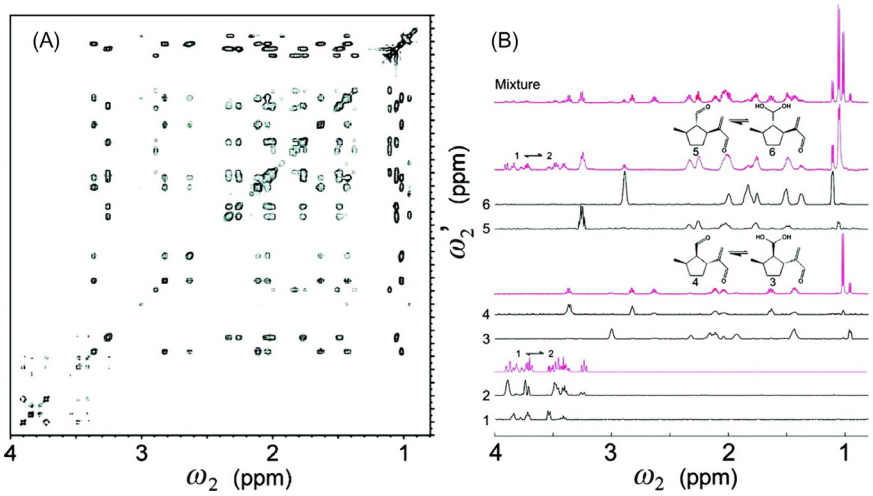

FIGURE 30.9 Covariance spectrometry. (A) Aliphatic section of covariance proton TOCSY spectrum of defensive secretion of a single walking stick insect. (B) One-dimensional ^{1}H NMR spectrum of the mixture. The six black spectra are covariance TOCSY traces extracted from covariance TOCSY of panel A using the DemixC approach. The bottom three red spectra are reference 1D spectra of purified components. Each reference spectrum contains two species, R-glucose (trace 1) and β-glucose (trace 2); dialdehyde and diol forms of the anisomorphal (traces 4 and 3, respectively); and the peruphasmal (traces 5 and 6, respectively) monoterpenes. Chemical structures of the anisomorphal and peruphasmal and their corresponding geminal diols are shown as insets. *Reproduced from: Robinette SL, Bruschweiler R, Schroeder FC, Edison AS. NMR in metabolomics and natural products research: two sides of the same coin. Acc Chem Res 2012;45:288−297.*

In the case of novel metabolites, identification may be accomplished with the help of databases that contain fragmentation spectra prediction or spectral simulation functions.

The use of LC-MS enables identification and quantification of highly polar compounds, including organic acids, fatty acids, amino acids, and steroids, without the need of prior derivatization. LC-MS analysis is generally performed using soft ionization techniques, such as electrospray ionization and atmospheric pressure chemical ionization, which do not cause fragmentation of the molecular ions, and thus allows the molecular formulae determination. However, the identification process in the metabolite analysis is both analytically and computationally challenging due to the large diversity of compounds measured, the large variation in fragmentation patterns for different types of experiments, and the low reproducibility of the data generated by different instruments. In order to cope with these challenges, various databases and software tools have been developed that identify mass signals (see Tables 30.3 and 30.4 for representative tools).

The use of high mass accuracy spectrometers is prerequisite in metabolite structural characterization. However, marker identification cannot be solely based on high mass accuracy data. The safer way is to combine high mass accuracy data with database search in the general mass spectral databanks (e.g., METLIN), the specialized spectral databanks (e.g., Lipidmaps) and biochemical databases (e.g., HMDB or ChemSpider), or others. Using this approach a shortlist of potential candidates can be achieved, which can then be shortened further by performing MS/MS experiments, applying isotope ratios, and application of the nitrogen rule. Other parameters, such as polarity and retention time, can also be applied to assist in metabolite identification. The ultimate confirmation of the compounds' identity is by performing coinjection with known standards. A schematic representation of the steps followed for biomarker identification is given in Fig. 30.10.

30.8 PLANT METABOLOMICS

Plants are the major providers of most human needs. An intimate relationship between the human and plant world has evolved over generations of experience and practices. Plants are the source of food, fuel, shelter, clothing, and medicine, as well as other useful derived materials for polymers, adhesives, waxes, dyes, fragrances, and animal feedstocks. They produce a diverse array of chemical compounds, and it is estimated that as many as 200,000−1,000,000 metabolites are produced by the plant kingdom [29], while each plant could contain as many as 5000−25,000 chemical entities [30]. Thus, it is not surprising that plants have become subjects of interest in numerous metabolomics studies conducted for various applications in agriculture, nutrition, food safety, environmental science, and traditional and herbal medicine.

Early plant metabolomics research focused mainly on the analysis of genetically modified plants by gene deletion or gene transfer [31]. In the last decade, high throughput metabolomics analysis of complex mixtures in extracts of

TABLE 30.3 List of Representative Software for MS Data Processing in Metabolomics

Software/web tool	Description
MathDAMP: http://mathdamp.iab.keio.ac.jp/	A freely available software to facilitate the visualization of differences between metabolite profiles acquired by hyphenated mass spectrometry techniques.
MetaboAnalyst: http://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml	A Web-based metabolomic data processing, statistical analysis, and functional interpretation software. It allows functional enrichment and pathway analysis.
MetAlign: https://www.wageningenur.nl/en/show/MetAlign-1.htm	A freely available software program for the preprocessing and comparison of full scan nominal or accurate mass LC-MS and GC-MS data.
MAVEN: http://genomics-pubs.princeton.edu/mzroll/index.php	An open-source cross platform metabolomics data analyzer packages to reduce complexity of metabolomics analysis, featuring multifile chromatographic aligner, peak-feature detector, formula predictor, pathway visualizer, and isotopic flux animator.
MZmine 2: *mzmine.sourceforge.net/*	An open-source software for MS data processing, focusing on LC-MS data; platform independent; user-friendly, flexible, and easily extendable.
XCMS online: https://xcmsonline.scripps.edu/index.php	A freely available software, which provides high-quality metabolomic analysis in a format that allows users to upload LC/MS metabolomic data.
AnalyserPro http://www.spectralworks.com/analyzerpro.html?	A data deconvolution software application for LC-MS and GC-MS data. It utilizes proprietary algorithms to detect obscured components.

TABLE 30.4 List of Representative Databases for MS Data Analysis in Metabolomics

Name of Database (Address)	Description
MassBank: (www.massbank.jp/?lang = en)	A publicly available database for compound identification and structure elucidation, including spectrum search, quick search of compounds, etc.
MMCD (Madison Metabolomics Consortium Database): *fiehnlab.ucdavis.edu*	A database containing information about small molecule data from GC-MS, NMR, direct injection MS, LC-MS, and LC-MS/MS platforms.
MMD (Manchester Metabolomics Database): *dbkgroup.org/MMD/*	The database provides knowledge on 42,687 endogenous and exogenous metabolites obtained from a wide range of sources.
HMDB (Human Metabolome Database): www.hmdb.ca/	A freely accessible database containing a comprehensive collection of human metabolome data. It supports extensive text, chemical structure, and query searches.
PubChem: https://pubchem.ncbi.nlm.nih.gov/	A database of chemical molecules and their activities against biological assays, useful for search of properties, chemical structure, name fragments, chemical formula, molecular weight, etc.
METLIN: https://metlin.scripps.edu/	A metabolite database for metabolomics to assist in a broad array of metabolite research and identification. Repository of MS/MS metabolite data is accessible to public.
KEGG (Kyoto Encyclopedia of Genes and Genomes): (www.genome.jp/kegg/kegg1.html)	A database resource containing a collection of databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances.
Lipidmaps: http://www.lipidmaps.org/	A multiinstitutional effort to identify and quantitate all of the major and minor lipid species in mammalian cells using systems biology approach and mass spectrometry.
ChemSpider database: http://www.chemspider.com/	*ChemSpider* is a free chemical structure database providing fast text and structure search access to over 34 million structures from hundreds of data sources.

plant has been actively conducted, and its potential use is well recognized. Metabolomics technique has been exploited to survey phytochemical diversity among herbs used in traditional medicine, and has been shown to be useful in discriminating varieties based on chemical composition, thus allowing growers to implement standards for quality control. For example, ^{1}H NMR-based metabolomics has been used to discriminate and identify the marker compounds of three morphologically resembling species of medicinal Pennyworts (Apiaceae), and to describe the effects of light exposure to the metabolic profiles on these plants. Saponins, asiaticoside, and madecassoside, along

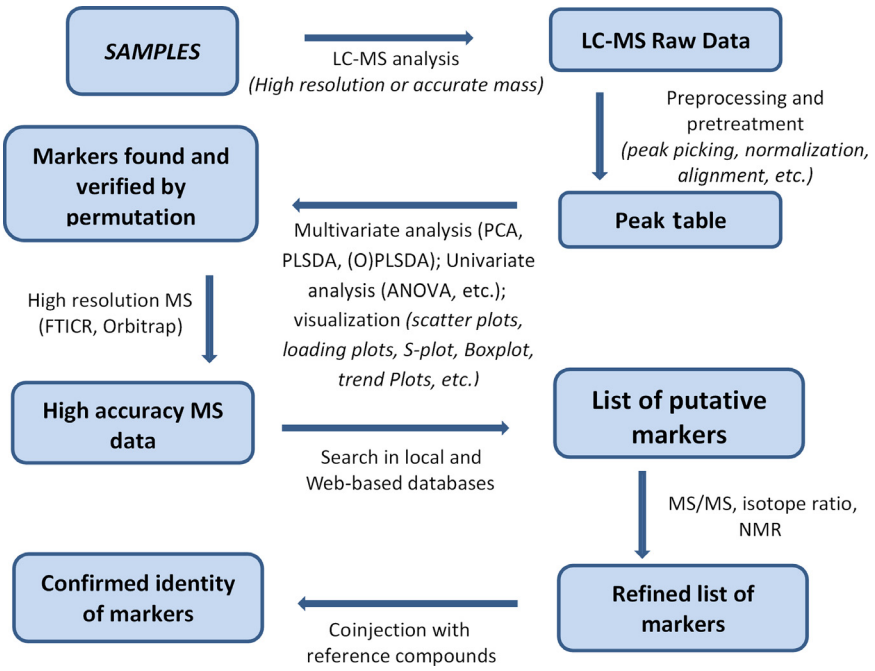

FIGURE 30.10 Schematic representation of the steps of biomarker identification in MS-based metabolomics.

with the chlorogenic acids were the metabolites that contributed most in distinguishing them (see Fig. 30.4) [32]. The effects of climatic conditions on the metabolites in green tea (*Camellia sinensis*) have been studied using NMR-based metabolomics and revealed that high temperature, long sun exposure, and high precipitation stimulate theanine synthesis during the spring season [33].

The correlation of a plant's metabolome with various biological activities has been reported. Metabolic profiling using NMR spectrometry showed that the free radical scavenging activity of guava leaves (*Psidium guajava*) was dependent on the harvesting time, which correlated well to the content of catechuic and protocatechuic acid [34]. It was also shown that the samples of *N. sativa* seeds originating from different countries can be efficiently classified using NMR [11]. The effects of drying methods in postharvest treatment of medicinal herb, *Cosmos caudatus*, utilizing ^{1}H NMR metabolomics approach has also been investigated, and revealed that air, oven, and freeze drying methods retained the major constituents (mainly flavonoids), but alter their concentrations [35]. In an effort to boost the yield of medicinally important indole alkaloids, the study on transgenic *Catharanthus roseus* using NMR-based metabolomics was able to confirm that overexpression with octadecanoid-derivative responsive *Catharanthus* AP2-domain (ORCA-3) and geraniol 10-hydroxylase (G10H) genes can significantly increase the accumulation of strictosidine, vindoline, catharanthine, and ajmalicine, but had limited effects on anhydrovinblastine and vinblastine levels [36].

Metabolite fingerprinting followed by principal component analysis is a powerful tool to differentiate plant samples. This approach can be used to trace the origin of samples based on their genetic factor, geographical location, climatic condition, or agricultural practice. It can also be used to identify the effect of postharvest treatment, and to track down the biochemical changes taking place during maturing process of plant or postharvest degradation. The knowledge can be used to design the strategy to control the progression of the relevant transformation, thus prolonging the shelf-life and conserving the quality of the plant products.

30.9 METABOLOMICS IN HERBAL MEDICINAL (PHYTOMEDICINAL) RESEARCH

Medicinal plants and herbs have been the major inspiration for scientific investigation since the early days of modern science, particularly in the field of pharmacognosy. The main interest in most of the studies on these plants has been directed toward the discovery of novel bioactive compounds, with the hope to develop them into drugs. Such efforts have been rewarded with the availability of numerous useful drugs used today to combat human diseases. Even at the dawn of the 21st century, 11% of the 252 drugs considered as basic and essential by the WHO were exclusively derived from flowering plants [37]. In spite of this, plant-based traditional methods for treating diseases are still being practiced by as much as 80% of the world population today [38].

In recent years, traditional and herbal medicines (the term "phytomedicines" is also used mainly in Europe) have been regaining popularity, not only in developing countries, but also in the developed nations like the United States and Germany. This could be associated with the rising cost of conventional treatment and drugs, in addition to the accompanying detrimental side effects. Unfortunately, the long use and popularity of herbal medicine has not translated into its acceptability by the majority of general public and conventional medical practitioners. The main reason for this is due to the lack of scientific content, equivalent to that available for allopathic medicine. Moreover, the concept of the holistic approach in traditional herbal medicinal practices has not been well understood and received by the modern medical practitioners.

The underlying concept and administration practices applied in "traditional herbal medicine" are not comparable to that applied in "conventional medicine" [39]. Whereas the former adopts the multiple constituents for multiple targets in treatment, the latter adheres to the single constituents for single therapeutic target approach. It has been demonstrated that the outcome of administrating a pure compound (artemisinin) and that of a plant (*Artemisia annua*) extract containing the same chemical entity is essentially different. This difference was mainly caused by the complexity of a plant extract that introduces many variables to conventional phytomedicinal research, which could possibly contribute to chemical complexity and bioactivity [40]. The concept of synergistic effects, which is also understood as true over additive effects as often observed in experimental and clinical studies using phytopharmaceuticals, is not widely ascribed in modern medicine. It is only recently that modern medical therapy has begun to acknowledge this "synergy" concept and its uses in combination therapies in the treatment of several diseases [2].

The full potential of the herbal medicines will not be realized unless the chemical compositions present in herbal products are well defined. The credibility of the herbal medicines can only be established if they are appropriately standardized to ensure proper quality control, reproducibility, and accountability of the materials [41]. It forms a prerequisite for the reproducibility of the effect of the active ingredients on a batch to batch basis [42]. The lack of consistency on the efficacy and safety of herbal medicine consequently leads to the lack in public confidence toward its usefulness. The usual standardization of herbal medicine has been based on the detection of few plant chemical markers, or identification of one or two biomarkers of "pharmacologically active" constituents. Unfortunately, this does not provide a holistic view of the product, since the bioactivity is usually derived from the collective actions of multiple constituents. It is therefore imperative that all the phytochemical constituents in herbal medicines be comprehensively accounted and determined, so that their biological outcomes can be assured. This will also help to identify the possible side effects of the active constituents and finally enhance the quality control [43]. Establishing the standardization method and preparation of standardized extract are crucial before proper clinical tests on herbal medicine can be conducted.

Metabolomics is a systematic approach, which adopts a "top-down" strategy to look into the function of organisms as reflected by the end products of the metabolic network, and to detect the metabolic changes of a complete system caused by interventions. This approach agrees well with the holistic concept of herbal medicine, which embraces a complex medical science. On account of this, metabolomics offers a vast potential as the platform for standardization, and to enrich the scientific basis of traditional herbal medicine.

NMR-based metabolomics is one of the ways to develop quick and reliable quality control, as well as a metabolite profiling procedure in order to ensure quality and consistency of herbal medicines. Fingerprinting based on NMR spectrometry is robust, relatively fast, and easy to use, requiring simple sample preparation steps. This will allow verification and quality control exercises to be done in a much quicker and more accurate manner than the currently practiced methods [44]. Other fingerprinting methods, such as FTIR, GC-MS, and LC-MS, may also be adopted with some advantages and disadvantages (as discussed earlier). Adoption of these new methods may change the status and scenario of herbal medical practices in health care system.

The use of biomarkers can be a key to establishing "evidence-based alternative medicines." A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic interventions" [45]. It is a distinct biochemical, genetic, molecular characteristic, or substance that can indicate a particular biological condition or process. It can be used to provide information on the presence and progression of the disease state and the effect of therapeutic interventions on them. In drug discovery and development, the use of predictive biomarkers can assist in the drug approval process and clinical trial design [46]. The same concept may also be applied for herbal medicine. Identification of biomarkers could lead to the prediction of metabolic alteration due to interventions (or treatment) of herbal medicine, and the action mechanism involved during the episode.

Metabolomics has been applied in the study of metabolic impacts of plants, either in their raw or extract forms, on animal or human models. For example, the effect on metabolite profile through consumption of cruciferous vegetables (CV; i.e., broccoli and cauliflower) has been investigated on human subjects. *S*-Methyl-L-cysteine sulfoxide was identified as a urinary biomarker of CV consumption [47].

A metabolomics approach utilizing LC-MS-method on biochemical parameters and profiling of the components in ginseng roots was used to study its antidiabetic effect on Goto−Kikazaki rats (a type 2 diabetes [T2D] animal model). The study demonstrated that the therapeutic effects of the root extracts on hyperlipidemia and hyperglycemia are age-dependent of the plant, and this could be linked with the variation in both the ratios and concentrations of specific bioactive ginsenosides at different growth ages. This study introduced novel systems biology-based approaches for linking biological responses with active components in herbal extracts [48]. In the study of pathogenesis and the effect of *Centella asiatica* extract on streptozicin-induced diabetic of obese rats (T2D mimic), the metabolites of the urine and serum samples were analyzed based on an NMR-based metabolomics approach. Identification of the metabolites and quantification of their changes upon a long-term treatment of CA extract on obese diabetic rats, revealed the reversal of the glucose, lipid, tricarboxylic acid cycles, and amino acid metabolic disorders back toward the normal state [49].

Some herbal preparations, especially in the traditional Chinese medicinal system, contain multiple plant ingredients. Huang−Lian−Jie−Du−Decoction (HLJDD), a representative antipyretic and detoxifying prescription is composed of a blend of four famous traditional Chinese medicinal herbs, namely *Rhizoma coptidis*, *Radix scutellariae*, *Cortex phellodendri*, and *Fructus gardeniae*, formualted in a specific ratio. Pattern analysis of the ^{1}H NMR data (Fig. 30.11) disclosed that HLJDD could relieve stroke in rats suffering from ischemia/reperfusion (I/R) injury by

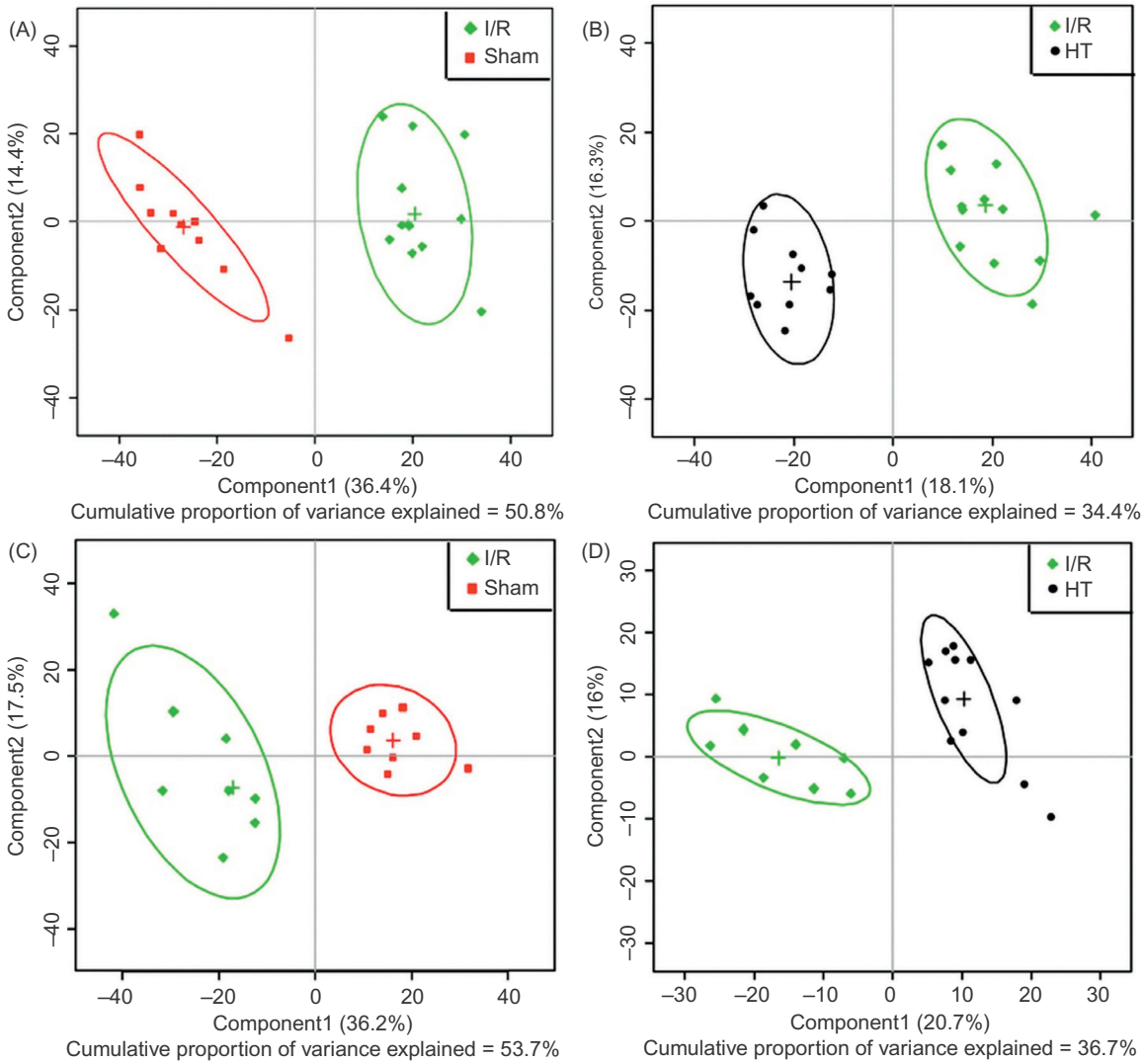

FIGURE 30.11 Scores plots from PLSDA analysis of NMR data from serum and brain tissue extracts of ischemic stroke rats: serum of sham and ischemic reperfusion (I/R) groups ($n = 9,11$, respectively) (A), and of I/R and HLJDD-treated (HT) groups ($n = 11,10$, respectively) (B); brain tissue extracts from sham and I/R groups ($n = 8$) (C), and from I/R and HT groups (D) ($n = 8,11$, respectively). *From Wang PR, Wang JS, Yang MH, Kong LY. Neuroprotective effects of Huang-Lian-Jie-Du-Decoction on ischemic stroke rats revealed by ^{1}H NMR metabolomics approach. J Pharm Biomed Anal 2014;88:106−116.*

ameliorating the disturbance in metabolic processes including the energy, membrane, mitochondrial, neurotransmitter, and amino acid metabolisms. The overall outcomes are the alleviation of oxidative stress from reactive oxygen species and the inflammatory damage [50].

Notwithstanding the value of the efforts in understanding the underlying biochemical implication of herbal treatment, most conventional studies on medicinal plants have been fragmented. The data on metabolite profiles of the plants involved has not been included in most of the studies and discussions. Such information will be crucial in ensuring the reproducibility of the current results. The use of holistic metabolomics approach in these studies will pave the way to understanding of the mechanism of action of the multicomponent system in herbal medicinal practices. Furthermore,some of the techniques developed in metabolomics may be applicable in the clinical study settings.

30.10 METABOLOMICS IN DRUG DISCOVERY AND DEVELOPMENT

Conventional drugs will remain the prime approach in disease intervention for some time to come. However, in recent years the number of new molecular entities introduced by the pharmaceutical industry as new drugs has declined dramatically [51]. The failure of drug candidates during Phase II and III clinical trials has been blamed for the cause of this, which has also inflicted time and financial losses to the pharma industry. The FDA through its Critical Path White Paper in 2004, has set the development of biomarkers in the areas of genomics, proteomics, and metabolomics as the highest scientific priority in the drug development efforts [51]. It was also suggested that biomarkers should be employed in preclinical development for the early detection of the likely-to-fail candidates. Among the "omics" technologies, metabolomics was shown to be useful in assisting the preclinical development phases of discovery, pharmacology and toxicology.

Drug safety is an important issue in medicine [52]. There have been numerous cases related to adverse effect of drugs, which led to their removal from the shelves, and ended up with legal suits. There is also a huge variation in drug interactions amongst individuals, depending on their age, gender, and genotype. The application of metabolomics in drug toxicity studies has been an area of interest in drug development programs. Discovery of biomarkers relevant to the toxic effects of new drug candidates at the earlier (preclinical) stage could save the overall cost of drug development [53]. Metabolomics offers an inexpensive and convenient ways to monitor drug dosage and clearance in patients, as well as to monitor drug toxicity and adverse reactions. In relation to this, a ^{1}H NMR metabolomics database has been created by the Consortium for Metabolomics Toxicology (COMET) in order to assist in vivo toxicological assays on drugs at preclinical and clinical levels [54]. Various statistical/chemometric software for organ toxicity prediction were developed based on this database, which could further help in the identification of organ-specific toxins.

The diversity of metabolite signatures for acute organ toxicity evaluation by employing NMR- or LCMS-based analytical methodologies has been known from several studies. For example, in one study, gentamicin-induced renal toxicity was found to be associated with elevated levels of glucose and reduced levels of trimethylamine *N*-oxide (TMAO), xanthurenic acid, and kynurenic acid in urine [55]. Metabolomics in conjunction with toxicogenomics revealed the mechanism responsible for the liver damage induced by the high doses administration of pentamethyl-6-chromanol (PMCol), an anticancer drug, by the inhibition of glutathione synthesis, and alteration of drug metabolism pathways [56]. The effect of viral infection on metabolic state has been investigated on cultured human fibroblasts infected by influenza virus-A, herpes simplex virus type-1, and human cytomegalovirus, and revealed that substantial metabolome alteration was induced by all the viruses [57–59].

In the search for bioactive natural products, LCMS-based metabolomics was employed to de-replicate bacterial strains of *Micromonospora* spp. and *Verrucospora* spp., isolated from tropical ascidians. The study confirmed that analysis of secondary metabolites provided finer resolution of strain differentiation than genetic sequence [60]. Further application of the LC/MS-PCA approach led to the discovery of novel polyenepyrones from marine-derived *Streptomyces* spp. grown on solid media.

Understanding the mode and mechanism of drug actions is central to optimizing their use, and the discovery of new therapeutics with novel targets. Metabolomics has been used to characterize the antibacterial properties of triphenylbismuth dichloride (TPBC), an antibacterial agent successfully used against device-associated infections [61]. Using extracellular metabolites profiling based on NMR spectroscopy, a unique TPBC-mediated change in the metabolites of *Staphylococcus aureus* was identified, which indicated that TPBC blocks bacterial pyruvate catabolism (Fig. 30.12).

Global understanding of drug action at molecular, cellular, and whole-organism levels may now become achievable through bioinformatics (including metabolomics) methods that mine and integrate the data available from different screens. This holistic approach will enhance drug discovery, as well as facilitating the better understanding of human health at system level. Metabolomics approach is increasingly important in improving the clinical trial outcomes in the context of drug discovery and precision medicine.

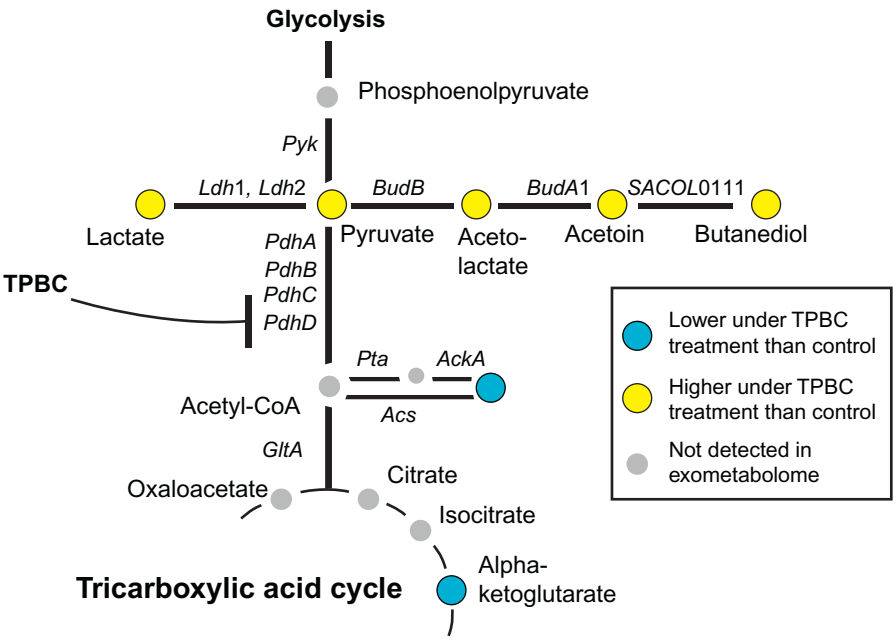

FIGURE 30.12 Schematic representation of the pyruvate metabolic pathways in *Staphylococcus aureus*. *Reproduced with permission from: Birkenstock T, Liebeke M, Winstel V, Krismer B, Gekeler C, Niemiec MJ, et al. Exometabolome analysis identifies pyruvate dehydrogenase as a target for the antibiotic triphenylbismuthdichloride in multiresistant bacterial pathogens. J Biol Chem 2012;287:2887–2895.*

30.11 SUMMARY

Metabolomics is an emerging and rapidly evolving technology tool, which involves quantitative and qualitative metabolite assessments. It offers tremendous promise for diverse applications in various fields such as medical, environmental, nutrition, and agricultural sciences. In pharmacognosy, metabolomics provides unique and improved means of characterizations that permit biological sample authentication, better understanding of herbal medicinal practices, and in drug discovery. Metabolomics allows system level metabolic analysis, which could be used in detection of disease-related biomarkers. Moreover, it provides a snapshot of the functional genetic status of an organism, which allows the possibility to understand the relationships between metabolic changes, the related biochemical pathways, and gene functions.

Application of metabolomics in biological sciences has grown exponentially since the turn of the 21st century, indicating the potential importance of this technique. In this chapter we have discussed rudimentary issues and the strategies involved (especially the LC-MS and NMR-based) in metabolomics, from which readers can gain some basic concepts and philosophy. This in turn will help generate ideas for its application in their own research. Specific topics relevant to pharmacognosy, including the plant metabolomics, metabolomics in herbal medicine, and metabolomics in drug discovery and development were included in this chapter. Additionally, safety and efficacy issues hampering the public acceptance of traditional and herbal remedies were discussed. The issues may be resolved through understanding of their holistic nature, which could be realized by the application of metabolomics in the relevant studies.

The holistic approach of metabolomics is also perceived to be the tool for pursuing "system biology," and may eventually lead to setting a new paradigm in health and the health care system. Metabolomics is a developing field, and there is still plenty of room for improvement, refinement, and expansion for its application.

30.12 REVIEW QUESTIONS

1. Describe the basic concept of metabolomics as a tool for research in pharmacognosy.
2. Describe the reasons why metabolomics could become useful in understanding the holistic concept of herbal medicine.
3. Explain the rationale behind the belief that metabolomics approach is different from the conventional methods in pharmacognosy.
4. What are the factors that can cause variations in metabolomics analysis?
5. Describe the way to develop a potent medicinal plant into a herbal drug.

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Chapter 31

Novel Targets in Drug Discovery

N. Laurieri[1] and R. Delgoda[2]
[1]University of Oxford, Oxford, United Kingdom, [2]University of the West Indies, Mona, Kingston, Jamaica

Chapter Outline

Learning Objectives

- To introduce the basic principles and standard methods of target discovery
- To provide illustrative examples of recently discovered targets
- To assess emerging trends and future prospects in drug development

31.1 STRATEGIES AND TECHNIQUES IN TARGET DISCOVERY

The current paradigm in drug discovery is based on the functional modification of a target protein using small-molecule chemical modulators (inhibitors, activators) or biological biopharmaceuticals (antibodies). The process of drug discovery and development usually covers a long period of time (10−17 years), but with an overall probability of success lower than 10% [1] (Fig. 31.1A).

The identification of a new "druggable" target is the first crucial stage in the multistep drug discovery pipeline, and a robust rationale is required to validate likely disease-related targets. The term "target" has various connotations in the general context of drug discovery: it usually refers to a specific protein whose functional modulation has a high potential to improve disease outcomes. "Targets" could also be considered in a broader sense in that target selection might comprise a whole therapeutic area: this connotation is mainly employed in the context of systemic and multifactorial diseases, such as central nervous system (CNS) pathologies.

The target discovery process is composed of three main steps: the provision and elaboration of cellular and animal models in preclinical studies, the identification of a putative target, and the final validation of the proposed target (Fig. 31.1B). Lindsay exhaustively analyzed two distinct broad approaches in target discovery, namely, "molecular" approach and "systems" approach, which have been widely adopted in an alternative, but complementary manner [2]. Samples from clinical cases or appropriate cell line models are generally employed in the molecular strategy and most of the validated targets are intracellular proteins. In contrast, the "systems approach" looks at the disease process in whole organisms, such as clinical patients or *in vivo* animal models, and has been extensively chosen for diseases whose phenotypes could only be investigated at a systemic level and where the molecular basis may be unknown for a complex of interacting factors.

The majority of current treatments have been traditionally discovered through the systems approach and subsequent biochemical studies have allowed the determination of the molecular targets against which the drug reacts. In the last decade or so, the "molecular approach" has predominated since extensive investigations in the molecular biology domain have successfully led to the understanding of several cellular mechanisms underlying important disease phenotypes.

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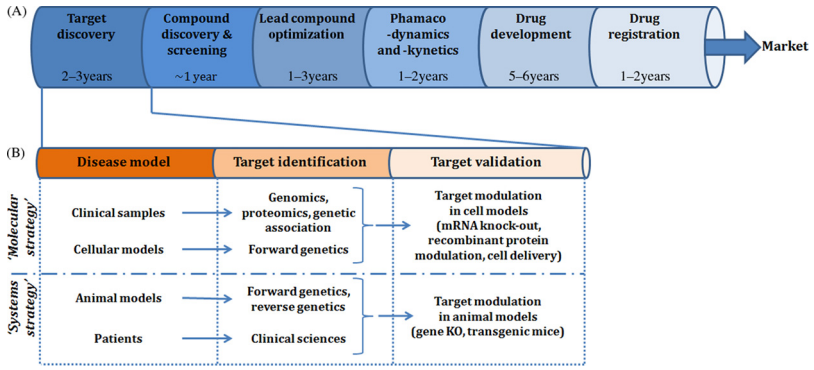


FIGURE 31.1 Drug discovery process. (A) *De novo* drug discovery constitutes a multistep process and the target discovery stage initiates this long-time procedure. (B) Molecular and systems-based approaches in target discovery are composed of three steps: the preparation of disease models or tissue samples, target identification, and target validation.

31.1.1 Disease Models

Ideally, healthy and diseased patients would be the most appropriate subjects to both perform clinical investigations and obtain tissue samples for drug discovery. However, this methodology is often impractical and could give rise to ethical issues. Cellular and animal models are therefore frequently chosen in an attempt to reproduce the relevant disease phenotypes. Evident limitations have to be taken into account for these models: for instance, the difficulty to reproduce *in vitro* the complex intracellular interactions, the lower biochemical resemblance between immortalized cell lines and primary clinical samples, or the genomic differences between humans and animal models. Additionally, the replication of long-term environmental factors in animal models, such as those at the origin of cardiovascular, nervous, and cancer diseases, constitutes a challenging, but intractable research field.

31.1.2 Target Identification

In recent years, genomics, proteomics, and genetic techniques have been commonly employed as effective tools to understand the cellular function of a protein and identify putative targets whose modulation can facilitate disease regression.

Genomics compares the levels of gene expression in normal cells *versus* disease samples using microarray techniques and relies on the fact that genes are differentially expressed in cells from healthy and pathological tissue [3]. Proteomics further tries to advance the knowledge of protein function in disease by measuring protein expression amounts and activity levels in pathological samples in comparison to normal cells; however, its utility in target discovery has been sometimes problematic for technical inaccuracies in quantifying proteins with complex post-translational modifications [4].

Also, investigating the relationship between single nucleotide polymorphisms and a disease in both control and unhealthy patients (technically called genetic association) can provide valuable results to localize possible disease-related genes in complex multigenic disorders [5]. In genetic association, target identification can be approached *via* two complimentary directions: (1) modifying a disease phenotype to identify the gene (*forward genetics*) and (2) mutating a gene to examine the phenotype alteration (*reverse genetics*) [6].

Finally, a recent review has comprehensively illustrated how the need for identifying novel drug targets has also involved the domain of natural products with the development of innovative biochemical techniques [7].

31.1.3 Target Validation

The final aim in target discovery is to prove that modulating a functional putative target in cells and animal models ameliorates the disease phenotype. A selection of different techniques can be used for this purpose. Cellular expression of the gene for the target protein could be initially modulated either directly (*via* antisense/small interfering RNA molecules) or at a transcriptional level (*via* antisense nucleic acids, ribozymes, or zinc fingers) [8]. Alternatively, direct inhibition of the putative protein could be achieved using different tools: dominant negative proteins, antibodies, RNA aptamers, or selective small-molecule inhibitors. The *in vitro* and *in vivo* delivery of successful therapeutic candidates is another major goal in the final stage of drug discovery, and might suffer from severe limitations as regards efficiency, toxicity, and selectivity [9]. In the end, viable animal studies with both transgenic and control animals, usually mice, are normally the decisive factors in making decisions to proceed further in drug development prior to initiating expensive clinical human studies. Indeed these data are required from regulatory authorities before a clinical trial would be given ethical approval.

TABLE 31.1 Physiological Processes and Diseases Targeted by Current Drugs Acting on Heart

Physiological Processes	Clinical Diseases
Myocardial contraction	Heart failure
Beat rate and rhythm	Cardiac dysrhythmias
Blood flow and metabolism	Coronary insufficiency

31.2 SELECTED EXAMPLES OF NOVEL TARGETS

In this section, examples are provided of studies illustrating the discovery of novel potential drug targets in different common diseases. These studies cover wide methodological diversity relative to their physiological domains, propose innovative mechanisms of action (MOA) for future drugs, and show how the "molecular" and "systems" approaches have been appropriately adopted in each case. This section is not intended to provide an exhaustive analysis of all recently discovered targets but gives a flavor of the range of methodologies used.

31.2.1 Heart Disease

Three physiological processes are the main objects of the drugs acting on heart, and each class represents a distinctive clinical disease (Table 31.1). Heart failure (HF) has the major prevalence with 23 million cases worldwide and the lifetime risk to develop HF is approximately 20% in the United States [10]. The high occurrence, elevated risks of morbidity and mortality, and the considerable associated healthcare costs characterize HF as one of the major public health and socio-economical issues.

HF mainly occurs when the cardiac output from the left ventricle is inadequate to meet the metabolic demands from peripheral body organs. Causes include damage to the myocardium itself, a circulatory volume overload, or excess pressure, which consequently produces water and Na^+ retentions *via* activation of the renin−angiotensin−aldosterone system (Fig. 31.2A). HF can be new (*de novo*), transient, chronic (CHF), or acute decompensated (ADHF). The sites of action of most of the current drugs used to treat HF in various complementary ways are illustrated in Fig. 31.2A.

Many disappointing attempts to reduce morbidity and mortality in CHF and ADHF using current treatments have encouraged the development of drugs toward new critical targets within the underlying cellular pathways [11]. Most of the innovative therapeutic targets are not involved in the modulation of the cascade consequences from such a cardiac dysfunction, but are mainly implicated in distinct etiologic processes: impaired cardiac contractility, Ca^{2+} cycling defects, ventricular remodeling, and management of high left ventricular pressure [12] (Fig. 31.2B).

In general, when the use of conventional diuretics and vasodilators is not sufficient to overcome both a low cardiac output and peripheral hypoperfusion, drugs increasing myocardial contractility (inotropes) have generally been indicated. However, principal concerns derived from current inotropic drugs (digoxin, dopamine, and phosphodiesterase 3), such as increase of myocardial oxygen demands (MVO_2), harmful augmentation of intracellular Ca^{2+} concentration levels, rise of heart rate, hypotension, arrhythmias, and mortality, have stimulated significant investigations for novel potential targets to treat the impaired myocardial contractility in HF.

A recent study adopting the "molecular approach" is paving the way for the development of novel positive inotropes. In physiological cardiac myocytes, myosin is a cytoskeletal motor protein which forms Ca^{2+}-dependent cross-bridges with the protein actin in the sarcomere to undergo a force-generating power stroke (Fig. 31.2B). Preliminary *in vitro* studies showed that a panel of chemical small molecules screened against biochemically reconstituted sarcomeres were able to increase the rate, duration, and amount of myocyte contractions *via* direct activation of myosin without collateral effects on Ca^{2+} homeostasis or MVO_2. The best compounds were subsequently characterized using *in vivo* rat and dog models for HF: an increase of the systolic ejection time and an improvement of the cardiac efficiency were observed with no collateral effects on energetic MVO_2 [13]. Finally, the most powerful cardiac myosin activator (CK-1827452) was tested in both healthy volunteers and patients with stable chronic HF in a Phase I study [14]. The systolic ejection time, stroke volume, and final cardiac output were significantly improved. These results supported subsequent clinical investigation on the lead myosin activator (commercially named *omecamtiv mecarbil*) with several Phase II trials in order to further validate a promising treatment for HF [15] (Fig. 31.2C).

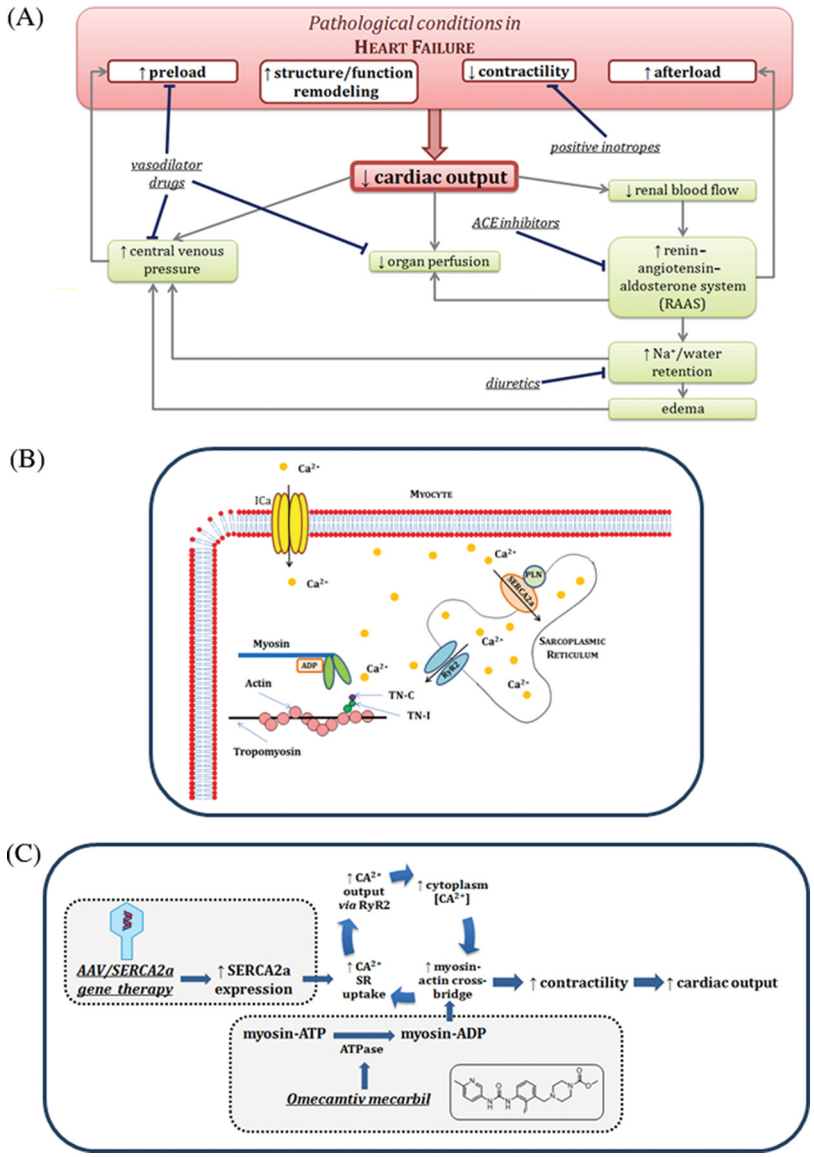


FIGURE 31.2 Conventional and innovative targets in heart failure treatment. (A) Simplified scheme showing the pathological factors in heart failure and the sites of action of the most commonly used drugs (underlined text). (B) Scheme of cyclic Ca^{2+} handling in cardiac myofilaments. Myosin (thick blue filament) contains two heads having ATPase activity. Thin black filament is made up of actin, tropomyosin, and troponin (TN). Troponin-C (TN-C) protein binds to Ca^{2+} released by the sarcoplasmic reticulum *via* the ryanodine receptor 2 (RyR2). TN-I inhibits actin–myosin binding until Ca^{2+} binds to TN-C. SERCA2a mediates Ca^{2+} uptake in the sarcoplasmic reticulum upon separation of the phospholamban protein (PLN). (C) Mechanism of action of novel therapeutic agents for treating heart failure. AAV/SERCA2a constitutes a gene therapy and omecamtiv mecarbil is a small-molecule ATPase modulator: they both have inotropic effects on myocardial contractility, thereby increasing the cardiac output from left ventricle.

In another study, the impaired contractile performance of a failing heart appeared to be targetable through a different cellular pathway. The reduction of Ca^{2+} concentrations in the sarcoplasmic reticulum (SR) of cardiac myocytes was found to cause impaired myocardial contractions in HF patients and contribute to the progression of the disease. Abnormal Ca^{2+} handling in SR seemed to result from a lower expression of a Ca^{2+} pump protein (sarco/endoplasmic reticulum Ca^{2+} ATPase [SERCA2a]) in the SR membrane; SERCA2a normally drives selective transfer of Ca^{2+} from myocyte cytosol into SR before Ca^{2+} is again delivered to the cytoplasm through the membrane protein Ryanodine receptor 2 (RyR2): this process enables the Ca^{2+}-dependent contraction of myosin–actin cross-bridges [16].

A "systems approach" was adopted to investigate how the restoration of SERCA2a activity in myocytes affects contraction with a view to developing a new treatment for HF. Transgenic mice with induced cardiac myocyte-specific overexpression of SERCA2a showed improved myocardial contractility, and restored levels of energy metabolism [17,18]. There was strong evidence that the increased inotropy resulting from SERCA2a overexpression did not have deleterious side effects such as those from standard pharmacological inotropes [18,19]. The preclinical positive results again supported pursuing clinical trials in patients with HF.

Initial attempts to target SERCA2a using small-molecule modulators were unsuccessful since SERCA2a protein is not an isolated protein but a part of an integral membrane complex with the protein phospholamban (PLN) (Fig. 31.2B). A gene therapy approach was alternatively attempted to restore physiological levels of SERCA2a enzymes

in damaged myocytes. In particular, in other HF models (rodents, pigs, and sheep), the transfer of the *SERCA2a* gene was attempted into cardiac myocyte nuclei using a recombinant adeno-associated type 1 virus (AAV1) vector, which was shown to have a favorable safety profile in humans. The successful selective restoration of SERCA2a levels in these HF animal models resulted in a significant improvement of cardiac contractility and energetics [19].

In the study "Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID)," the administration of AAV1/SERCA2a (MYDICAR) *via* percutaneous intracoronary infusions into subjects with HF allowed the restoration of SERCA2a enzyme levels without unfavorable side effects [20,21] (Fig. 31.2C). Phase II clinical trials are ongoing in order to investigate other associated aspects of SERCA2a gene therapy: in particular the long-term clinical outcomes, the quantification of SERCA2a expression levels in myocytes, and effects on left ventricular remodeling.

In addition to this gene therapy, chemical small molecules have been also sought as selective allosteric activators of SERCA2a protein using a reconstituted membrane system model. Although enzyme activation *via* allosteric structural modulators is an unusual goal in drug discovery, the initial positive results obtained from high-throughput screening were encouraging enough to pursue further with preclinical investigations on HF animal models [22].

Treatment of HF at both initiation and progression stages still constitutes a clinical challenge. There is a compelling need to expand the cardiovascular repertoire of drug targets and investigate clinical outcomes using better-designed randomized clinical trials [12].

31.2.2 Central Nervous System

The functional complexity of the CNS compared to any other organ in the body represents a challenge in pharmacology for treating brain disorders. Understanding how a drug affects individual cells can give a clear idea about the likely effects of a drug on the whole organ for systemic organs such as heart, liver, or kidney. This is not the case in the CNS. Generally, the relationship between a drug's effects at the neuronal level and its results on the functions of the entire brain and the whole organism still remains unpredictable. Thus, investigations on CNS disorders do not tend to involve the "molecular approach" in research to discover targets, although molecular and animal model studies have been strongly linked. Single gene disorders such as Huntingdon's Disease and Duchenne Muscular Dystrophy have also been important in identifying potential drug targets.

Overall, the intricacy of the brain machine is precisely regulated by a panoply of specialized neuronal mediators (neurotransmitters, neuromodulators, neurotrophic factors) which are involved in many chemical transmission pathways [23]. These biomolecules control both fast-end events and long-term adaptative processes through excitable and "unexcitable" neurons, for instance, glial cells [24]. In the last three decades, a variety of drug target proteins, such as ion channels, receptors, enzymes, and transport proteins, has been investigated in neuropharmacology and considerable knowledge has rapidly accumulated [25]. However, the occurrence and social impact of neurodegenerative diseases in aging populations has prompted a huge research effort in recent years. Alzheimer's and Parkinson's diseases constitute the most frequent brain disorders with about 24 and 7 million cases, respectively, worldwide [26,27]. In both diseases, the neuronal death appears to be caused by insoluble aggregations of misfolded proteins [28].

As a case of study, this review will focus on Alzheimer's disease (AD). Originally defined as *presenile dementia*, AD aetiology has been characterized so far by two molecular features in the CNS: the formation of amyloid plaques, which are amorphous extraneuronal deposits of β-amyloid proteins (Aβ), and the development of cellular neurofibrillary tangles (NFTs), which consist of oligomerizations of phosphorylated tau proteins into paired helical filaments (PHFs) [29] (Fig. 31.3). Efforts to block the formation of these two neurotoxic aggregations have recently been of intense interest, since no aetiologic treatments exist and current therapies (mainly cholinesterase inhibitors) are unable to retard AD progression [30].

A number of cellular pathways which are closely related to the formation of amyloid plaques or PHFs have gained great attention in research in recent times. Firstly, aggregating Aβ peptides ($A\beta_{40}$ and $A\beta_{42}$) are the products of a specific proteolysis of the amyloid precursor protein (APP) by transmembrane β- and γ-secretase enzymes; on the contrary, α-secretases cleave APP at diverse sites so that neuroprotective nonaggregating moieties (sAPPα) are generated [31,32] (Fig. 31.3). The membrane protein TACE (tumor necrosis factor-α [TNF-α]-converting enzyme) promotes the activity of α-secretase enzymes; however, this stimulation appears to be reduced in AD patients from the lower amounts of sAPPα detected in their cerebrospinal fluid [33].

In a recent study, a cascade of protein interactions underlying APP cleavage *via* TACE has been discovered (Fig. 31.3). Transgenic TG2576 mice, which have been widely used as animal models for AD, were shown to develop Aβ plaques in the brain ($TgA\beta^{pos}$) using positron emission tomography imaging [34]. This abnormal accumulation of

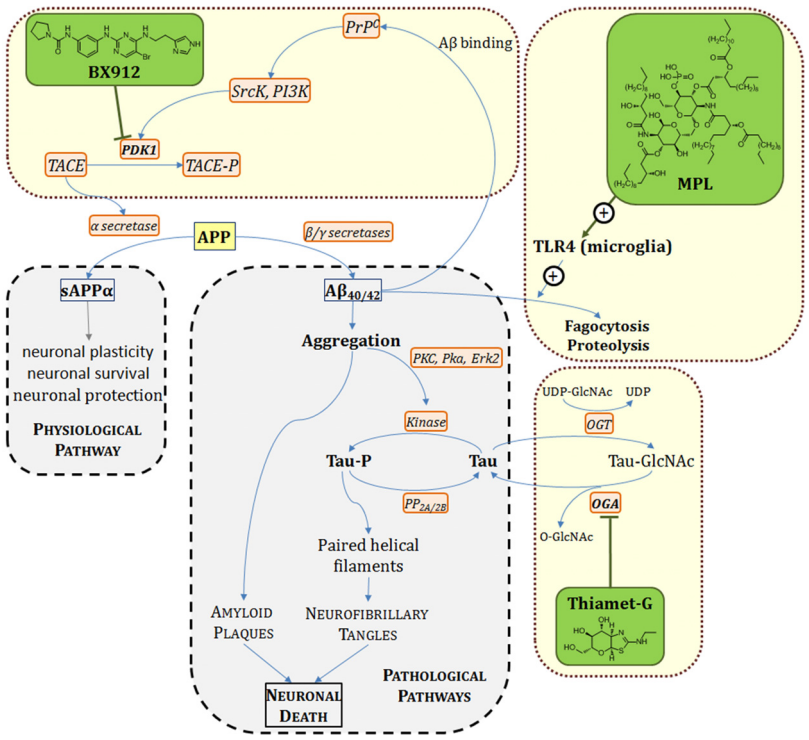


FIGURE 31.3 Pathogenesis of Alzheimer's disease and potential therapeutic pathways. Proposed physiological processing of APP by α-secretase enzymes gives rise to sAPP biomolecules which exert neuroprotective and trophic functions in neurons. Cleavage of APP at different sites by β/γ-secretases generates Aβ peptides which are amyloidogenic and provoke neurodegeneration in AD patients. The restoration of the physiological pathway or the indirect inhibition of the pathological processes by using an appropriate selective chemical modulator (BX912, MPL, and Thiamet-G) are proposed as novel therapeutic approaches to treat Alzheimer's disease. Novel target proteins (PDK1, TLR4, and OGA) are bolded; enzymes are labeled in italics in boxes.

amyloid plaques was closely related to the inactivation of TACE through selective phosphorylation by 3-phosphoinositide-dependent kinase-1 (PDK1). An increase of PDK1 activity was detected in primary cultures of hippocampal neurons from TgAβ^{pos} mice, but not in mice without Aβ plaques (TgAβ^{neg}). Two other signaling pathways in neurons (PrP^{C}-induced Src kinase and phosphatidylinositol 3-kinase [PI3K]) were shown to stimulate PDK1 activity, as suggested by parallel kinase-specific inhibitor studies with primary neuron cultures from TG2576 mice.

Direct inhibition of PDK1 in TgAβ^{pos} mice was successfully achieved using injections of a selective small-molecule inhibitor (BX912) or a PDK1-directed short interfering RNA molecule. Lower levels of phosphorylated TACE enzymes were consequently measured and the concentrations of Aβ peptides in the cerebrospinal fluid and the number of amyloid plaques in the brain diminished as compared to untreated TgAβ^{pos} mice: an increase of the neuroprotective sAPPα species was observed. Symptomatic signs in treated TgAβ^{pos} mice were also evaluated: memory and cognitive impairment and performance in behavioral tasks significantly improved.

Ultimately, the threefold increase of PDK1 activity in cerebrospinal fluid samples from AD patients reinforced the therapeutic relevance of inhibiting PDK1 to restore the physiological production of neuroprotective sAPPα peptides *via* TACE. Seeking safe small-molecule PDK1 inhibitors makes a compelling case to advance this pharmacological research.

Another study has proposed the downstream removal of the neurotoxic Aβ$_{40}$ and Aβ$_{42}$ peptides as a novel potential therapeutic approach to prevent the formation of amyloid plaques [35]. Impairment in Aβ degradation, rather than enhanced production of Aβ peptides, appears to better characterize AD pathophysiology [36]. In the CNS, microglial cells essentially participate in many intraneuronal signaling processes and can be stimulated by Aβ aggregations not only to release pro-inflammatory neurotoxic mediators, but also to induce Aβ clearance *via* phagocytolysis [37].

Toll-like receptors (TLRs) on the surface of microglia were shown to bind Aβ peptides. Stimulating these TLRs with selective agonists accelerates Aβ clearance both in *in vitro* and *in vivo* studies [38,39] and transgenic AD mice models without the *TLR4* gene showed increased levels of fibrillar Aβ in the brain [38]. Michaud and his colleagues exploited the selective property of microglial cells to capture and degrade Aβ peptides *via* TLRs with a view to discovering a potential drug target for AD. The selective stimulation of the TLR4 receptor in microglia *in vitro* using a lipid devoid of endotoxin activity, namely monophosphoryl lipid A (MPL), appeared to induce the phagocytosis of Aβ species without collateral harmful inflammatory responses (Fig. 31.3).

Additionally, weekly intraperitoneal injections of MPL into transgenic mice (APP$_{swe}$/PS1) with unpaired expression of aggregating Aβ peptides provoked both a considerable reduction in the number and size of Aβ plaques and a significant improvement in cognitive and behavioral functions. Different hypotheses to explain how peripheral administration of MPL both decreases Aβ levels in the brain and diminishes cognitive impairments have been proposed for future

investigations. Overall, stimulation of TLR4 *via* the MPL ligand in AD patients represents a promising way forward for a novel effective treatment of AD.

A third cellular pathway to possibly address neuronal death in AD has recently been studied: the oligomerization of phosphorylated tau proteins into PHFs in neurons gives rise to NFTs with apoptotic effects [40] (Fig. 31.3). Specific kinase proteins (GSK-3β, CDK5) are responsible for the hyperphosphorylation of tau proteins. The chemical modification causes tau to dissociate from the cytoskeleton microtubules and subsequently to form neurotoxic aggregations. Besides, the extracellular Aβ plaques appear to activate the formation of reactive oxygen species which in turn trigger other kinases (PKC, PKC, Erk2) which have also been implicated in tau phosphorylation [40].

In addition to the phosphorylation process, protein tau is also subject to other posttranslational modifications; in particular, the glycosylation of tau with an *O*-linked *N*-acetylglucosamine moiety (*O*-GlcNAc) has gained particular interest in AD research [41]. The glycosyltransferase, uridine diphosphate-*N*-acetyl-D-glucosamine:polypeptidyl transferase (OGT) conjugates *O*-GlcNAc from uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) to particular serine/threonine residues of tau protein. Inversely, the removal of the *O*-GlcNAc moiety from glycosylated tau species is achieved by a glycoside hydrolase, *O*-GlcNAc-ase (OGA) [42] (Fig. 31.3). Results from initial screenings of OGA inhibitors in cellular and animal studies were consistent with the hypothesis that phosphorylation is likely to compete with *O*-GlcNAc in balancing the posttranslational modifications of tau [43].

In a recent study, transgenic JNPL3 mice, which serve as an animal model for AD, were used to determine the possible neuroprotective effects of increasing the amounts of *O*-GlcNAc-tau compared with phosphorylated tau [44]. JNPL3 mice are characterized by a well-established progressive neurodegeneration which is associated to augmented levels of tau phosphorylation and oligomerization. Long-term administration of a selective small-molecule OGA inhibitor, thiamet-G, in these animal models significantly increased the amount of *O*-GlcNAc-tau species as compared to untreated mice, and gave neuroprotective effects as suggested from a decreased motor neuron loss. *In vivo* immunohistochemistry and *in vitro* biochemical analyses also revealed that the neuronal protective effects in thiamet-G-treated mice were clearly associated with reduced levels of tau aggregations in both brain and spinal cord, but surprisingly apparently independent from the tau phosphorylation state. The absence of measurable adverse side effects following continuous administration of thiamet-G in JNPL3 mice suggested focusing on the OGA enzyme is a worthwhile novel potential target to reduce neurotoxic tau oligomerizations in AD patients.

Overall, other molecular mechanisms underlying synaptic pathophysiology and memory dysfunction continue emerging incessantly in studies on both Alzheimer's and Parkinson's diseases in order to discover novel therapeutic targets for slowing the neurodegenerative progression and possibly improving the neuroprotection [45,46].

31.2.3 Infections and Cancer

At the beginning of the 20th century Paul Ehrlich coined the term chemotherapy to describe the use of synthetic chemicals which destroy infective microorganisms without major effects on the host. Nowadays, in the collective consciousness, chemotherapy is associated with drugs which inhibit the growth of cancer cells. The development of chemotherapeutic agents has constituted the hallmark in discovering drugs which are toxic for pathogenic microorganisms or tumor cells.

As regards bacterial infections, *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), remains one of the leading causes of death worldwide from a single infectious disease [47]. However, the long treatment duration and adverse effects of current antitubercular drugs (isoniazid, rifampicin, ethambutol, pyrazinamide), the emergence of multidrug resistant (MDR) Mtb strains, and the lethal synergy with HIV/AIDS underlie the compelling need for novel TB drug targets with original MOA and improved efficiency. After 40 years of unsuccessful research for novel drug candidates, the TB field has recently experienced some positive outcomes with the approval of bedaquiline [48] and delamanid [49] for MDR cases, each with an innovative MOA: the former is an inhibitor of the ATP production which normally supplies highly energetic compounds for Mtb and the latter reduces the biosynthesis of mycolic acids which constitute essential components of the mycobacterial membrane.

Generally, the discovery of novel anti-TB targets in preclinical researches tends to move through three complementary approaches, each with particular pros and cons, as exhaustively detailed in a recent review [50]: firstly, biochemistry/medicinal-chemistry studies about proteins which are critical for Mtb survival; secondly, screenings of compound libraries versus Mtb whole-cell cultures; finally, selections of effective compounds against Mtb-infected macrophages. A copious panel of original targets from different cellular pathways are emerging for possible TB treatments: iron homeostasis control, membrane transport, degradation of damaged proteins, ATP synthesis, production of mycolic acids, and cholesterol catabolism for intracellular fuel (Fig. 31.4A) [50].

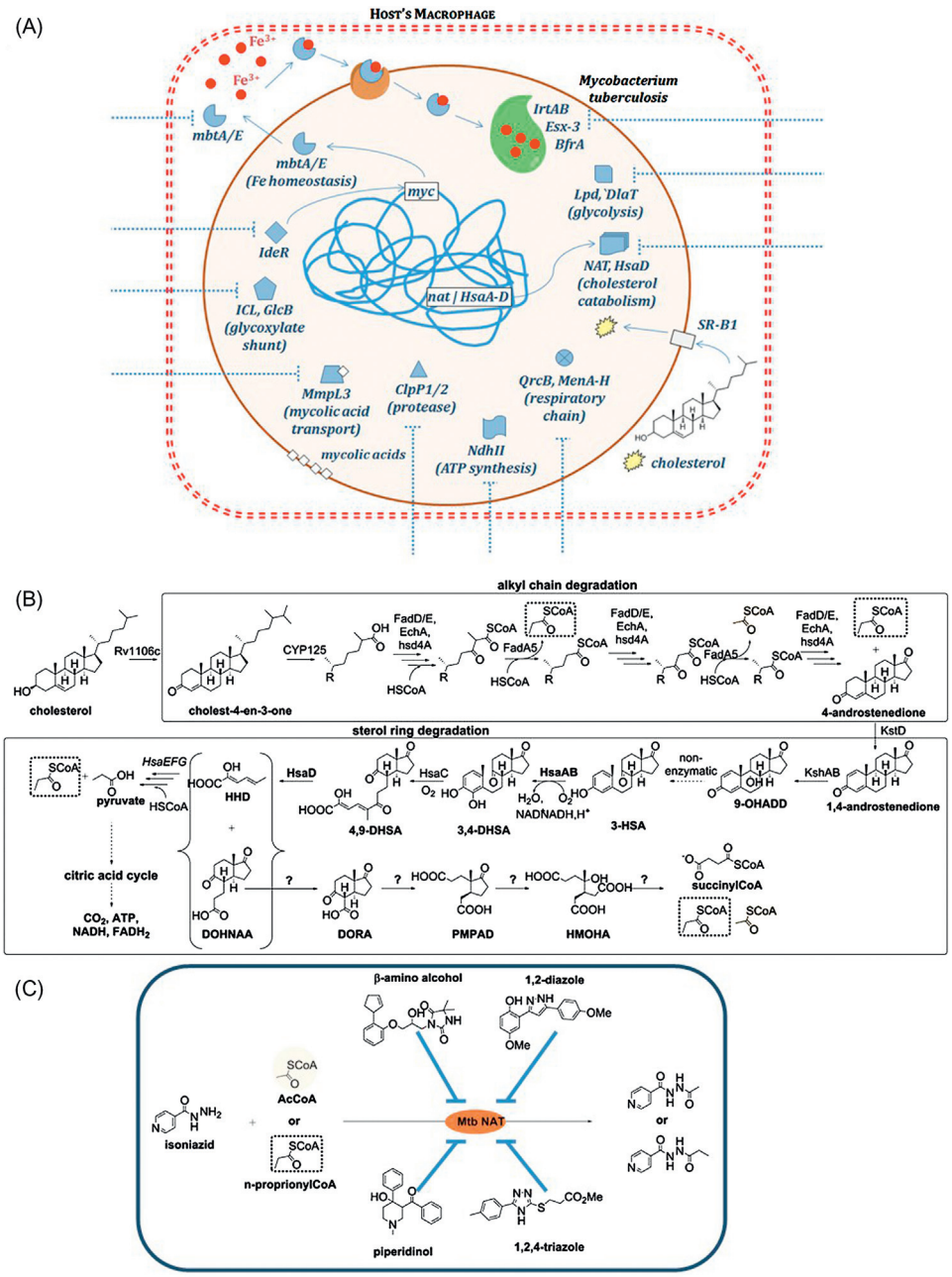


FIGURE 31.4 Emerging targets in tuberculosis treatment. (A) Simplified scheme of a *Mycobacterium tuberculosis* (Mtb)-infected macrophage and possible drug targets. Numerous advances have been made in understanding both the pathogenesis of tuberculosis (TB) and Mtb physiology. Newly identified TB drug targets are shown and their physiological roles are labeled; screening chemical libraries against a single target and optimizing selective inhibitors constitute the mainstay of current investigations to develop more efficient antitubercular drugs. (B) Proposed pathways for cholesterol degradation in Mtb. The possible metabolites derived from the alkyl side chain and sterol ring degradations are presented. Enzymes known or predicted to operate at each step are labeled. Sterol ring catabolites are converted into CO_2 *via* the tricarboxylic acid cycle and HsaD enzyme has been proposed as potential novel target, whereas the products from the alkyl chain degradation, namely, *n*-propionyl CoA and AcCoA, can be also employed as substrates for NAT enzyme with deleterious effects on Mtb survival in macrophages. The fate of DOHNAA is only hypothesized. (C) Chemical reactions catalyzed by Mtb NAT using an acyl-CoA species and the antitubercular drug isoniazid as substrates. The compounds identified as inhibitors of Mtb NAT following high-throughput screening of a 5000 drug-like chemical library are shown.

In a recent multifaceted study, the *arylamine N-acetyltransferase* (*nat*) gene product from Mtb appears to be involved in both mycolic acid biosynthesis and macrophage's cholesterol degradation, which constitute essential biochemical pathways for Mtb survival in macrophages. Historically, two polymorphic *nat* loci were initially identified in humans (*nat1* and *nat2*) and the involvement of human NAT proteins as phase-II drug metabolizing enzymes in the detoxification or metabolic activation of xenobiotics has constituted for a long time a major field of investigation in pharmacogenetics. At least one *nat* gene was subsequently discovered in a wide panel of species from the eukaryotic kingdom [51] and also in many prokaryotic species, including Mtb. The ability of the Mtb NAT enzyme to inactivate the antitubercular prodrug isoniazid *via* hydrazine *N*-acetylation laid the foundations for proposing Mtb NAT as a good antitubercular target [52]. Moreover, deleting the *nat* gene in a range of mycobacterial strains had detrimental effects on cell wall synthesis, which prevented the organism from surviving in host macrophages and increased the sensitivity to antibiotic treatments [53].

In the Mtb genome, *nat* was then found to be part of a genetic cluster which is involved in cholesterol catabolism. Indeed, four gene products (HsaA/B/C/D) deriving from this operon cleave the sterol rings from cholesterol in Mtb [54]; NAT protein is functionally linked to this process in that the products resulting from the degradation of the cholesterol alkyl chain, namely, acetyl- and *n*-propionyl-coenzyme A, are specific substrates of Mtb NAT [55] (Fig. 31.4B). Moreover, both NAT and HsaD enzymes were found to be completely active at temperatures of about 50°C, which seemed consistent with the fact that Mtb could resist in host macrophages regardless of the marked temperature increase in TB patients [55].

The research for selective inhibitors against Mtb NAT was therefore essential to establish the pharmacological potential of targeting NAT in TB. Both the availability of automatable enzymatic assays and the accessibility of pure recombinant NAT proteins from different prokaryotic and eukaryotic species (including human NAT1 and NAT2) allowed the implementation of a high-throughput screen of over 5000 chemicals against NATs [56,57]. The comparison of the results obtained from a duplicate screening was useful to establish the kingdom/species/isoenzyme selectivity of the main hit inhibitors. Some small molecules were demonstrated to specifically inhibit Mtb NAT [58,59] and one of these chemicals, a 1,2,4-triazole compound, was also shown to prevent mycobacterial growth with disrupting effects on cell wall morphology similar to those observed after *nat* deletion [60] (Fig. 31.4C). The recent crystal structures of all the proteins involved in the operon (Mtb NAT, HsaA/B/C/D) have a great potential to contribute to the understanding of the physiological role of these enzymes and to the design of selective inhibitors for therapeutic purposes.

More recently, the close link between human NATs and cancer (especially in carcinogenesis, tumor-related polymorphisms, oncocytogenetics, and abnormal overexpression) led to investigation of their functions in both physiological or pathological conditions and their potential therapeutic role in tumors [51]. In particular, proteomic analyses have identified human NAT1 as one of the most overexpressed proteins in invasive ductal and lobular breast cancers in both women and men, with decreasing levels in later stage tumors [61,62]. Besides, uncontrolled cellular proliferation was observed upon induction of NAT1 overexpression in noncancerous human breast cell lines [63]. Therefore, selective NAT1 inhibitors, as released from the double screening previously mentioned, have constituted valuable tools to evaluate human NAT1 as a therapeutic, diagnostic, or prognostic biomarker as a whole.

A recent review has comprehensively presented numerous studies in medicinal, structural, and computational chemistry, which have been undertaken to improve the potency of some lead human NAT1 inhibitors; the long-term aim has been the development of chemotherapeutic agents for drug-resistant estrogen-receptor (ER)-positive breast cancers [51]. Two drug-like compounds, a rhodanine and a naphthoquinone, were found to act as selective human NAT1 inhibitors in both enzymatic assays and breast cancer cell lysates overexpressing human NAT1 (namely ZR-75-1), and various analogues have been further developed in medicinal-chemistry studies [57,64,65] (Fig. 31.5A).

Particular attention was paid to the naphthoquinone hit compound because of its unique colorimetric properties: it undergoes a distinctive color change from red to blue upon binding to human NAT1 and its close murine homologue (mouse NAT2), but not other mammalian NAT isoenzymes, neither in buffer at physiological (pH = 7.5). It was the first example of a selective noncovalent sensor to detect a potential protein biomarker in its native form without the need for a tag or an antibody [64]. Virtual modeling studies using a high-resolution crystallographic NAT1 structure [66] and a naphthoquinone derivative, indicated that the interaction between the naphthoquinone inhibitor and human NAT1 relies on selective ionic interactions between the conjugate base of the compound ($pK_a = 9.16$) and the guanidinium moiety of an active site residue, arginine 127 ($pK_a = \sim 12.5$) [67] (Fig. 31.5B). Ongoing investigations in organic chemistry and pharmacology are developing more color-sensitive NAT1 inhibitors in order to eventually exploit these probes for detecting human NAT1 in breast cancer samples [68] (Fig. 31.5C).

Research continues to seek improved drugs with higher tumor selectivity and wider windows of therapeutic index than the drugs prescribed in current practice; thus targeted therapies with limited side effects are the Holy Grail.

A fairly recent molecule of interest (although it may not be fully acknowledged as a target per se) has emerged in various cancers following observations of the different roles played by the cytochrome P450 (CYP) enzymes. These proteins constitute an ubiquitous superfamily of enzymes found across all biological kingdoms and are expressed as 57 isoforms in humans. Human CYPs are classified according to their sequence similarities, and involved in xenobiotic metabolism (primarily families CYPs1-3), as well as biosynthesis of important steroids and their precursors (CYPs17, 19, 24). Their involvement in carcinogenesis through the bioactivation of numerous exogenous and endogenous substrates, their upregulation and overexpression in various tumors in comparison to normal surrounding tissue, and their possible role in drug resistance, have all led to strategies that include the development of CYP-mediated prodrugs, selective inhibitors, gene and immune therapy. Natural products have also been employed in the first two types of strategies and as such are sketched out here.

The involvement of CYP1 enzymes, in particular CYPs1A1 and 1B1, in cancer is multifaceted. The activation of environmental pollutants into noxious forms capable of binding DNA and thus initiating cancers is exemplified by the benzo[a]pyrene activation pathway where CYP1 enzymes (particularly CYP1A1 and CYP1B1) form the ultimate carcinogen, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BDPE) [69]. CYP1 has been identified as crucial in the formation of the tetrahydro-BDPE in human lung cells [70]. Numerous natural and synthetic inhibitors of CYP1 family at *in vitro*, *in vivo*, and clinical trial stages have been examined (most noteworthy being kaempferol and resveratrol), although long-term considerations for CYP1 inhibitors are still being assessed [71].

The metabolism of 17β-estradiol to 4-hydroxyestradiol by CYP1B1 is followed by another enzymatic catalysis yielding estradiol-3,4-quinone, which in turn is capable of binding DNA. This observation has linked CYP1 enzymes (in particular CYP1B1) with estrogen receptor (ER) positive cancers, such as breast, endometrial, and uterine cancers [72]. The preferential expression of CYP1B1 in different cancer tissues (also in ER-positive tumors) relative to normal surrounding tissues has made it an attractive target for developing novel prodrugs which can be activated by this enzyme selectively in tumor tissue with mitigated adverse side effects from impact on normal cells [73]. CYP1B1 activated anticancer prodrugs which are under development include trans-resveratrol, aryl oximes, duocarmycin analogues, and benzothiazoles.

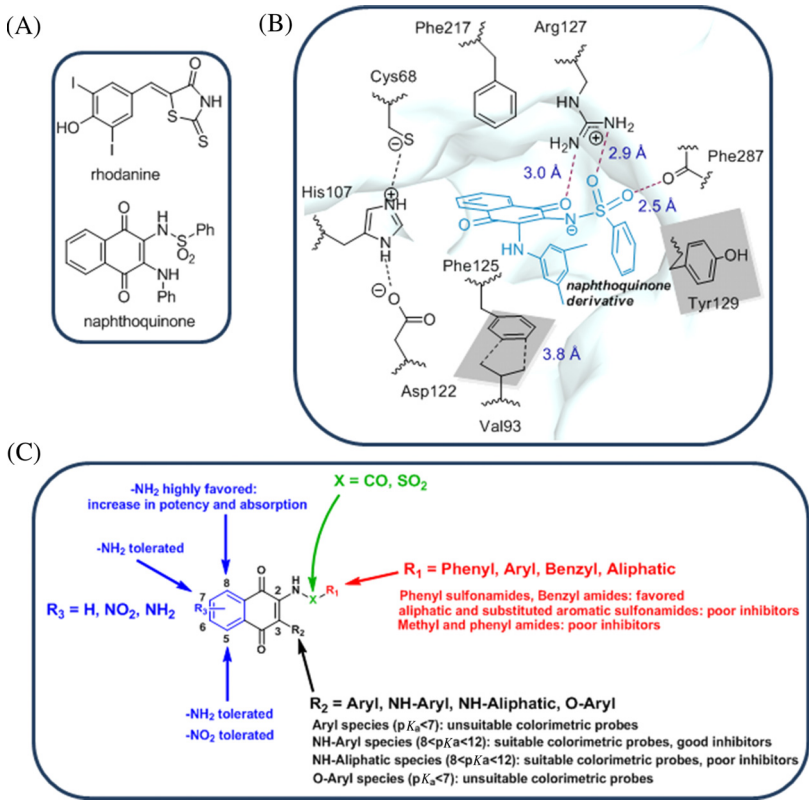
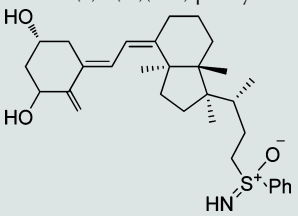
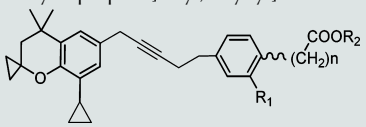
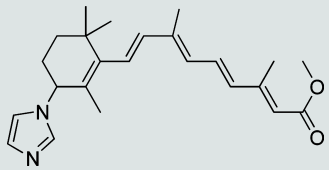
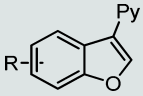
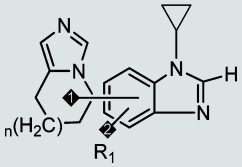


FIGURE 31.5 Biochemical characterization of human NAT1 selective inhibitors. (A) Compounds identified as inhibitors of pure human NAT1 following a high-throughput screening of 5000 drug-like compounds against a panel of pure recombinant eukaryotic and prokaroptic NAT proteins. The naphthoquinone and rhodanine chemicals were also identified as human NAT1 inhibitors in lysates from estrogen-receptor-positive breast cancer cell cultures (ZR-75-1). (B) Schematic representation of a naphthoquinone derivative inhibitor as modeled in human NAT1 active site. The residues involved in inhibitor binding are labeled. Hydrogen bonds are drawn as dashed lines and the Van der Waals surfaces in the enzyme's pocket are shown as gray planes. The surface representation of the catalytic pocket in the background is based on the crystal structure of human NAT1 (pdb code: 2PQT). (C) Summary of the structure–activity relationship studies carried out on the human NAT1 naphthoquinone inhibitor. The effects of different substituents around the naphthoquinone core are detailed.

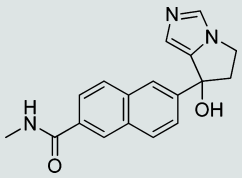
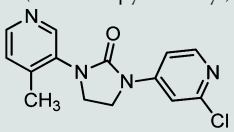
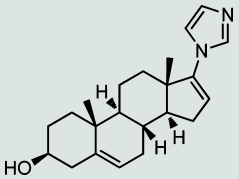
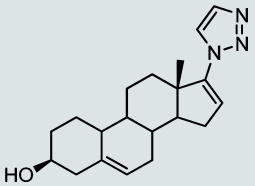
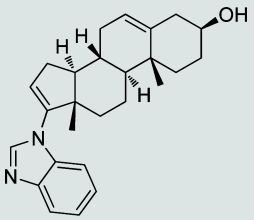
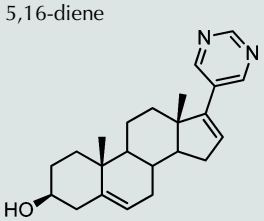
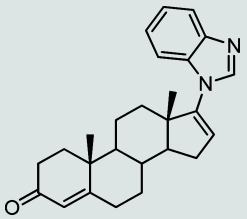
The distinctive roles played by CYP24 in vitamin D metabolism, by CYP26 in trans retinoic acid activated vitamin A, by CYP19 in estrogen biosynthesis, and by CYP17 in the upregulation of androgens, such as testosterone, have allowed the opportunity for developing selective inhibitors in relation to renal, skin, breast, and prostate cancers, respectively. A multitude of inhibitors under current development at various stages, including benzofurans and benzoimidazole derivatives, have recently been detailed and a few examples are highlighted in Table 31.2 [74].

TABLE 31.2 CYP Inhibitors Under Current Development

CYP Inhibitor	Inhibited CYP *source* (substrate tested)	IC50/Ki Compound (nM)
MK-24-(*S*)-S(O)(NH) phenyl sulfoximine 	CYP24 *Recombinant in V79 cells* ([^{3}H-1β]-1α, 25(OH)$_2$D$_3$)	7.4 ± 4.2
4-[(8-cyclopropyl-3,4-dihydro-4,4-dimethylspiro[2H-1-benzopyran-2, 1′-cyclopropane]-6-yl)ethynyl]-2-fluoro-benzeneacetic/benzoic acid  Wherein R_1 = F or H n = 0 or 1 R_2 = H, alkyl chain of 1−6 C	CYP26 *P450RAI-1 transfected HeLa cells* ([^{3}H]-RA)	14−50
VN/14-1 	CYP26 *COS-1 cells transfected with hP450RAI* ([11, 12-^{3}H]-ATRA)	1.20 ± 0.07
Benzofurans  Wherein Py = 2-, 3-, or 4-pyridyl group R = substituted/unsubstituted phenyl group, or substituted/ unsubstituted aromatic heterocyclic group	CYP17 *Prepared from rat testis* (17α-hydroxyprogesterone)	N.D.
Pyrroloimidazolyl and imidazopyridinyl substituted 1H-benzimidazole derivatives  Wherein n = 0 or 1 R_1 = H, OH, N, or an alkyl group	CYP17, CYP19 and CYP11 *In vivo rat study* (PMSG, LHRH, ACTH)	N.D.

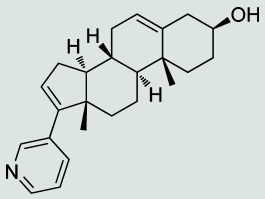
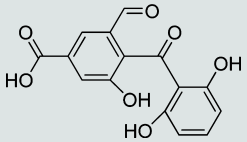
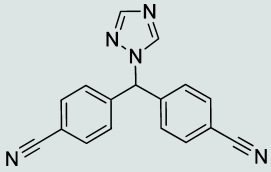
(*Continued*)

TABLE 31.2 (Continued)

CYP Inhibitor	Inhibited CYP *source* (substrate tested)	IC50/Ki Compound (nM)
TAK-700 	CYP17 *Recombinant P450c17* ([1,2-^{3}H]-17α-hydroxyprogesterone)	38
1-(2-Chloro-pyridin-4-yl)-3-(4-methyl-pyridin-3-yl)-imidazolidin-2-one 	CYP17 *Recombinant protein* (17-α-hydroxypregnenolone [21-^{3}H])	27
VN/85-1 	CYP17 *Prepared from human testicles* ([21-$^{3}H_3$]-17α-hydroxypregnenolone)	8 ± 1 K_i = 1.2
VN/87-1 	CYP17 *Prepared from human testicles* ([21-$^{3}H_3$]-17α-hydroxypregnenolone)	13 ± 1 K_i = 1.4
VN/124-1 or TOK-001 	CYP17 *P450c17-expressed in E. coli* ([21-$^{3}H_3$]-17α-hydroxypregnenolone)	300
5,16-diene 	CYP17 *P450c17-expressed in* E. coli ([21-$^{3}H_3$]-17α-hydroxypregnenolone)	500
4,16-diene 	CYP17 *P450c17-expressed in* E. coli ([21-$^{3}H_3$]-17α-hydroxypregnenolone)	915

(*Continued*)

TABLE 31.2 (Continued)

CYP Inhibitor	Inhibited CYP *source* (substrate tested)	IC50/Ki Compound (nM)
Abiraterone 	CYP17 *Prepared from human PC tissue* (^{3}H-17α-hydroxy- progesterone and ^{3}H-progesterone)	2.9–4
TAN-931 and derivatives 	CYP19 *Prepared from human placenta* ([1β, 2β -^{3}H]-androstenedione)	4.8–80.8 μg/ml K_i = 40,000
Letrozolole 	CYP19 *Prepared from human placenta* ([1β, 2β -^{3}H]-androstenedione)	1.2–11

Source: Adapted from Francis S, Delgoda R. A patent review on the development of human cytochrome P450 inhibitors. Exp Opin Ther Pat 2014;24:699–717. N.D.: not determined.

Our account about target discovery in the cancer field is necessarily brief, and more details can be found in a plethora of recent reviews. Current cancer chemotherapy comprises an eclectic combination of drugs and techniques which aim to specifically target cancer cells despite their smallest biochemical differences from normal cells. However, many rapidly invasive cancers remain essentially untreatable, and less toxic, but more selective chemotherapies are sought for early-diagnosed tumors. An array of different novel approaches for treating cancer as a whole have been proposed for future scientific researches: e.g., inactivating components of oncogene signaling pathways, restoring functions of tumor suppressor genes, employing tissue-specific proliferation inhibitors, developing engineered prodrugs for cancer, and enhancing host selective immune response.

Generally, 5-year relative survival rates for all cancers diagnosed have improved over the last three decades, going from 49% in 1975–77, to 55% 1987–89, and up to 68% in 2004–10 [75]. This reflects both the earlier diagnosis of certain cancers and improvements in treatment. Survival statistics vary greatly by cancer type and stage at diagnosis, and is calculated as the percentage of people alive at a fixed time period after a cancer diagnosis divided by the percentage of people expected to be alive in the absence of cancer according to normal life expectancy. Unfortunately, relative survival may not predict individual prognosis and should be interpreted with caution.

Overall, an earlier diagnosis and more accurate methods of predicting response to a specific therapy will continue challenging molecular laboratories and diagnostic industries; the identification of subgroup-specific tumor targets constitutes a valuable opportunity to develop patient-specific therapies in a stratified medicine approach.

31.3 DRUG DEVELOPMENT AND FUTURE PROSPECTS

The discovery of a new disease-specific target is followed in outline by two main stages of process: firstly, the identification of a novel chemical entity which can modulate the target function and act as a therapeutic agent; secondly, the development phase, wherein the safety and efficacy of the lead compound is established through different clinical trials, and appropriate formulations and administration are devised for patients. Although the process of drug development has evolved from managed serendipity to engineered selection, the identification of the best chemical candidate with good

efficacy and safety in humans remains a highly complex, pricey, and risky business. Despite enormous investments in pharmaceutical companies, the high failure rate in drug development over the past decades underlines the complexity of biological systems and the weak reliability of predictions based solely on molecular considerations [76].

The multifactorial nature of chronic diseases, which constitute the main focus of interest for pharmaceutical industries, encourages the combination of a molecular approach and a systems-based strategy in target discovery to mitigate against failure of a drug candidate. In the end, the actual "decision gates" process, as is usually adopted in developing a drug product in pharmaceutical laboratories and industries, has to date constituted a useful tool to prevent risks for humans and identify a successful and safe therapeutic molecule [77].

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31.4 REVIEW QUESTIONS

- Why is the identification of a good target critical in drug discovery?
- Outline steps and methods involved in target identification.
- Provide at least one example of current potential targets in heart diseases, ailments of the central nervous system, infectious diseases, and cancer. Outline reasons for their suitability as a good target.
- Outline stages of drug discovery following target identification.

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Chapter 32

Nanotechnology: Building and Observing at the Nanometer Scale

T.J. Booth[1] and M.A.B. Baker[2]

[1]*Technical University of Denmark, Kongens Lyngby, Denmark,* [2]*University of New South Wales, Kensington, NSW, Australia*

Chapter Outline

32.1 INTRODUCTION

Nanotechnology is, very simply, the study of things at the nanoscale, a millionth the size of a grain of sand. The word "νανος" (nanos) in Greek means "dwarf"; the leap from the scale of normal microscopy, at a millionth of a meter, to the nanoscale, at a billionth of a meter, has required the development of new tools to both observe and assemble molecules.

Working at the nanometer scale can bring additional complications, since at these small length scales molecular motors operate at the energy level of the thermal bath [1]. Behavior at this scale differs from macroscopic behavior, since at these length scales thermal fluctuations have a greater influence on the operation of molecular machinery, and on the dynamic structure of proteins. Thus some care is needed not to extend irrelevant macroscopic considerations such as friction and inertia to the nanoscale [2].

With this caveat in mind, nanoscience is still fundamentally the reduction of length scales from the micrometer scale to the nanometer scale. In the modern era we are able to both investigate biological systems and fabricate structures to interact with them. The last 20 years has delivered new techniques that can directly create nanoscale components. Some do this with miniaturized versions of existing macroscale techniques, where we buildup or remove material a single atomic layer thickness at a time [3]. Or, learning from biology and using a constructive perspective, we can use DNA oligomers to self-assemble precise 3D structures in solution under standard biological conditions [4]. Mastery of the nanoscale requires not only the ability to generate nanoscale structures, but also the ability to see them. Great advances have been made recently in the visualization of structures in precise nanometer detail [5]. Primarily this has been through advances in microscopy techniques that enable visualization of structures smaller than the wavelength of light [6].

It is our intention here to provide a necessarily brief and general overview of some of the key ideas in microfabrication and microscopy. The interested reader should consult Madou's "Fundamentals of Microfabrication and Nanotechnology" [7] for further detail.

32.2 HOW WE MAKE SMALL THINGS

32.2.1 Additive and Subtractive Processes

When producing a new object or material, there are two general approaches—we can either add material until the desired shape is achieved (e.g., adding a handle to a clay bowl to make a cup) or by taking material away (e.g., carving

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a large block of wood in order to make the same shape). The terms "bottom-up" and "top-down," coined in the nanotechnology context by the Foresight Institute in 1989 [8], are used when discussing nanotechnological processes. The "top-down approach" is the subtractive process of making smaller systems from larger initial systems—removing the parts of the system which we do not need until we make the system we want. The "bottom-up approach" is the complementary additive process where we build systems up from smaller parts. In very general terms, microfabrication processes are "top-down," whereas chemical synthesis is a "bottom-up" process.

Technological progress in "top-down" processes has resulted in the miniaturization of electronic systems and geometrically increasing performance at lower cost for over 50 years. Only just now is the rate of progress slowing, due to fundamental physical limits on the size of electronic systems, thermal management, and other considerations [9]. The key driving force behind this progress has been our ability to control the structure and properties of materials, particularly silicon, with a vast and increasing array of technologies collectively termed "microfabrication techniques."

32.3 MICROFABRICATION AND OPTICAL LITHOGRAPHY

Microfabrication is the application of a range of additive and subtractive techniques in order to produce useful structures with dimensions in the micrometer or submicrometer scale. Typically this involves silicon and other semiconductors, but microfabrication can also be applied to other materials such as polymers and metals.

32.3.1 Masks, Resists, and Selectivity to Patterning

Top-down fabrication requires the ability to select regions of an object where our additive and subtractive processing takes place. The canonical technique for doing this is lithography (from Greek λίθος, lithos, "stone" and γράφειν, graphein, "to write"), and particularly optical lithography. This uses ultraviolet light projected through a selectively transparent "photomask" to produce a physical or chemical change in a material and etch a pattern onto it. Sometimes this is used in multiple steps, to produce another mask that is then used to alter the selectivity of a second material and generate a pattern on that.

The photomasks used typically consist of a thin plate of glass covered in chrome metal. This chrome is selectively removed in some areas, producing a mask with both transparent and opaque regions. When UV light is shone through the mask, it selectively exposes areas of an object beneath. This object is usually covered in a light-sensitive material called a photoresist. Exposure of the photoresist to UV light through transparent areas of the photomask causes physical or chemical changes in the photoresist layer, leaving unexposed ("masked") regions unchanged. Typical photoresists are polymeric materials which can be spun coated onto laminar objects and baked in order to produce polymer films of typically $0.1-100\ \mu m$ thickness. They usually incorporate a photoacid compound—this compound undergoes photolysis on exposure to UV, breaking down into a strong acid. If the exposed resist is then placed in a "developer" bath—typically a mild alkaline solution—then these UV exposed and therefore acidic regions will be selectively removed by the developer bath, leaving behind the unexposed resist. Such resist-based masks are useful for both additive and subtractive microfabrication.

Lithography is used to define areas where top-down processing is to take place. A typical additive process is lift-off metallization. The patterned resist and substrate are coated with a thin film of deposited metal by, e.g., thermal or electron beam evaporation, or RF sputtering. The portion of the metal which lands on the unmasked substrate surface will be left behind when the portion of metal which lands on top of the resist is removed ("lifted off") from the surface along with the remaining resist via dissolution. This process is shown in Fig. 32.1.

Subtractive processes in microfabrication usually involve using the resist layer as a physical or chemical etch mask. During such processes, the resist will protect the surface of the substrate from an aqueous etchant solution or ionic bombardment while the exposed areas of the substrate are patterned.

Optical lithography based on chrome masks and mercury lamps can be used to pattern resist layers and therefore produce resulting structures with a size typically in the range of $1-2\ \mu m$. These are limited by the wavelength of light, the thickness of the resist layer, the degree of scattering encountered in the resist, the contrast which can be achieved between exposed and unexposed areas, the tendency of the photogenerated acid to catalyze further photoacid lysis, and the size of the polymer chains in the resist layer. Some of these limitations can be overcome by reducing the wavelength of the light used to expose the resist. Performing such lithography under vacuum (air has a UV absorption edge at 185 nm) or in liquids with high refractive index (enabling the use of high numerical aperture optics) has enabled the continuous scaling down of feature sizes to the present 100 nm or less.

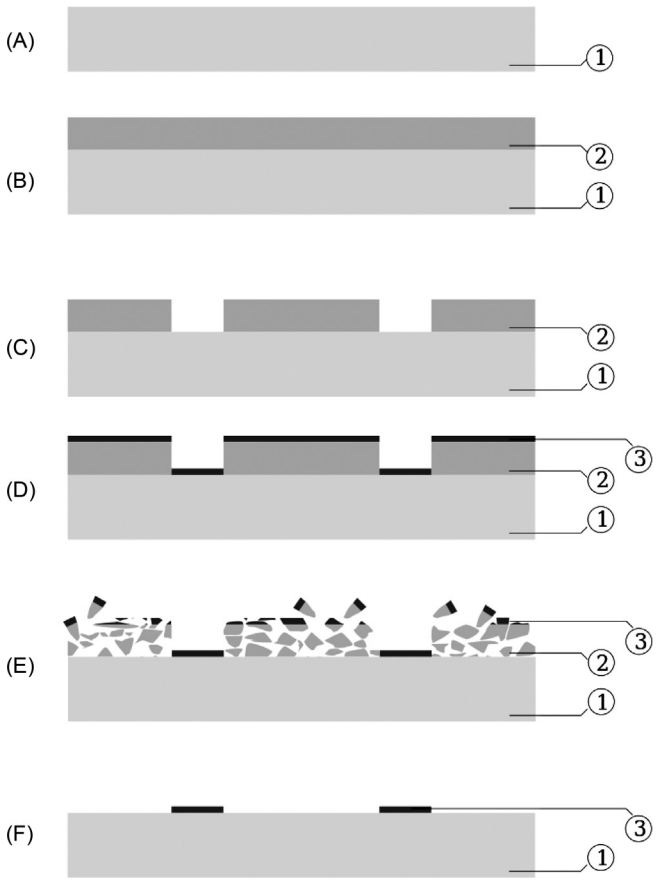


FIGURE 32.1 Lift-off metallization. (A): a substrate (1) is (B): coated in a photoresist (2). (C): the resist layer is exposed to UV and developed, leving some exposed areas of substrate, and other areas masked. (D): metal or other material (3) is evaporated uniformly over the surface. (E): the resist is removed, along with the excess metal. (F): the final patterned metal-on-substrate.

In research, custom low-resolution masks for optical lithography with features on the order of 5 μm can be made quickly and inexpensively using direct-write optical lithography: by rastering a laser across a chromium layer on a glass plate coated in lithographic resist and then using a chemical etchant to remove chromium in the exposed areas of the developed resist layer as above. When masks with resolutions on the order of 1 μm or smaller are required, an alternative technique must be employed. In situations where throughput is not as great a concern as flexibility and ultimate resolution, electron beam lithography is typically employed.

32.4 ELECTRON BEAM LITHOGRAPHY

Electron beam lithography is analogous to rastered direct-write optical lithography, except that the effective wavelength of the illumination is very small, enabling feature sizes of the order of 10 nm. This feature size is not wavelength limited (the de Broglie wavelength of 1−100 keV electrons used is 1−0.01 nm), but instead limited by scattering and the generation of secondary electrons in the resist. Electrostatic and electromagnetic optics are used to form and scan a focused electron beam across the surface of a lithographic resist in order to expose some areas of the resist to electrons. The prototypical resist for electron beam lithography is poly(methyl methacrylate)—PMMA. When exposed to electrons, the polymer chains in PMMA are broken up in a process known as scission, becoming shorter and more soluble in organic solvents. It is also possible to expose PMMA to larger doses of electrons, which has the effect of cross-linking the polymer chains together and making the exposed resist more difficult to remove. Electron beam lithography is often used in research and development due to its versatility and high resolution—; however, as an industrially applicable technique it is many orders of magnitude slower than competing optical lithographies.

32.5 ATOMICALLY PRECISE THICKNESS—2D MATERIALS

The final limit of our ability to pattern materials will arrive when we have control over the position and composition of every atom in our system. The production and isolation of graphene [10], a single atom thick layer of carbon atoms,

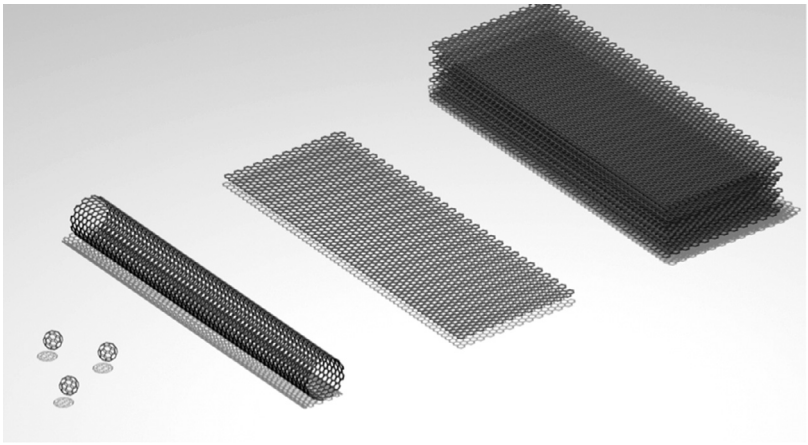


FIGURE 32.2 Carbon-based nanostructures. From left to right: zero-dimensional C_{60} (buckminsterfullerene), a one-dimensional single-walled carbon nanotube, a two-dimensional graphene sheet, and a three-dimensional stack of graphene layers, graphite.

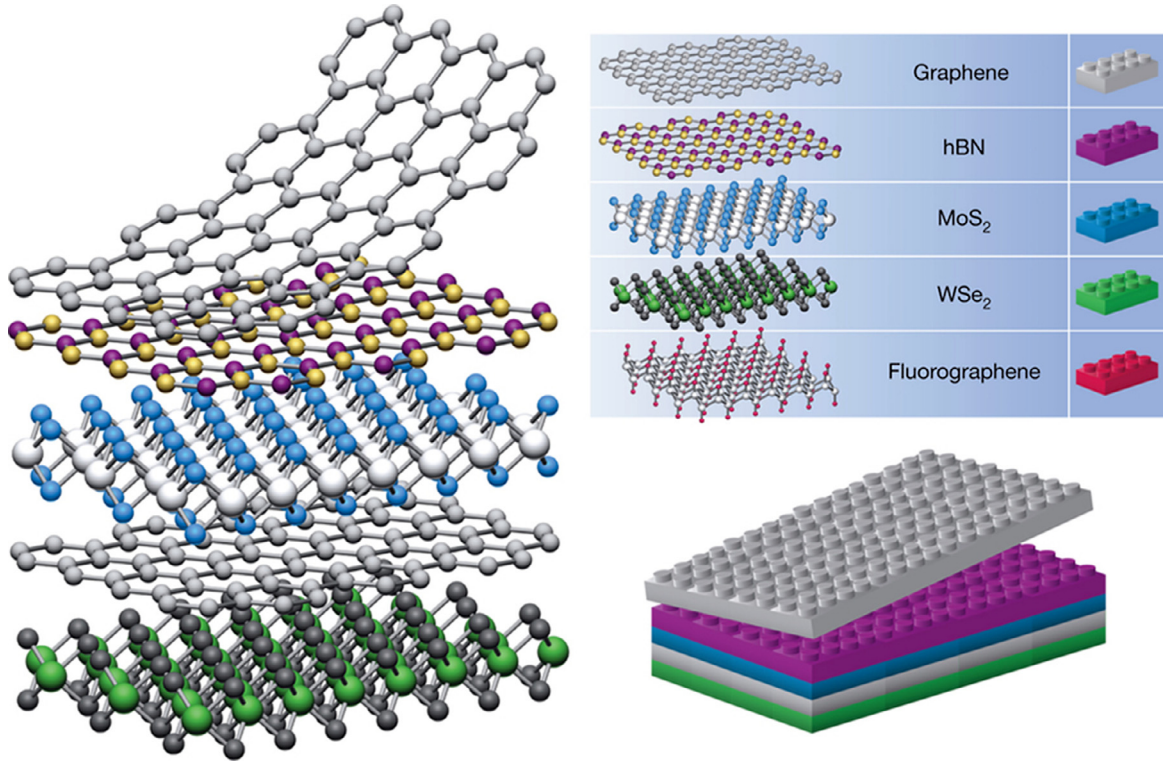


FIGURE 32.3 Stacking single atom layers of 2D materials into van der Waals heterostructures. *From [12] Geim AK, Grigorieva IV. Van der Waals heterostructures. Nature 2013;499:419–25.*

and subsequently the identification of a range of other single or few-atom thick crystals [11] has effectively solved this problem for one out of the requisite three dimensions (Fig. 32.2). By combining stacks of two-dimensional materials into so-called van der Waals heterostructures [12], we can produced layered structures with just the right layers (Fig. 32.3).

The challenge of patterning such 2D materials in the lateral dimensions remains, however. In general, microfabrication technologies are applied to these materials in order to pattern them—; it is fortuitous that most of the vast range of technologies we have developed for fabricating planar silicon structures are in general applicable directly to the lateral structuring of graphene and other 2D materials.

More recently, there have been efforts to pattern 2D materials at the atomic scale using the finely focused electron beams of the transmission electron microscope [13]. In these techniques, a lack of lithographic resists and the simultaneous patterning and imaging of systems enables an unprecedented degree of control over the final structure of the

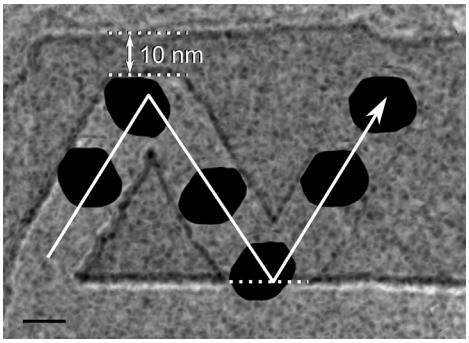


FIGURE 32.4 Montage of a silver nanoparticle cutting a nanoscopic equilateral triangle in a graphene sheet. Scale bar 10 nm. *From [16] Pizzocchero F, Vanin M, Kling J, Hansen TW, Jacobsen KW, Bøggild P, et al. Graphene edges dictate the morphology of nanoparticles during catalytic channeling. J Phys Chem C 2014;118:4296−302.*

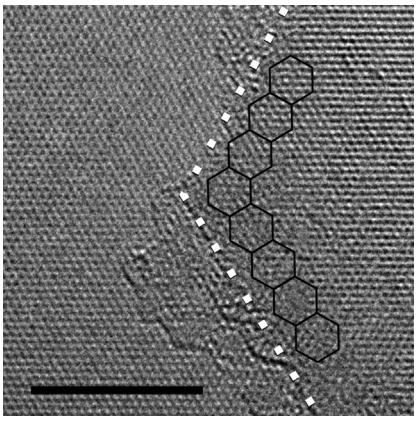


FIGURE 32.5 Atomic resolution TEM image of a graphene edge (white dotted line) produced by metal nanoparticle etching. The orientation of the graphene lattice is indicated by the array of hexagons. Scale bar 5 nm. *From [16] Pizzocchero F, Vanin M, Kling J, Hansen TW, Jacobsen KW, Bøggild P, et al. Graphene edges dictate the morphology of nanoparticles during catalytic channeling. J Phys Chem C 2014;118:4296−302.*

system. One exciting application of such technologies has been the development of nanopores based on graphene and other 2D materials [14]. Such nanopores consist of a single hole some 1−2 nm in diameter in an otherwise impermeable graphene sheet. Under certain conditions, DNA molecules can be made to pass through such nanopores, translocating one base pair at a time. This opens up the possibility for sequencing DNA based on measuring the ionic current through the nanopore, measured during the DNA translocation, or otherwise.

Atomic scale patterning of graphene in the lateral dimensions through the use of catalytic metal nanoparticles has also been demonstrated [15]. Tracks with well-defined widths and atomic scale roughness can be produced in this way, but such techniques have highlighted a fact which may constitute an insurmountable limit in terms of our ability to control the structure of materials on an atomic scale (Figs. 32.4 and 32.5). Fundamentally, the removal or addition of atoms from or to a system is a statistical process—it proceeds at an average rate determined by the activation energy and the temperature of the system. It is not possible to predict with certainty exactly if or when a specific atom will be removed or added. In most additive or subtractive processes we are either not interested in atomic precision, or we proceed with addition or subtraction until we are sure that there is no more material to add or subtract. When designing structures at the atomic scale, it will, however, be necessary to find and develop new means of harnessing the fundamentally discrete and stochastic nature of matter to produce the structures we require.

Biology is born from such variation. As such, evolution has refined molecular machinery to not only thrive in a stochastic and fluctuating environment, but also to use stochastic variation as principles driving assembly of molecular complexes. Thus biological machinery solves the challenge of assembling scaffolding and structure in a fluctuating environment, so in the next section we discuss how we can guide biological building blocks to design novel nanostructures.

32.6 BOTTOM-UP BIOLOGICAL ASSEMBLY

Biological assembly differs from nanofabrication as, instead of the directed deposition or depletion of structures, biological components self-assemble into the desired structure, driven only by interactions between the subunits. The DNA molecule has several features that make it particularly appealing for strategic self-assembly, in particular, because it has a well-understood, strong, and specific interaction with a complementary strand based upon sequence identity. Additionally, its diameter of $\sim$2 nm, helical pitch of $\sim$3.4−3.6 nm, and persistence length [17] of around 50 nm makes it highly suitable for construction on the nanoscale.

These factors coupled with the ease of synthesis of nucleic acids [18] and the ability to conjugate functional groups and other covalent modifications [19] have placed DNA self-assembly at the forefront of research in nanotechnology, since its inception fewer than 30 years ago by Ned Seeman who first realized that DNA lattices could be engineered with a structural purpose [20].

The pioneering work that enabled larger structures to be made with DNA, while retaining nanometer precision, was from Paul Rothemund, who invented DNA origami [21]. The principle of the DNA origami technique is to take a long scaffold strand and fold it over multiple times using staples strands of around 30 base pairs in length (Fig. 32.6). The staples fold the scaffold strand into different, specific patterns, and sites can be functionalized with biochemical linkers of interest, such as NTA, or biotin [23]. Coincidentally this long single-stranded scaffold was initially a viral DNA strand (from M13) as this was an easily accessible source for long single-stranded DNA. The staple strands are around 200 in number and specifically hold together different sections of the long strand, folding it into a structure.

To consider just how different biological self-assembly is from nanofabrication one can consider a set of children's Lego blocks. If you took this bag and shook it, you would never see the blocks build themselves into the final structure of the house, car, or pirate ship. However with biological building blocks this is indeed exactly what occurs; blind assembly into a final structure. The instructions for the assembly are contained structurally within the individual bricks, which provide a robust, reproducible, and simple method for nanoscale construction. DNA self-assembly also occurs in biologically relevant conditions, i.e., around pH 7.5 in aqueous solution, whereas nanofabrication typically requires clean rooms and does not, by default, interact with biological systems. Thus DNA scaffolding supplies the perfect platform for structural scaffolds with which we can interrogate biological systems.

From initial work on DNA systems, the applications of DNA nanotechnology are now diverse. For further reading see the review of Bath et al. [24].

32.7 HOW WE OBSERVE SMALL THINGS

Identically to surface etching when performing nanofabrication, if we want to image a surface, we are limited by the wavelength of light. This diffraction limit can be sidestepped by using alternative imaging sources, such as electron beams and electron microscopy, which are particularly suited for examining frozen biological samples at high magnification. This is becoming quite a mainstream method for studying cellular architecture at molecular resolution [25], but requires expensive microscopy and cryo protein preparations. Instead, we will focus below on high-resolution microscopy using light, with recent advances allowing us to break through the diffraction limit and resolve detailed nanoscale structures in living systems.

32.8 HIGH-RESOLUTION LIGHT MICROSCOPY

The invention of microscopes that could magnify over 100$\times$ ushered in the era of microscopy in the 17th century, and was driven by Antonie van Leeuwenhoek and Robert Hooke. This type of microscopy enabled microbiology, and was naturally limited by the diffraction limit of light. This depends on the wavelength of the light, and the resolution is typically half the wavelength, so for 600 nm light, it is impossible to resolve objects that are closer than $\sim$300 nm. The discovery of fluorescent proteins, initially green fluorescent protein from jellyfish [26], allowed the use of microscopy to label and see individual biological components, and now there is a plethora of different color fluorescent proteins that can be used to label multiple components of a cell in parallel. This enabled the functional study of biological systems using light, however, by definition this is not the nanoscale, but the microscale. Recent developments in light microscopy and single molecule microscopy (culminating in the Nobel Prize for Chemistry in 2014) have enabled creative solutions to push past this diffraction limit and use light microscopy to resolve objects at the nanoscale [27].

The diffraction limit of light sets a limit on the spatial resolution of an image, all detail around 500 nm is blurred and the structural detail is lost. This is much like trying to reproduce a photograph using only a thick paintbrush—the

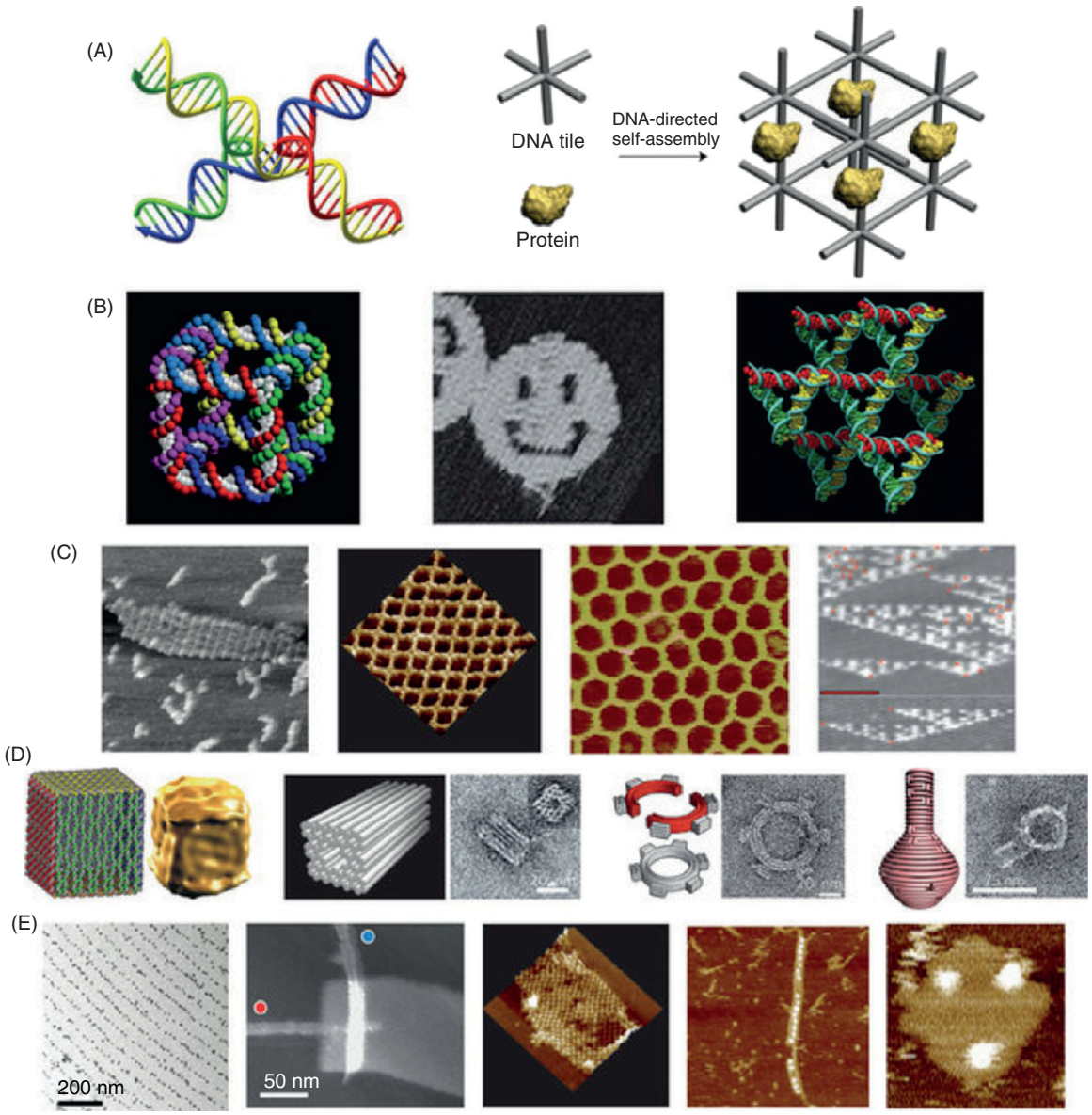


FIGURE 32.6 (A) Seeman's original proposal consisted of using immobile DNA junctions (left) to build 3D scaffolds that could be used to organize proteins (right). (B) Important milestones in structural DNA nanotechnology: the first wireframe 3D cube (left), DNA origami (center) and a 3D periodic structure composed of tensegrity triangles (right). (C) DNA periodic arrays composed of double-crossover tiles (left), 4×4 tiles (center left), three-point star tiles (center right) and double-crossover-tile-based algorithmic assembly of Sierpinski triangles (right). (D) Three-dimensional DNA origami: a hollow box (left pair of images), a multilayer square nut (center left pair), a square-toothed gear (center right pair) and a nanoflask (right pair). (E) DNA nanostructure-directed patterning of heteroelements: double-crossover tiles for the organization of gold nanoparticle arrays (left), DNA origami for the assembly of carbon nanotubes (center left), biotin−streptavidin protein patterning of 4×4 tiles (center), aptamer-directed assembly of thrombin arrays on triple crossover tiles (center right), and Snap-tag and His-tag mediated orthogonal decoration of DNA origami (right). *Taken from [22] Pinheiro AV, Han D, Shih WM, Yan H. Challenges and opportunities for structural DNA nanotechnology. Nat Nanotechnol 2011;6:763−72.*

overall image will be there, but the fine detail will be lost. Techniques to improve this resolution rely on clever methods that effectively reduce the size of the paintbrush. The first of these is stimulated emission depletion (STED) [28]. STED works by depleting the excited state in all but a small region, so that the emitting region of fluorescence is in fact much smaller than the diffraction limit. This is achieved by overlaying a saturating depletion laser that has a "donut" profile, over a standard Gaussian exciting laser. The donut effectively cancels out the intensity from the exciting laser, leaving a "hole" that is much smaller than the diffraction limit (Fig. 32.7).

STED relies on altering the emission and depletion of fluorophores, but requires complex equipment to provide the depleting and imaging lasers. Another method, more widely used, is to use specially designed fluorophores that can be

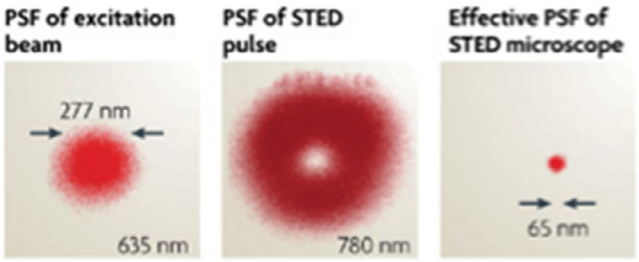
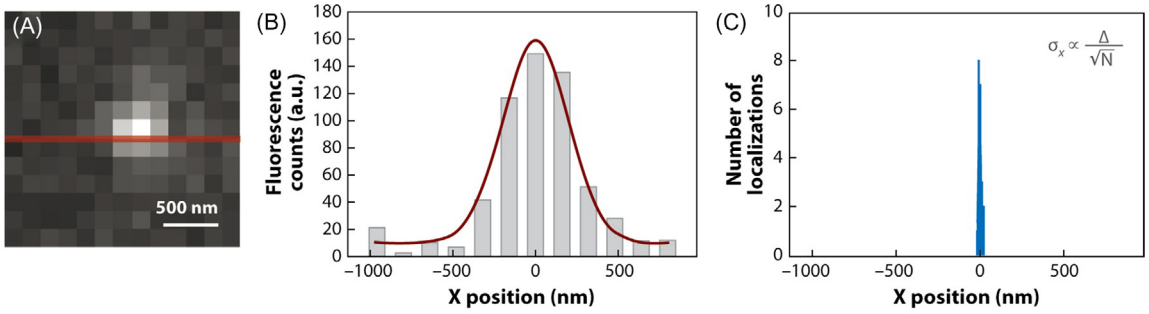


FIGURE 32.7 Point spread function (PSF) of STED microscopy. The focal spot of excitation light (bright red) is overlapped with a doughnut-shaped red-shifted light (dark red), which quenches excited molecules in the excitation spot periphery. This confines emission to a central spot. Scanning this central spot (called the zero) across the sample results in a subdiffraction image. *Taken from [29] Fernández-Suárez M, Ting AY. Fluorescent probes for super-resolution imaging in living cells. Nat Rev Mol Cell Biol 2008;9:929–43.*



Thompson MA, et al. 2012.
Annu. Rev. Biophys. 41:321–42

FIGURE 32.8 Superlocalization of a single fluorescent molecule. (A) Image of a single DCDHF-N-6 molecule (79) embedded in a PMMA thin film on a coverslip. (B) Cross section in the *x* direction through the center of the image in panel a. Each bin is a pixel (160 nm in width), and the counts in the pixel are the digital counts of photons recorded by the camera. The data is fit to a Gaussian function with a standard deviation of 200 nm, which is roughly equivalent to the diffraction limit. (C) Distribution of 50 obtained position localizations plotted on the same spatial scale as the data in panel b, showing a drastically smaller distribution (a standard deviation of 9 nm). Abbreviations: *PMMA*, poly(methyl methacrylate); *DCDHF*, dicyanomethylenedihydrofuran. *Taken from [30] Thompson MA, Lew MD, Moerner W. Extending microscopic resolution with single-molecule imaging and active control. Annu Rev Biophys 2012;41:321–42.*

turned on and off one at a time. This allows the location to be measured with precision, so that each fluorophore can be accurately localized, over many imaging cycles. This is known as photoactivatable light microscopy (PALM) or stochastic (STORM). These techniques rely upon temporal resolution being sacrificed in order to achieve greater spatial resolution.

We are able to locate one isolated fluorophore quite precisely, as we will not have confounding light from nearby fluorophores, and since the emitted light from a single fluorophore can be approximated by a Gaussian distribution known as a point spread function:

$$U(u,v) \approx A\exp\left(\frac{-(u-\mu_x)^2}{2s_x{}^2}\right)\exp\left(\frac{-(v-\mu_y)^2}{2s_y 2}\right) + B$$

where (u, v) are the coordinates in the sample plane, (μ_x,μ_y) is the true two-dimensional position of the molecule, A is the amplitude of the function, B is the background level, and s_x and s_y represent the width of the distribution in the x and y direction, respectively. By setting s_x and s_y as per the diffraction limit, we can resolve with $\sim$10 nm accuracy the true location of the particle (μ_x,μ_y). The accuracy depends only on the number of photons we can observe. The accuracy of position improves as $1/\sqrt{N}$, so with 100 photons we can localize the fluorophore position with 10 times greater accuracy (Fig. 32.8) [30].

When an image has multiple fluorophores, such as a whole cell, or a biological system with a high level of detail, then the individual point spread functions overlap, and we cannot resolve single fluorophores. However, since we can image one single fluorophore to around 10 nm precision, if we could turn each fluorophore on one at a time and record its position, we could build up a full map of each fluorophore in the system. This is precisely the mechanism by which these stochastic methods of ultraresolution are able to improve resolution.

The technological achievement that enabled this type of stochastic sampling of an image was the discovery of so-called photoactivatable fluorophores which could be driven into a dark state and then reexcited by excitation by a pump laser operating at 405 nm [31]. With these fluorophores, a cell could be labeled with a photoactivatable fluorescent protein at high concentration; however, when imaging, only a small subset of the proteins could be activated by the pump laser. Thus, at any one time only a few of the proteins would be activated, and they could be imaged and the location resolved to high accuracy.

Thus a high level of structural detail is seen, since details closer than 10 nm can be resolved (Fig. 32.9). The sacrifice comes in temporal resolution. Since only a few fluorophores are turned on at each moment, over many measurements, the time resolution is lost, but the structural detail and the spatial resolution is greatly improved.

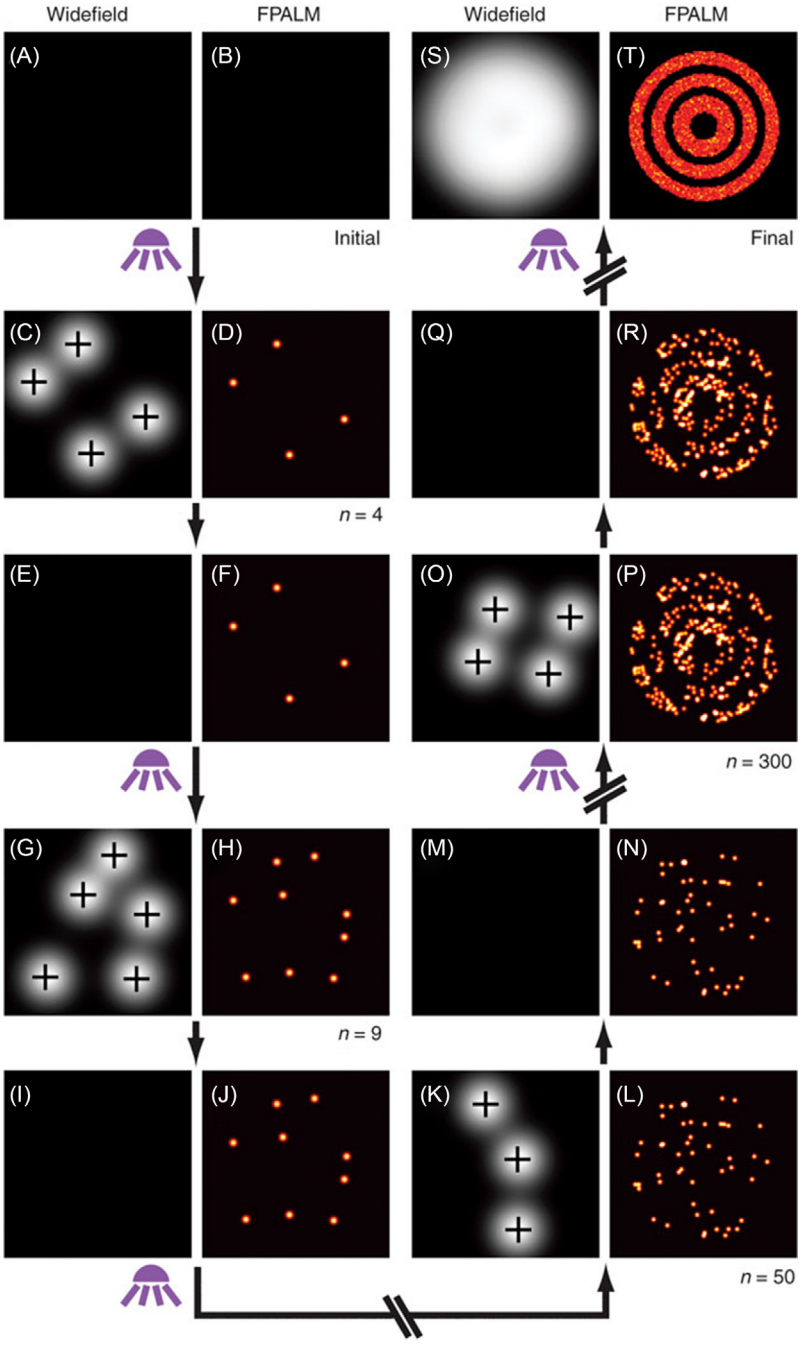


FIGURE 32.9 (A) Initially, photoactivatable probes are inactive and nonfluorescent under illumination by the readout laser (assumed to be continuous throughout) and (B) no molecules have been localized. After a pulse of illumination by the activation laser (purple light), (C) a sparse subset of molecules are activated and become fluorescent (bright spots) under the readout laser (on continuously). These active (fluorescent) molecules can be localized (black crosses) to start the buildup of the FPALM image, which is comprised of the plotted positions of all localized molecules (e.g., $n = 4$ in D and F). After the initial subset of (E) activated molecule bleaches, another subset of molecules is activated, (G) readout, and (I) bleached to continue building the FPALM image ($n = 9$ in H and J). After many cycles of (K, M, O, Q) activation, readout, and bleaching, the FPALM image begins to show structures (L, N; $n = 50$) and shows the underlying configuration of molecules as the density of localized molecules increases (P, R; $n = 300$). After a large number of photoactivatable molecules ($n = 10{,}000$) have been activated, imaged, and localized, (T) the FPALM image shows structures on subdiffraction length scales that are not resolvable in the (S) conventional widefield image. Images are simulated. *Taken from [32] Gould TJ, Verkhusha VV, Hess ST. Imaging biological structures with fluorescence photoactivation localization microscopy. Nat Prot 2009;4:291–308.*

Since the advent of the first photoactivatable fluorophores, there are now myriad fluorophores operating with different pump wavelengths and imaging wavelengths, creating a whole platform on which ultraresolution imaging can be based [33]. This enables imaging multiple components at the same time, looking at interactions occurring over nanometer length scales.

32.9 NONLIGHT TECHNIQUES: ELECTRON MICROSCOPY, X-RAY SCATTERING, AND FORCE MICROSCOPY

Other methods for visualizing structures at small length scales sidestep resolution limits associated with the diffraction limit by replacing light with electrons of much shorter wavelength. This is known as electron microscopy and can be performed using scanning electron microscopy, suitable for investigating the surface of materials [34], or transmission electron microscopy (TEM), which fires electrons completely through a sample, has much higher resolution, and is more suited to biological samples, especially when they are cryogenically frozen [25]. Recent advances in electron microscope cameras, namely that they directly detect electrons rather than photons, has led to phenomenal recent increases in TEM resolution [35]. To achieve even higher resolution, but requiring highly symmetric target structures, X-rays can be fired through a sample and the subsequent scattering can be used to identify crystal structures of proteins, or the radial symmetry of solution structures of proteins and nanostructures using small-angle X-ray scattering [36].

There is also a method for visualizing structures that directly images a surface due to contact with a cantilever, known as atomic force microscopy, or AFM [37]. This can be used to directly image interactions between the surface of the cantilever tip and the molecules on the surface, and can also measure structural properties of the molecules on the surface, such as the force required to bend or break these molecules.

32.10 CONCLUSION

We can now assemble biological components at the nanometer scale. This opens another 1000-fold level of detail over which we can construct, verify, and improve the structures we build. We still do not understand how nature controls self-assembly on these length scales with such precision, considering the inherent stochasticity of the environment, nor do we understand entirely how complex molecular architecture has arisen only from neighbor−neighbor interactions and subunit chemistry.

Enhanced understanding of these chemical interactions will enable us to employ such architectures in the fabrication of nanoscale systems. Top-down processes can only produce structures with a certain tolerance on the dimensions of structures. Bottom-up and self-assembly processes are effectively capable of infinitely fine tolerance of produced structures, but have limited degrees of control on where and when such structures are produced, due to the inherent stochasticity of such systems. In the future, a “synthetic” approach will no doubt be required, where we employ top-down techniques to define active regions for bottom-up and self-assembly processes to occur, closing the gap between the two paradigms.

Fabricating nanoscale structures and artificially synthesizing biological nanomachinery demonstrate that we are able to not only understand but also adapt nature’s blueprints for design. This will usher in the next era for nanotechnology, and enable the design of bespoke molecular nanomachines that promise to transform society through their applications to medicine, industry, construction—ultimately anywhere that further miniaturization is of benefit.

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Chapter 33

Ethical Aspects of Working With Local Communities and Their Biological Resources

I. Vandebroek
The New York Botanical Garden, Bronx, NY, United States

Chapter Outline

33.1 INTRODUCTION

Ethical issues can arise during any stage of research involving human participants. This chapter is written from the perspective of a field researcher (ethnobotanist) who is collaborating with indigenous and farming communities, here united under the umbrella term local communities, with the goal to record local knowledge (LK) of the biological resources they use to sustain themselves. Many local communities who are living in remote rural or impoverished urban areas possess substantive knowledge about their surrounding biological resources, while often facing limited participation in aspects of mainstream society involving land rights, access to markets and healthcare, or legal representation. The debate has been ongoing for more than two decades about how to best protect the rights of these communities during research that deals with their knowledge and use of biological resources. International law, professional societies of researchers, and international organizations have achieved substantial milestones in addressing how to best safeguard these communities' rights to be properly informed about research objectives and potential implications of a project, provision of prior informed consent (PIC), and sharing of benefits resulting from commercial development of their biological resources. However, this field of ethics is still evolving and even though its principles are clear (to obtain PIC from local communities and ensure their involvement on mutually agreed terms (MAT)), practical implementation not only differs from country to country, but faces important unresolved issues [1]. This chapter does not deal with ethics in clinical trials, even though important concepts addressed here are of equal relevance to the latter, including PIC, protection of human subjects against harm, and mindfulness in research.

The question may arise how this chapter is useful to ethnopharmacologists or pharmacognosists who do not conduct field research with humans but focus solely on the laboratory analysis of plant extracts and their bioactivity. The impetus for laboratory studies may have come from LK about the medicinal usefulness of a biological resource. It is important that laboratory-based scientists learn about the culturally sensitive context of the biological resources they are investigating, and how the rights of self-determination of the cultural groups that have traditionally used these resources are best protected. In an increasingly globalized world, we all need to stay informed about cross-disciplinary

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commonalities in ethics. Developing a culturally sensitive mindset is of vital importance for ethical actions by researchers, such as acknowledgment that LK is inherently linked to cultural heritage, and the need for joint publication of research results, as well as giving back to local communities. In the past, biological and cultural harms have resulted from research undertaken without the consent of local communities. The next generations of researchers, field- and lab-based alike, are expected to build harmonious and beneficial relationships with local communities when researching or handling the biological resources utilized by these communities.

33.2 LOCAL COMMUNITIES, LK VERSUS TRADITIONAL KNOWLEDGE

Local communities possess different types of knowledge about their environments and biological or genetic resources, including ecological, technological, theoretical, and practical knowledge about plants, animals, and the environment at large. These different types of knowledge are part of a larger body of LK systems that encompass knowledge, beliefs, traditions, practices, institutions, and worldviews [2]. The existence of LK systems is not restricted to any particular cultural group. Rather, all communities possess LK, including rural and urban communities, settled and nomadic peoples, original inhabitants, and migrants. LK can be seen as an umbrella for both traditional and indigenous knowledge [3].

Preference for the wording LK, traditional knowledge, or indigenous knowledge may vary according to country, field of study, and political point of view. For example, people struggling to gain recognition for their rights as indigenous people may logically prefer to use indigenous knowledge instead of LK. In this chapter, LK is used as a neutral, inclusive term that represents all types of knowledge, held by any community, regardless of its political implications, degree of cultural specificity, or geographic location. It is important to keep in mind, however, that researchers should be sensitive to local peoples' preferences of self-identification and be allies in their right of self-determination.

LK represents the human capital of local communities and is their main asset to exert control over their lives. LK systems are developed in a community over time, adapted to the local environmental and cultural context, dynamic, and constantly changing, based on continuous experimentation with the environment, and transmitted and molded over centuries of use. They serve the purpose of sustaining a community and its culture, as well as the biological resources useful to a community [3]. As such, LK carries important political and ethical implications that should not be overlooked during research and require a sensitive approach by researchers.

33.3 INTELLECTUAL PROPERTY RIGHTS

Intellectual property is a fundamental human right of all peoples (Universal Declaration of Human Rights in 1948). Everyone has the right "to the protection of the moral and material interests resulting from any scientific, literary or artistic production of which he is the author." For many generations, local communities have been involved in the discovery, improvement, and conservation of genetic resources for their benefit [4]. However, ownership of this knowledge carries little legal protection. *Unimproved* genetic and biochemical resources have been historically regarded as the common heritage of humankind. This means they are ownerless and were considered freely accessible to anyone. On the other hand, private ownership, and legal protection in the form of intellectual property rights (IPR), exists for *improved* products based on these genetic resources. This depicts a bleak scenario in which individuals or companies in the North have been able to obtain genetic resources free of charge from resource-rich countries in the South, patent the genes and chemicals in these resources without fair compensation of the providers, and sell the patented improved products back to the countries where the source material originated. The dilemma here is that IPR are applied to protect transgenic plants, plant varieties, and earlier also to protect isolated natural biochemicals from plants, but not the LK, nor the wild plant species associated with this knowledge. The field of IPR is rapidly changing and laws vary from country to country. Until recently, natural biological substances could be patented in the United States if they were sufficiently "isolated" from their naturally occurring states. Examples are patents on insulin or vitamin B12. Today, patenting isolated natural biochemicals is no longer possible. In 2013, the US Supreme Court ruled that mere isolation of a *product of nature* is no longer sufficient to satisfy the criterion of *invention*. In patent law, three criteria are used to judge if something is patentable: novelty (refers to the degree of *newness* or absence of an existing knowledge base), nonobviousness (which involves an inventive step), and utility (meaning there is a market for the invention).

LK faces difficulties satisfying the requirements for patent protection. This knowledge is often verbally transmitted without written records, and is not held by individuals but collective in nature, meaning that as an intangible cultural component it belongs to a community or an entire cultural group. Also, cultures do not operate in a vacuum. It is a universal human trait to share experiences and information. Exchange of biological resources and LK frequently occur through trade and intermarriages, especially between neighboring cultural groups. Western IPR law is based on

individual property ownership, a concept that is often alien and potentially detrimental to many local communities [4]. Furthermore, the cost of applying for, defending, and enforcing a patent or other protective mechanisms is often prohibitive for local communities.

Possibilities for protecting LK under IPR regimes include patents, petty patents, plant patents, plant variety certificates, trade secrets, geographic indicators (certificates of origin), traditional knowledge registries, and *sui generis* protection systems [4]. However, applying for any of these mechanisms of protection requires access to technology and probably also the involvement of people and institutions outside local communities, including government officials and IPR specialists. In addition, these mechanisms require access to sums of money likely to be several dimensions higher than the cumulative income of all members of a community. For many, if not most, local communities, these mechanisms are not only alien, but will likely remain out of their economic reach for the foreseeable future.

33.4 THE LEGAL FRAMEWORK OF FIELD RESEARCH WITH LOCAL COMMUNITIES

Research with local communities and their biological resources takes place within a legal framework shaped by United Nations treaties such as the Convention on Biological Diversity (CBD) (1992) and the Nagoya Protocol (2010). The interpretation of this legal framework into national requirements for research or development differs from country to country and has been repeatedly scrutinized and revised over the years. Laws and guidelines are constantly evolving. Therefore, it is important that researchers who wish to obtain approval for their research projects from national governments consult with national focal point persons to obtain the latest information about the documents required for application (see https://www.cbd.int/countries/ and search under country profiles).

The United Nations Declaration on the Rights of Indigenous Peoples in 2007 represented an important milestone in the path towards ethical research practice. While not legally binding, it constitutes an important standard for engaging with indigenous peoples and protection of their LK. By virtue of their right to self-determination, indigenous communities are entitled to collectively and autonomously decide about what happens in their communities and territories, and to give or withhold their consent to any proposal made for research [5].

33.4.1 The Convention on Biological Diversity (1992)

The CBD is a multilateral treaty that recognizes conservation of biological diversity as "a common concern of humankind" and an integral part of sustainable development. The CBD represents the first attempt by the international community to address biological diversity as a whole in a global legal instrument. The CBD was established at the Earth Summit in Rio de Janeiro in 1992 out of global concerns for the decrease in biological diversity due to human activity, and the need to counter this trend for the benefit of future generations. The CBD entered into force on December 29, 1993, with three major objectives: (1) The conservation of biological diversity; (2) the sustainable use of the components of biological diversity; and (3) the fair and equitable sharing of benefits arising from the utilization of biological diversity. The CBD reaffirmed that States hold sovereign rights over their biological resources. It further requires that access to and transfer of biological resources can only occur with the PIC of Member States and communities who use these resources as part of their LK systems. The CBD assigns national sovereignty to, but not ownership over, biological resources. It obliges states that have ratified this convention to: (1) Acknowledge that communities have a vested interest in their LK; (2) recognize that communities need to stay involved in potential industrial uses incorporating their LK; and (3) share commercial benefits arising from the utilization of this LK. One of the difficulties with the CBD is that it does not convey any property-like rights to LK holders, but only recognizes their cultural relation and interest therein, as well as their right to receive benefits [6].

The CBD has established the concept of *access and benefit sharing* (ABS) [5]. Since the Convention's entry into effect in 1993, ABS has been one of the most controversial regulatory and public policy issues from an international and national point of view. ABS agreements typically form part of bioprospecting endeavors, in which LK is used to identify and subsequently screen commercially valuable genetic and biochemical resources. While most countries (168 in total) are signatories of the CBD, relatively few (57 in April 2014) have implemented national measures regarding ABS. Moreover, the countries that do have national ABS frameworks in place use different mechanisms to regulate access. One of the many dilemmas countries face is how to promote basic research without weakening control over access to genetic resources by third parties, and how to strengthen those controls without limiting research [7]. Many a scholar conducting basic research has experienced a great deal of confusion and frustration when attempting to follow national access procedures because of unclear rules and differing approaches for basic and applied research. The development and implementation of national ABS legislation and regulatory measures is solidly in the hands of national

authorities. Therefore, national authorities represent the best source of information for researchers as they can explain and clarify the legal framework and provide details about the needed documentation to obtain government approval for research.

33.4.2 The Nagoya Protocol (2010)

Nearly two decades after the CBD entered into force, a large number of its member countries continue to face challenges for the adoption and implementation of functional national ABS laws and measures. The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the CBD, which entered into force on October 12, 2014, is a supplementary agreement to the CBD. It provides a legal framework for benefit sharing regarding genetic resources and LK associated with these resources.

The Protocol goes further than the CBD in establishing an obligation to take measures in accordance with domestic law for obtaining PIC or prior approval and involvement of local communities for access to genetic resources where they have the established rights to grant access to those resources.

The Nagoya Protocol drew on an analysis of existing legal and other instruments related to ABS at national, regional, and international levels. This included access contracts, experiences with their implementation, as well as compliance and enforcement mechanisms. Article 5 of the Nagoya Protocol requires that benefits arising from the utilization of genetic resources, their subsequent applications and commercialization, are to be shared in a fair and equitable way with the party providing such resources. Article 5 states that such sharing shall be upon MAT. These MAT can be specified in an ABS agreement that details the benefits to be shared. The Protocol reiterates the fact that benefits shared on MAT may be monetary and/or nonmonetary. Those benefits should be shared with the indigenous and local communities that are holders of genetic resources under domestic legislation.

The Nagoya Protocol requests that the signing parties (the countries providing genetic resources) establish compliance measures to ensure that genetic resources and associated LK are utilized based on PIC and in accordance with MAT. These measures are one of the innovative mechanisms set out by the Protocol. The Protocol leaves a great degree of discretion to provider countries as to the types of measures they may adopt to meet this obligation. The Protocol only predicates that countries shall designate one or more checkpoints to monitor and enhance transparency on the utilization of genetic resources.

Unresolved issues of the Nagoya Protocol pertain to its application related to genetic resources and LK that were acquired before it entered into force (previous acquisition) and whether it includes knowledge that is publicly available or so widespread that it is common knowledge not connected to one particular community [1].

33.5 BEYOND THE LEGAL: ESTABLISHING ETHICAL RESEARCH PARTNERSHIPS WITH LOCAL COMMUNITIES

Ethical considerations are an essential part of the research process. They begin with the understanding that local communities are full-fledged partners in research instead of "a source of informants and information." I avoid using the word "informant" in my own research, even though the literature frequently uses this term, because it hints at a more extractive type of relationship between researchers and local communities. Instead, I prefer the word "participant," because it indicates a partnership, and sets a clear standard for the equity of collaborations with local communities. Other misleading terms, including "Western" and "traditional," are discussed by McClatchey [8].

It is essential to listen to, and incorporate, the voices of local communities during the conception of a project, and to plan the project in accordance with community ways of communicating and decision-making. Decision-making can be a lengthy process and involve consensus discussions. It is imperative that researchers do not rush this process, even if it contradicts with the often narrow time windows granted to them by their funding sources or universities.

Ethics continue to matter long after a project ends. Ethical questions live on in decisions to publish (Will the community be acknowledged in coauthorship with researchers?), in expectations from community members after the project ends (What happens next with the project outputs? Will there be any follow-up? How is community ownership of project results guaranteed?), in considerations about intangible knowledge recorded as project results that may be developed later into tangible products for commercial gain by third parties (Should certain results be withheld from publication?). These and other questions and the answers to them, if overlooked or ignored, can wreak much havoc to well-meant projects (see also [9] for five critical questions that every ethnobiologist should ask prior to research).

33.5.1 Mindfulness

Attention to imbalances of power is a crucial element in the collaborative experience with local communities. Researchers should be aware of, and reflect on, how the project, its results, and even their presence in the community, can impact the lives of the people they are collaborating with. Many communities live in areas of high interlinked biological and cultural diversity. Commonly, these communities also face searing poverty and other inequalities such as lack of integration into market economies, access to biomedical healthcare and schooling, and struggles about territorial or land rights. Researchers who have not fully reflected on these inequalities may unintentionally reinforce power imbalances, e.g., by prioritizing their own goals over the challenges faced by participants. Reflectivity on the part of the researcher includes recognition of asymmetrical power relationships in which the researcher holds significantly more power, and the ways in which power shapes the researcher–participant relationship.

The International Society of Ethnobiology describes mindfulness as "a continual willingness to evaluate one's own understandings, actions, and responsibilities to others." Researchers should cultivate an attitude of constant self-questioning about the research process, about ethical issues that can arise at various stages of research, and about the consequences of their decisions and actions, even those that are well-intended [5]. *Mindfulness* is the core value in the Society's Code of Ethics that is available in eight languages at http://bit.ly/1P4CQy7. This Code provides a framework for establishing ethical and equitable relationships with local communities. It contains 17 principles and 12 practical guidelines that "embrace, support and embody the concept, and implementation of traditional resource rights."

33.5.2 Prior Informed Consent

The essential step before commencing research, and before each individual interview, regardless of it being casual or a survey, is to obtain PIC. Obtaining PIC means that the researcher explains the project aims, and the possible risks and benefits of participating in the project, in a clear and simple language. Communities have the right to refuse to participate in a project, and participants have the right to refuse to answer any question that makes them feel uncomfortable, or to stop participating at any time. Prior to an interview, each participant should receive a copy of the consent form that describes this information in detail. The form should also specify if the participant will receive any monetary compensation or a gift after the interview. PIC can either be recorded as a signature (written consent) or verbally. The type of consent chosen greatly depends on the local context. Members of local communities who are not accustomed to signing documents might find the procedure of written consent intimidating and suspicious. For persons who lack literacy, requesting written consent may be downright insensitive. In immigrant communities with many undocumented persons, requesting written consent may provoke fear of identification. It is the responsibility of the researcher to be thoroughly informed about the local context, ask advice from community leaders, and choose the type of consent that promotes a trusted relationship.

33.5.3 Continued Communication Is Key

Effective communication is essential to building lasting collaborations. Community members should know at any time what to expect, and what not to expect, from a project. When research does not go as planned, and changes in research directions are contemplated, it is important that this is done in close consultation with the community. It is advisable to tone down unrealistic expectations about research outcomes, such as the fast development of a new clinically tested pharmaceutical from a medicinal species, or other goals that take substantial time to develop without guaranteed success. In an ideal research team, community members are trained as para-scientists who can learn the ropes of scientific methods such as plant collection, interviewing, recording of geographic coordinates, and assist researchers during fieldwork. It offers local youth the opportunity to get familiar with science, and possibly inspiration to pursue further studies. Hereby, the project directly builds local capacity and increases the chances that communities take ownership of the results. When the project ends and researchers leave, it is the community who ultimately decides on the long-term fate of the project.

33.5.4 Publish or Perish?

Researchers who work with local communities can be caught between a rock and a hard place when it comes to publishing research results. On the one hand, science is based on the premise that research data is made widely available for scrutiny. On the other hand, participants may not be comfortable with the publication of certain aspects of their LK,

e.g., knowledge about sacred medicinal recipes. Researchers should carefully consider and reconcile their different roles as scientists and community allies when it comes to publishing. Coauthorship of community members on publications is one way to acknowledge local peoples' invaluable contributions to a project.

One of the approaches to protecting LK is that of *defensive disclosure*, whereby information is deliberately put in the public domain and serves as *prior art*. When there is proof of prior art, third parties can no longer patent an invention. Open or closed LK registries, and community guidebooks summarizing project results in local languages, are examples of prior art. Community guidebooks offer the additional advantage of returning project results to the community [10].

33.5.5 Requirements From National Governments and Home Institutions

In-country permit applications commonly require proof of previous consultation with local communities and their leaders as one of several steps in obtaining government permission for field research. The verbal consultation process usually leads to a written agreement. In my own work in Bolivia, a "convenio" (agreement) was drafted that stipulated mutual expectations by the community and the research team. The document was reviewed by all parties involved in the project, revised, and subsequently stamped and signed by community representatives and researchers. This written agreement also represented an important instrument for the community to formulate their expectations of the project as tangible results, including a community guidebook about the plant resources they used as medicines, a cultural heritage component that had not yet been formally recorded [10].

International organizations, professional societies, and granting agencies worldwide (e.g., WHO, American Anthropological Association, European Union) have developed clear ethical guidelines that research projects must adhere to. In the United States, universities require researchers to apply for Institutional Review Board (IRB) approval prior to initiating research. An IRB is a committee established to review and approve research involving human subjects. The purpose of the IRB is to ensure that research be conducted in accordance with all federal, institutional, and ethical guidelines. Researchers applying for IRB approval are required to take Human Subjects Training to understand the protection of the rights and welfare of human participants in research. In addition, in preparation for an IRB application, researchers must provide detailed information about their strategies to recruit participants, minimize potential risk and harm to participants, maintain confidentiality of research data, offer benefits for participants (monetary or nonmonetary compensation), and use photo and/or video materials. Project flyers and consent forms are also scrutinized by the IRB. Numerous other countries have equivalent regulations or guidelines, and ethics committees that oversee them, but deficiencies in ethics review, training, and monitoring are not uncommon [11].

33.6 WHAT ABOUT ETHICS IN LABORATORY RESEARCH?

Scientists who are not doing fieldwork are not exempt from learning about, and applying, ethical guidelines and principles associated with field-based research. The central concept of mindfulness applies to laboratory research of plants used by local communities, and includes reflection on questions such as: Was plant material obtained in accordance with the Nagoya Protocol and national legislation on plant collection and ABS regulations? Were local community members thoroughly informed about the project, its outcomes, potential benefits, and risks of the study? Did community members give their PIC before providing information on plant uses? Already 25 years ago, even before the CBD was developed, ethnopharmacologist Elaine Elisabetsky [12] pleaded for an ethical framework for ethnopharmacologists. When pharmacological or chemical studies of a biological resource (plant, animal, or mineral) are undertaken with the intention to corroborate its traditional use, local communities play a pivotal role in the research process, even if plant collection or interviewing was not done by the laboratory-based researcher. In these cases, the rights of the communities should remain at the forefront at all times, and feature in all decisions about project deliverables, including publications and benefit sharing. In other words, being a laboratory-based researcher working only indirectly with LK does not imply exclusion from the intricacies of the debate on LK and IPR, safeguarding PIC, or foregoing ABS regulations, nor from claiming research results as being solely the scientific merit of the laboratory.

33.7 CONCLUSION

In collaborations involving local communities and their biological resources, the key to ethical research is in establishing successful, lasting partnerships. This requires a shift in mindset, in which local community members are no longer seen as passive bystanders and informants from whom data can be readily extracted. Instead, an ethical collaboration

requires that both researchers and local community members operate on an equitable basis, in which scientific knowledge and LK are considered complementary sets of data within their own epistemological and cultural frameworks. In getting to that state of mind, I have summarized the following do's and don'ts:

Do's:

1. Do your due diligence: learn about the local cultural context, consult the social sciences literature. Build lasting contacts with the community and the country.
2. Commit yourself to ethical professional guidelines. Be mindful; constantly question your assumptions and behavior. Be aware of your own cultural biases.
3. Inform yourself thoroughly about required documents for project approval at different levels (university, community, country).
4. Mention community members as collaborators in publications. Establish the best ways and formats to give back the results to the community when the project ends.

Don'ts:

1. Take shortcuts in project planning or outreach. Prepare a realistic planning of project activities that includes time needed to obtain permits and giving back the results to the community.
2. Assume that it is enough to focus on obtaining research results and neglect ethical aspects of the project that are not routinely a part of your discipline.
3. Ignore the importance of PIC, which includes informing community leaders and interview participants about their rights and risks of participating in the project.
4. Skip feedback of project results to the community, even if you are pressed with time.

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Chapter 34

Factors to Consider in Development of Nutraceutical and Dietary Supplements

P.K. Mukherjee[1], R.K. Harwansh[1], S. Bahadur[1], V. Duraipandiyan[2] and N.A. Al-Dhabi[2]
[1]*Jadavpur University, Kolkata, West Bengal, India* [2]*King Saud University, Riyadh, Saudi Arabia*

Chapter Outline

Learning Objectives

- Botanicals useful as nutraceuticals and dietary supplements
- Major aspects for development of nutraceuticals and dietary supplements
- Identification and authentication of the plant material
- Metabolomics approaches for food science and nutrition research
- Quality control, standardization, and scientific validation of botanicals
- Pharmacovigilance and regulatory affairs of nutraceuticals and dietary supplements

34.1 INTRODUCTION

Nutraceuticals can be defined as diet supplements that deliver a concentrated form of a presumed bioactive agent from a food, presented in a nonfood matrix and used in dosages with the purpose of enhancing human health care [1]. Since ancient times, natural products have been used as a prominent source of therapeutic agents for the cure and mitigation of diseases in humans and animals [2]. Hippocrates (460−370 BC) stated "Let food be your medicine and medicine be your food." In the 20th century, the term nutraceutical was introduced to describe a union between nutrition and pharmaceutics, with both being major contributors to human health and wellness [3]. There is growing research on the biologically active functional compounds exhibiting promising fields for application of such compounds in food and nutritional products, thus developing added value for manufacturers and benefits for consumer health. Nutraceuticals and functional foods have similarities in nature but have some difference in the format by which they are consumed. Nutraceuticals are usually consumed in a dosage form similar to pharmaceuticals (capsules, pills, tablets, etc.), while functional foods are always consumed as normal foods and have several potential health benefits [4].

The European Nutraceutical Association defines nutraceuticals as nutritional products which have effects that are relevant to health. In contrast to pharmaceuticals, however, these are not synthetic substances or chemical compounds

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formulated for specific indications. These are products that contain nutrients (partly in concentrated form) and are assigned to the category of food. They can be bought over the counter in pharmacies, supermarkets, specialist shops, and via the Internet. According to the European Food Safety Authority, botanicals can be considered as a type of food supplement, but not strictly as nutraceuticals, due to their nonfood origin. As defined by the American Nutraceutical Association, a nutraceutical is any substance that is a food or a part of a food and provides medical or health benefits, including prevention and treatment of disease. Such products can be in the form of isolated nutrients, dietary supplements, and specific diets, to genetically engineered designer foods, herbal products, and processed foods, such as cereals, soups, and beverages [5].

Dietary supplements are defined in the US Drug Supplement Health and Education Act of 1994 as a product (other than tobacco) that includes one or more of the dietary ingredients like a vitamin, a mineral, an herb or other botanical, an amino acid, or any other substance used to supplement the diet by increasing total dietary intake, metabolite, constituent, extract, or combination of any of the above [6].

During development of nutraceuticals and dietary supplements, there are several important aspects which should be considered for their global acceptance, including efficacy, safety, and stability [7]. Furthermore, the production- and consumption-related issue of nutraceuticals are that the composition and contents of active constituents in natural plants vary depending on season, climate, temperature, humidity, soil, and several other factors. So the collection, identification, and maintenance of uniform quality, standardization, metabolite profiling, efficacy, safety, bioavailability, stability, regulatory issues, etc., are significant factors to be considered for better understanding of the health-promoting effects of the nutraceuticals [6].

34.2 BOTANICALS AS NUTRACEUTICAL AND DIETARY SUPPLEMENTS

Botanical-based products are the backbone of the health food market and various types of products have been developed and promoted. They include whole foods with benefits beyond basic nutrition, like grains, beans, pulses, cereals, fruits, spices, vegetables, tea, coffee, etc. (Fig. 34.1). Typical products in this category would include rosemary, green tea, and garlic extracts [8]. Several dietary supplements have been marketed in different pharmaceutical dosage forms, such as tablets, capsules, powders, or liquids, that are derived from plants and used as primary food sources [9].

According to the World Health Organization (WHO), about 5.6 billion people (80% of the world's population) depend on medicinal plants for their primary health care needs, and they are most widely used for the treatment of various acute and chronic diseases [10]. Botanicals have been traditionally used for promotion and management of human health issues, e.g., *Ginkgo biloba*, *Allium sativum*, *Camellia sinensis*, *Coffea arabica*, *Daucus carota*, *Emblica officinalis*, *Dillenia indica*, *Glycine max*, *Moringa oleifera*, and *Vitis vinifera*. Several botanical preparations (raw materials and finished products) have a certain history of human exposure either as foods or from other sources [11]. Thus, evaluation of a botanical preparation should exploit all existing knowledge and should be scientifically validated [12]. Several aspects need to be considered for development and promotion of nutraceutical and dietary supplements. There are several factors which have a significant role in the development and promotion of botanicals as nutraceuticals and functional foods (Fig. 34.2).

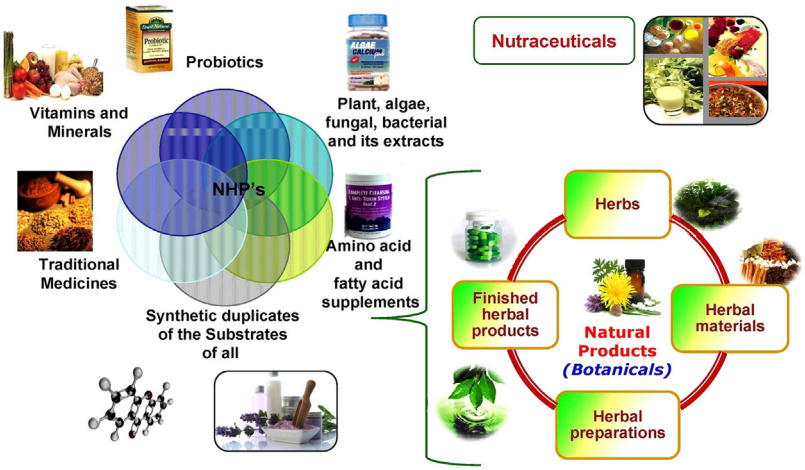

FIGURE 34.1 Botanicals used as nutraceuticals and dietary supplements.

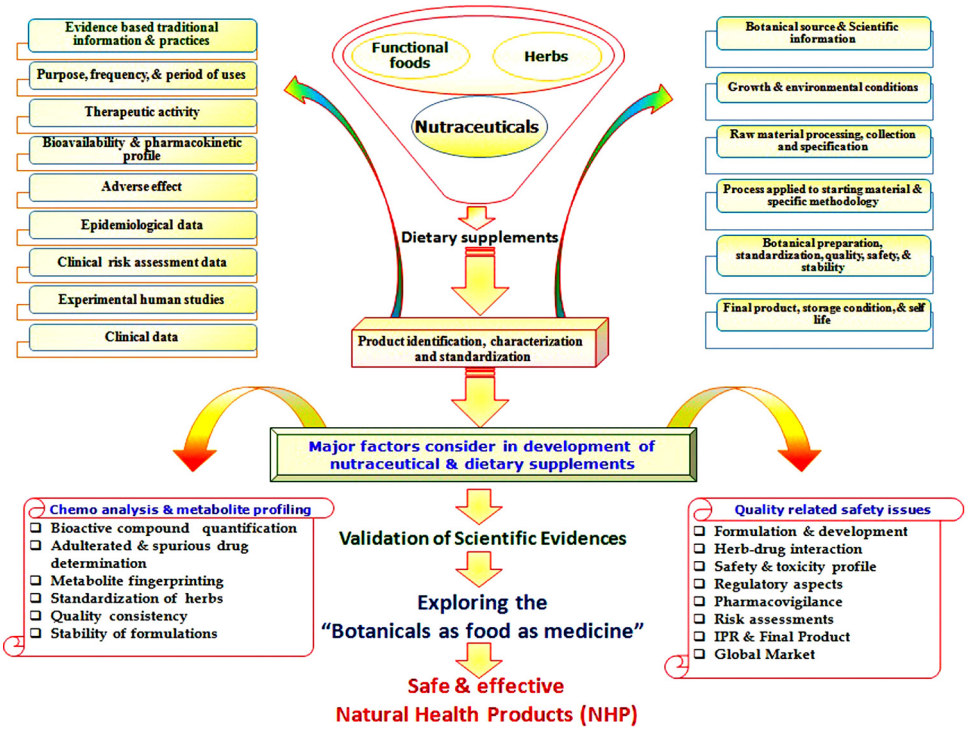

FIGURE 34.2 Factors for development and evaluation of herbs used in development of nutraceuticals.

34.3 DEVELOPMENT AND PROCESS VALIDATION OF BOTANICALS AS FUNCTIONAL FOOD

All botanicals and their derived products should be properly characterized and standardized through analytical techniques. The available data on the individual products should be obtained on a sufficient number of batches for the evaluation of natural variability and the establishment of suitable specifications [11]. Details of sampling schedules should be considered with appropriate deliberation for analytical and batch to batch variability in the nutraceuticals preparation. These are some of the important processes which need to be validated very carefully in each and every step [13].

34.3.1 Botanical Source

- Identity
- Scientific name (plant family, genus, species with name of authority, and if relevant, variety, and chemotype), common names/local names
- Part(s) of the plant used
- Geographic origin (continent, country, region)
- Environmental conditions

34.3.2 Growth Conditions

- Wild or cultivated
- Good agricultural practice
- Site of collection, time of harvest, stage of growth
- Drying, fermentation
- Storage conditions
- Pre- and postharvest phytosanitary treatments (e.g., use of pesticides)

34.3.3 Raw Material (e.g., Dried Plant Material)

- Specifications according to standard reference (e.g., Pharmacopeias) including:
- Identity tests e.g., macroscopic examination, microscopy, FT-IR, thin layer chromatography (TLC), high-pressure liquid chromatography (HPLC), gas chromatography (GC)

- Quantitative tests to determine
  - Constituents relevant for the beneficial effects
  - Constituents of toxicological relevance

34.3.4 Process Applied to Starting Material

- Steps of preparation (e.g., extraction process, solvents)
- Methods used
- Specific precautions (light, temperature sensitivity)

34.3.5 Botanical Preparation

- Standardization criteria (markers: toxic or physiologically active constituents, other relevant constituents; plant−extract ratio)
- Specifications (level/range for markers)
- Physicochemical properties of the relevant constituents (stability)
- Purity criteria (e.g., microbiological, mycotoxins, pesticides, heavy metals, residual solvents, other contaminants) either by chain control or analysis
- Level and nature of excipients
- Formulation methodology
- Storage conditions

34.3.6 Final Product (Food or Supplement Containing the Botanical Preparation)

- Fate in food or formulated product (e.g., stability)
- Industrial food processing
- Preparation for consumption

34.3.7 Information Required for the Intended Application

A crucial aspect is needed in considering the development of dietary supplements as natural health products (NHPs) for the management of human health. The knowledge and information required on the intended use of the product and their consequences are illustrated below [13].

34.3.7.1 Intended Use

- Description of the product, i.e., food or food supplement as consumed
- Product composition (list of ingredients, concentration of active ingredients)
- Purpose of the product, anticipated health effects
- Proposed use: frequency, duration, level of consumption, population/defined target groups

34.3.7.2 Dietary Exposure

- Estimated dietary exposure of the active ingredient(s) via product and habitual diet taking into account geographical/cultural variation
- Estimated dietary exposure in nontarget groups taking into account geographical/cultural variation
- Assessment of consequences of aggregate exposure (combined exposure from other products in which the active ingredient(s) is/are added and/or present naturally)

34.3.7.3 Dietary Consequences

- Assessment of the nutritional consequences of the introduction of the new product

34.4 IDENTIFICATION AND AUTHENTICATION OF THE PLANT MATERIAL

The botanical identification and authentication is the first crucial step to avoid confusion, admixtures, or adulterations in the botanicals. Identification should be carried out combining various methods, including macroscopic and microscopic examination, chemical fingerprinting, and DNA-based characterization [14]. The useful active compounds in plants for nutraceuticals preparation are among the huge diversity of secondary plant products that are often specific for certain plants or plant groups [11]. Characters and individuality that can be used to identify the plant material, which can be grouped into different categories.

34.4.1 Macromorphology

In this category all the visible characters present in the plant specimens should be described. All significant characters including roots, leaves, stems, flowers, and fruits must be consulted.

34.4.2 Microscopy

The plant material used may not be available as a whole but traded as parts of the plant. Microscopic characters comprise the structure of hairs on plant surfaces, the presence of crystals in the tissues, or the occurrence of specialized cells. These types of characteristics can be most commonly found in the monographs of various pharmacopoeias or other compilations.

34.4.3 Phytochemical Characteristics

A fingerprint profile of all the compounds is usually characteristic for an individual plant or part of a plant. The analyzed compounds may or may not be the bioactive substances, but quite often are characteristic marker substances only. The chemical fingerprints obtained by various chromatography techniques including TLC/HPTLC/HPLC of nutraceutical preparations should be also documented.

34.4.4 DNA Sequence

As the genetic composition is unique for each species and is not affected by age, physiological conditions, and environmental factors—DNA-based markers are also used in identification of inter/intraspecies variation. Random amplified polymorphic DNA-based molecular markers have been found to be useful in differentiating species of medicinal plants. From the last two decades an important approach for plant identification has emerged based on their individual plant DNA sequences. The DNA sequence is unique for an individual and similar between closely related individuals. Some DNA regions are conserved within a specific taxon but differ between taxa. These parts of the DNA sequence can be used to study the relationship of taxa (phylogeny) or to identify a specific taxon, often referred to as DNA barcoding. Therefore, these can also be applied in processed products, as is the case in plant extracts [15].

34.5 METABOLITE PROFILING AND CHEMO-ANALYSIS

Metabolite profiling not only identifies the metabolites but also compares the nature of compounds. The output of sensors (analytical detectors) is known as “profiling,” which are classified and statistically analyzed to mark their differences [16]. Metabolite profiling involves identification of metabolites as the analysis is based on their spectral peaks and calibration curves. Metabolomics studies may help in the scientific validation and documentation of dietary supplements through characterization of several metabolites and biomarkers in the nutraceutical preparation [17]. Metabolome investigation comprehensively examines the entire range of metabolites in a sample by the mutual application of various analytical techniques [18]. These techniques include mass spectrometry (MS), nuclear magnetic resonance (NMR), HPLC, capillary electrophoresis (CE), HPLC-NMR, HPLC-MS, GC-MS, and CE-MS to perform the comprehensive analysis of food products [19,20].

Comprehensive analyses of nutraceutical preparations are required for both pharmaceutical and industrial purposes through metabolomics techniques [21]. There is a need to focus on potential applications of metabolomics in several major areas of nutraceutical and dietary supplement development, e.g., (1) component analysis; (2) quality/authenticity assessment; (3) monitoring the consumption; and (4) physiological monitoring in food intervention [22].

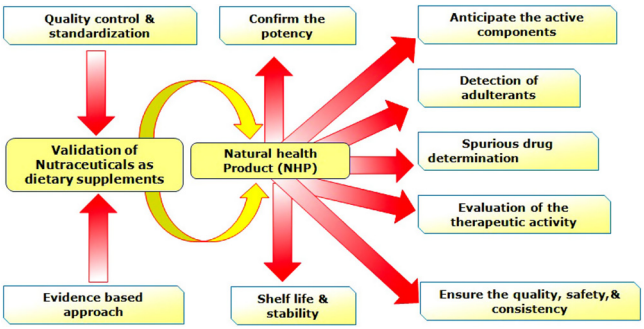

FIGURE 34.3 Scientific approaches for marker profiling.

The standardization and chemoprofiling of herbal drugs includes authentication, harvesting the best quality raw material, assessment of intermediate and finished products, and detection of harmful and toxic ingredients (Fig. 34.3). Some specific markers are required for the quantitative studies of herbal products. 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. Marker components may be classified as active principles, active markers, and analytical makers, while biomarkers may be defined as pharmacologically active. The 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 a single or several marker compounds by a developed method is required for quality control purpose [15].

34.6 REGULATORY ASPECTS, STANDARDIZATION, AND SCIENTIFIC VALIDATION

Regulatory affairs establish common principles and responsibilities that provide a strong scientific database, efficient organizational arrangements, and procedures to underpin decision making for nutraceutical preparations. The main objective of regulatory affairs is to provide the basis for the assurance of high quality of food products which can increase consumer's interest for ensuring the efficacy, quality, and safety. Regulatory frameworks vary from region to region as detailed in the chapter on regulations in this book. In Europe, the Committee on Herbal Medicinal Products is an excellent model for how scientific evaluation of herbal medicines can be harmonized and accepted through science-based standards to ensure public health [23].

Some parameters for the understanding the development of herbal drug regulation in a given nation are general policy structure, drug registration system, development of pharmacopoeia, national monographs, inclusion in essential medicine list, and drug type (OTC or prescription). Most countries, with the exception of Bhutan, Sri Lanka, and the Maldives, have herbal drug regulation and registration systems. Korea, Indonesia, India, Myanmar, Sri Lanka, Thailand, China, Malaysia, and Vietnam have National Monographs for herbal drugs. Pharmacopoeias for herbal medicines are developed in most counties. In Bhutan, India, Thailand, China, the Philippines, Republic of Korea, and Vietnam, the essential medicine list includes herbal drugs [24].

In Canada, NHPs sold are subject to the NHPs Regulations, which came into force on January 1, 2004. Under this regulation, all producers of NHPs need to apply for licenses before selling them in Canada [24].

In India, the Department of Indian Systems of Medicines and Homoeopathy was established in March 1995 as a separate department in the Indian Ministry of Health and Family Welfare and renamed as the Department of Ayurveda, Yoga & Naturopathy, Unani, Siddha and Homoeopathy (AYUSH) in November 2003 with a view to providing focused attention to the development of Education and Research in AYUSH. The Department has been elevated to an independent Ministry of AYUSH in 2014. The Drugs & Cosmetics Act of 1940 lays down various rules for production and marketing of herbal products. Schedule T of Drugs and Cosmetics Act, 1940, specifically deals with the good manufacturing practice (GMP) for AYUSH drugs [25].

In China, Chinese herbal products are governed by the State Food and Drug Administration (FDA) and can be registered as functional food or drugs. Regulatory approval of functional food is the responsibility of the Department of Food License, whereas that of Chinese herbal drugs is controlled by the division of Traditional Chinese Medicines (TCMs) & Ethno-Medicines under the Department of Drug Registration. Drugs in China cover not just chemical/synthetic drugs but also traditional medicines. The supervision on TCM in China is as strict as that of chemical drugs and biological products. Registration of TCM is subject to strict technical evaluation, safety data, and clinical trials [24]. The Codex Alimentarius has developed some specific guidelines on food supplements [26]. The WHO model covers a

definition of health supplements, provisions to establish for risk assessment, labeling requirements, a negative list of ingredients, provisions for nutrition and health claims, and standards for GMP for food supplements.

In the United States, dietary supplements do not need approval from the FDA before marketing. Companies that manufacture or distribute dietary supplements containing "new dietary ingredients" are required to submit premarket safety notifications.

But the FDA can take any regulatory action to remove unsafe products from the market, including products containing new dietary ingredients for which there is inadequate evidence of safety in a premarket safety notification [24]. The FDA has published guidelines on "substantiation" of "structure/function claims" for use by manufacturers who want to substantiate those claims through the application of a substantiation standard of competence with reliable scientific evidence to claim about the benefits and safety of dietary supplements [27]. The entire nutraceutical and dietary supplement must be manufactured under current good manufacturing procedures established by regulation in 2007 [28]. In July 2011 the FDA issued guidelines on how to comply with the regulatory requirements to provide a premarket safety notification for dietary supplements. This draft contains discussions on how to determine the identity of plant-based ingredients and how to explore the history of use or other evidences to demonstrate the safety of plant-based ingredients [29]. Therefore, there is an urgent requirement for adherence to GMP to assure the quality of nutraceuticals and dietary supplements [30].

34.7 PHARMACOVIGILANCE

Pharmacovigilance is the process of monitoring, evaluating, and communicating drug safety, which has 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 evaluation during clinical trials. Although these methods were developed for monitoring modern medicines they are also applicable for evaluating the safety and toxicity of nutraceuticals products. The Medicines and Healthcare Products Regulatory Agency define some significant problems in the regulation of herbal medicines in the United Kingdom [31] which include (1) lack of knowledge about the products being used; (2) limited use of yellow card adverse drug reporting scheme; (3) lack of 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 GMP standards for manufacturing. Pharmacovigilance is a very essential tool for developing reliable information on the safety of nutraceuticals and herbal medicines [32]. The recent information with "phytovigilance" (the term used for pharmacovigilance of herbal drugs) raises the suspicion that there is a tendency to unequal treatment of herbal medicine. In so many countries, manufacturers do not require any regulation to demonstrate the safety and efficacy of herbal products in human trials before marketing and there are no specific warnings about known or unknown adverse drug effects on labels [33]. The WHO has recognized the importance of the use of herbal medicines and developed some guidelines for the monitoring of herbal safety within the existing pharmacovigilance framework [34]. WHO started International Drug Monitoring Programme in 1968, for identification of the earliest possible pharmacovigilance signals. The program now has more than 80 member countries from all parts of the world contributing individual case safety reports (ICSRs) to the WHO Global ICSR Database System, VigiBase. The VigiBase data resource is the largest and most comprehensive in the world, and it is developed and maintained by the Uppsala Monitoring Centre on behalf of the WHO. By May 2015 over 11 million reports were contained in this database. VigiBase is a computerized pharmacovigilance system, in which information is recorded in a structured, hierarchical form to allow for easy and flexible retrieval and analysis of the data. The case reports in the WHO database do not identify the patient or reporter. Its purpose is to provide the evidence from which potential medicine safety hazards may be detected [35].

In view of establishing the safety of herbs, initiating the pharmacovigilance program will assist in understanding and the prevention of adverse effects or any other possible related problems [32]. Effectiveness is usually established through data obtained from animal studies, preclinical, and clinical trials involving humans, and in vitro testing to ensure compliance with acceptable standards [36]. Several functional foods and nutraceuticals have been exploited for boosting and promoting human health. There are some potential factors which play an important role in the development and promotion of nutraceuticals as dietary supplements. These factors should be considered for better understanding of the health-promoting effects of nutraceuticals, and for understanding the safety related quality issues and bioavailability. For the globalization of nutraceuticals and functional food products, there is a need for harmonization of regulations for their adaptation worldwide.

34.8 REVIEW QUESTIONS

1. What are nutraceuticals and dietary supplements?
2. How do botanicals act as a nutraceuticals?
3. What are the major factors to be considered for the quality evaluation of nutraceuticals and dietary supplements?
4. How can metabolomic studies be used as a standardization tool?
5. What are the regulatory aspects of nutraceuticals and herbal medicines?
6. What about the safety aspects for nutraceuticals?

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Chapter 35

The Global Regulatory Framework for Medicinal Plants

D. Picking

University of the West Indies, Mona campus, Jamaica, West Indies

Chapter Outline

Learning Objectives

- Provide the reader with an overview of the regulations applicable to commercial medicinal plant products from a global perspective
- Explore the underlying reasons for regulation
- Provide an overview of existing regulatory frameworks and categories
- Compare the current regulations at a national and regional level
- Identify the major role being played by the World Health Organization
- Identify the significant challenges which remain to achieving regional and international harmonization

35.1 WHY REGULATE?

35.1.1 Medicinal Plant Use Worldwide

The majority of people in developing countries continue to rely on medicinal plants for their primary healthcare, with the World Health Organization (WHO) estimating 80% prevalence [1–3]. These figures are supported by recent research in countries such as Jamaica, where 1-year prevalence figures of 72.6% were reported in urban and rural settings [4]. With the world population currently estimated to be 7.3 billion, and around 80% residing in developing countries, this equates to approximately 4.7 billion people using medicinal plants in these countries [5,6]. In more developed countries, the estimates for medicinal plant use are lower, but growing, with current figures of up to 30% prevalence in countries such as the United States and United Kingdom [5,7–11]. Globally, this equates to approximately 5.1 billion people using medicinal plants or medicinal plant (MP) products as a source of primary health care.

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35.1.2 Worldwide Trade in MP Products

Data from COMTRADE, the database of the UN Statistics Division, for the period 1991–2003, identified, in descending order, the United States, Germany, and Hong Kong, as the overall highest ranking centers for the trade in medicinal and aromatic plants. This compilation, covering 110 countries, identified, based on trade volumes, Hong Kong, the United States, and Japan as the top three countries for imports, and China, Hong Kong, and India, as the top three for exports. China exported one-third of the total figure for global exports, which was four times as high as those exported from India [12].

Recent data shows significant and consistent growth for the worldwide trade in MP products. The estimated output of Chinese materia medica was US$83.1 billion in 2012, a 20% increase on the previous figure for 2011 [3]. The US National Institute of Health survey identified US$34 billion of spending on complementary and alternative medicine in 2007 with US$14.8 billion of this spent on MP products [13].

35.1.3 Key Drivers in MP Product Demand

According to the WHO, key drivers for increasing demand include [2,3]

- concern about the adverse effects of pharmaceutical drugs
- the perceived safety and efficacy of MPs
- questioning of orthodox medicine and concern about physician and prescription costs
- increased risk of long-term chronic illness, for which pharmaceutical drugs are seen to be ineffective
- increased access to health information, and MP products, through the internet

35.1.4 Quality and Safety Challenges

Growth in demand for MP products in developed countries, and the continued reliance on MPs in developing countries, has raised concerns amongst national health authorities, particularly concerning quality and safety, in a rapidly growing and largely unregulated market [14–16].

35.1.5 Quality Control Challenges

MPs, whether cultivated or wild crafted, are inherently complex, and their production and primary processing has a direct influence on quality control, and ultimately safety and efficacy. The WHO has issued a number of guidelines to meet these critical challenges, including:

- good agricultural and collection practices (GACP) for MPs [17]
- good manufacturing practices (GMP) for herbal medicines [18]
- guidelines for assessing quality of herbal medicines with reference to contaminants and residues [19]

Without effective quality control and regulation patient outcomes can be negatively affected by:

- incorrect identification leading to the sourcing of the wrong plant, or the incorrect part of the plant being be supplied
- plant material contamination, e.g., with microbial toxins
- plant material contaminated with illegal pesticides, fumigation agents, radioactivity, toxic metals, and environmental pollutants
- unscrupulous companies deliberately substituting plant material
- unscrupulous companies deliberately adulterating plant material or adding substances, such as synthetic drugs, to boost clinical efficacy
- unscrupulous companies supplying plant material which does not meet established shelf-life standards
- poor manufacturing standards leading to inclusion of foreign matter, or cross-contamination with other plant materials
- information about MP products may be inaccurate or deliberately misleading
- absence of contemporary technology approaches to assuring quality and safety in industry

The UK government agency responsible for the safety of medicines, the Medicines and Healthcare Products Regulatory Agency (MHRA), has identified cases of MP products being adulterated with pharmaceutical drugs to boost

efficacy. Examples include the illegal inclusion of a prescription-only corticosteroids in a topical "herbal" cream for eczema, imported from India [20].

35.1.6 Safety Challenges

The quality issues listed above are responsible for a significant proportion of reported problems. However, other risks include those associated with:

- direct side effects from MPs
- reactions resulting from overdose, inappropriate dosing, tolerance, and dependence
- hypersensitivity, allergic, and idiosyncratic reactions
- mid-term and long-term toxic effects, including liver, renal, cardiac, neurotoxicity, genotoxicity, and teratogenicity
- medicinal plant–drug interactions

The reporting and significance of MP–drug interactions to patient safety is discussed in more detail in the Section 35.2.7.

35.2 REGULATORY CATEGORIES AND FRAMEWORKS

In reviewing the global regulatory framework for MPs, it is useful to start by identifying the key categories into which MP products may fall and the regulatory frameworks that currently exist.

35.2.1 Regulatory Categories

MPs are classified differently in different countries. Table 35.1 lists some of the main regulatory categories that currently exist internationally.

The regulation of MPs is complicated and varies in terms of both how the plant is used and where it is used. For example, a single MP such as *Curcuma longa* L. (Zingiberaceae) (turmeric) is sold and used as a culinary spice in most countries of the world, being one of the main ingredients found in curry. However, the plant may also be sold as an MP

TABLE 35.1 Regulatory Categories Available for Medicinal Plant (MP) Products [21–24]

Regulatory Category	Description
Prescription medicine	Medicines purchased with a prescription from a registered physician.
Over-the-counter medicine (OTC)	Medicines purchased without a prescription from a physician.
Herbal medicinal product (EU)[a]	Any medicinal product, exclusively containing as active ingredients one or more MP substances or one or more MP preparations, or one or more such MP substances in combination with one or more such MP preparations.
Herbal substance (EU)[a]	Whole, fragmented, or cut plants, plant parts, algae, fungi, lichen in an unprocessed, usually dried, form, but sometimes fresh.
Herbal preparation (EU)[a]	MP substances subjected to treatments such as extraction, distillation, expression, fractionation, purification, concentration, or fermentation. Include powdered plant substances, tinctures, extracts, essential oils, expressed juices, and processed exudates.
Dietary supplement	Substance intended to supplement the diet and contains, either alone or in combination, a vitamin, a mineral, a MP, or an amino acid.
Functional food	Foods with added biologically active substances such as phytochemicals and probiotics and foods containing minerals, vitamins, fatty acids or dietary fiber. Health claims relate to "enhanced function" and "reduced risk of disease."
Additional categories	MP products classified differently to those listed above include: cosmetics, traditional medicines (TM), homeopathic remedies.

[a]*European Union (EU) Definition.*

TABLE 35.2 Claim Categories [21]

Claim Category	Description
Medical	Specified to treat, cure, or prevent a disease, or to restore, or to modify physiological functions.
Health	Statement, suggestion, implication that a product carries a specific health benefit, but not nutritional claims, nor medicinal claims.
Nutrient content	Indication that a particular product is rich in a nutritional component, such as fiber or fat.
Structure/function	Links a nutritional/phytochemical component to an effect on a structure or function of the body.

product, with claims supporting its use, ranging from that of a functional food to an over-the-counter (OTC) medicine, which may or may not have been evaluated through clinical trials.

35.2.2 Claim Categories

In addition to the regulatory categories for MP products (Table 35.1), many countries have established guidelines and regulations regarding health-related claims that may be made about these products (Table 35.2).

Such claims can take the form of product labels, leaflets provided with the product, and websites. In many countries the type, or the extent, of the claim(s) will define the regulatory category into which the MP product is placed. Turmeric may make nutritional claims, based on the plant being a rich source of key nutrients. The claims might position the product as a functional food based on the presence of specific nutrients, e.g., as a food rich in polyphenols, a group of phytochemicals known to have antiinflammatory properties. And finally, turmeric might be supported with specific medical claims, based either on documented traditional use, biological assay screenings, or ultimately, clinical trials, in which a particular, standardized preparation of the plant is shown to treat, cure, or prevent a specific disease, or to restore or modify physiological function (Table 35.2).

35.2.3 Pharmacopoeias

Pharmacopoeia is a Latin word derived from the Greek term "pharmakopoiia," meaning "art of preparing drugs." Pharmacopoeias constitute legal frameworks that contain descriptions of medicines used in national health care systems, noting their formula, analytical composition, physical constants, main botanical, and chemical properties useful for identification, mode of preparation, assay methods to regulate purity, content range of the active principle(s), preservation of quality, shelf-life, and biological potency.

In a survey of WHO member states, 34 countries (24%) indicated that they had national pharmacopoeias which included monographs for MP products. Table 35.3 provides examples of several such national and regional pharmacopoeias [25].

Some countries do not have their own pharmacopoeia but identify other pharmacopoeias that are recognized by their regulatory authority. In Canada, e.g., the Canadian Natural Health Products Directorate currently recognizes a number of pharmacopoeias, including [33,34]:

- Ayurvedic Pharmacopoeia of India (API)
- British Pharmacopoeia (BP)
- Pharmacopoeia of the People's Republic of China
- United States Pharmacopoeia (USP)

The importance of pharmacopoeias with MP entries is highlighted by the disparity in the commercial availability of MPs between those countries with and without a written history of TM and MP use. For example, sub-Saharan Africa and the Indian Ocean Islands, with an oral tradition of TM and, until recently, no formal pharmacopoeias, are home to approximately 60,000 plant species, yet account for only 83 of the world's 1100 leading commercial MPs [35,36]. In contrast, China and India, with extensive documentation of TM and MP use, are the world's two largest exporters of raw MP material [37].

TABLE 35.3 Examples of Pharmacopoeias [25]

Country/Region (reference)	Description
UK [26]	The British Pharmacopoeia Commission Secretariat (BPCS), of the Medicines and Healthcare Products Regulatory Agency (MHRA), maintains the British Pharmacopoeia (BP), the official collection of standards for UK medicinal substances. BPCS also supports the regulatory work in the fields of medicinal plant (MP) medicines by providing new monographs for traditional MP products. The 2013 edition of the BP lists 98 MP monographs.
China [27,28]	The 2010 Pharmacopoeia of the People's Republic of China is the 9th edition and includes traditional Chinese medicines (Volume I) and Western chemical drugs (Volume II). Volume I contains 2165 monographs for single medicines (plant, fungal, animal, and mineral), fixed combinations (patent medicines), and simple preparations, giving information on the sources, identification methods, preparation processes, and utilization, and major indications for use, dosage, and cautions.
EU [29]	The 7th edition of The European Pharmacopoeia, 2012, contains 240 MP monographs and includes active substances, excipients, and preparations of chemical, animal, human, or MP origin, homeopathic preparations and homeopathic stocks, antibiotics, and dosage forms. MP monograph requirements include the history of the product, nomenclature, producer details, description of manufacturing process and controls, characterization—elucidation of key phytochemical structures, impurities, batch analysis, reference standards, storage specifications, and stability analysis.
India [21,30]	The Indian Pharmacopoeia (IP) is published by the Indian Pharmacopoeia Commission (IPC), following the requirements of the Drugs and Cosmetics Act, 1940. In 1966, 161 MP monographs were listed dropping to 27 monographs in 1985, and increasing to 93 monographs in 2012. India has two further national pharmacopoeias, the Ayurvedic Pharmacopoeia of India (API) and the Unani Pharmacopoeia of India (UPI). Both are legally binding. In addition, a national database on MPs is maintained by the Foundation for Revitalization of Local Health Traditions [31].
USA [32]	The US Pharmacopoeial Convention (USP) sets standards for the identity, strength, quality, and purity of medicines, food ingredients, and dietary supplements manufactured, distributed, and consumed worldwide. The standards are enforceable in the United States by the Food and Drug Administration (FDA), and are used in more than 140 countries. The Dietary Supplement Compendium (DSC), listed within the USP, includes nearly 800 monographs—documenting identity, strength, quality, and purity—for dietary supplements, dietary ingredients, and other components from the USP and The National Formulary (USP–NF) and the Food Chemicals Codex (FCC).

The development of pharmacopoeias raises a number of questions. For example, how do new MP preparations, and preparations that have not previously been documented, become regulated products? And with the documentation of indigenous traditional knowledge (TK), how does this information, in comparing usage, methods of preparation, and dosing, become integrated with other existing systems of medicine?

35.2.4 MP Monographs

A monograph is an individual description for each of those medicines published within pharmacopoeias or separately.

Monographs are documented descriptions of a medicinal ingredient, or ingredients, which make health or treatment claims. Information is sourced from a broad spectrum of sources, from scientific to documented traditional use. The WHO, as part of its Traditional Medicine Programme, established guidelines for the preparation of MP monographs [38], and published five volumes of its own monographs [39–43].

35.2.5 Essential Drug Lists

In 1977 the WHO created a list of essential drugs, aimed at satisfying the basic health care needs of the majority of a population within a country. Since the formation of the original list, updates have taken place every two years. The WHO Model List of Essential Medicines does not currently include MP products [44].

Many countries have gone on to create their own lists, with increasing numbers of these including MP products. A WHO survey identified 22 member states (16%) with MP products included in their national essential drug lists, including China, India, Mali, and Thailand [21].

35.2.6 TK and Traditional Use

The term "traditional knowledge" (TK) is defined by the World Intellectual Property Organization (WIPO) as a "living body of knowledge that is developed, sustained, and passed on from generation to generation within a community, often forming part of its cultural or spiritual identity" [45].

TK plays a significant role in the international regulation of MP products, in which regulators should be able to differentiate between those products with a long and documented history of use and those for which traditional use has not yet been established.

35.2.7 Pharmacovigilance

The WHO defines pharmacovigilance (PV) as "the science and activities relating to the detection, assessment, understanding, and prevention of adverse drug reactions (ADR)" [46]. The key objective is to monitor the safety of drugs once they are available to practitioners and patients, and to detect adverse events that may not have come to light during clinical trials. Although developed for monitoring pharmaceutical drugs, PV is a key aspect of the ongoing evaluation of MP product safety [47].

China established an ADR monitoring system in 1989, for both western and TCM drugs, managed by the Chinese State Food and Drug Administration (SFDA), with 10−15% of ADR reports received relating to TCM drugs [48].

A number of international PV reporting systems now exist, including the WHO's International Drug Monitoring program, VigiBase, managed by the Uppsala Monitoring Centre in Sweden. VigiBase collects adverse effects data from more than 100 countries worldwide, including China and the United States (through the Poison Information Centres). In December 2010, VigiBase contained approximately 6 million ADR reports, of which approximately 12,679 (0.2%) were MP product-related reports [47−49].

35.3 REVIEW OF CURRENT REGULATIONS

35.3.1 Global Versus Regional Versus National Regulations

The global trade in MP products is an international phenomenon with increasing numbers of products being used in countries that are different to the country of origin. Yet, while the global market for MP products grows, no global, harmonized, regulatory framework currently exists. In addition, progress in MP product regulation, internationally, is not occurring at an equal pace to product globalization and diversification [3,50].

A global trade in MP products is also a key driver for ensuring that international treaties on biodiversity and the protection of endangered species are met [3,50].

Regulations have tended to evolve regionally or nationally, with the result that different legal environments exist for the same, or similar, MP products. This raises a number of challenges for manufacturers, namely, having to navigate different legal frameworks in different countries and regions and ensuring that harmonized standards are met, particularly for quality and safety [3].

35.3.2 The Roles of the WHO and IRCH

The WHO plays a significant role in harmonizing MP product regulation at an international level. The International Regulatory Cooperation for Herbal Medicine (IRCH) was established in 2006 to provide a global platform for communication between respective regulatory bodies with the stated mission, "to protect and promote public health and safety through improved regulation for herbal medicines." As of November 2014 there were 28 members and three regional/subregional bodies [3,51,52].

35.3.3 Country Comparison

Legislation and regulation vary considerably, with individual MP products being sold without restriction or regulation in one country, classified as a food or dietary supplement in another, whilst classified as a medicine, with therapeutic claims and indications, based on traditional use, in others. Significantly different perspectives exist, from country to country, on the evaluation of benefit–risk, risk management, and patient information [38,50].

In a number of regions, such as Africa, the use of TM products is based on an oral tradition. A 2002 WHO survey reported that many of the MP products available lack any form of regulation and oversight, yet are sold openly in markets, stores, homes, and, in some cases, pharmacies, as OTC medicines and as dietary supplements. Most member states in the region had not established regulations for locally available and imported MP products. Only 8 of the 34 countries surveyed had any form of regulation in place and, in addition, most lacked intellectual property right legislation and biodiversity laws [21,53]. However, by 2010, the number had grown to 20 countries regulating and monitoring the use of MP products. Two countries in particular, Mali and Ghana, stood out for doing the most to develop and establish regulations in the region [3]. Whilst Mali has no national policy in place, a number of MP products are prescribed within the national health system and the Department for Traditional Medicine registered seven standardized MP products (safety and efficacy, including clinical data) which are included on the national essential drug list [14,54]. Ghana established a national program in 2000, with MP products registered as OTC medicines, and also developed a national pharmacopoeia. Up to 2013, the number of registered MP products in Ghana reached 340, sold in pharmacies as OTC medicines, in special outlets, and by licensed practitioners [3,14].

Countries, with a long history and well-documented use of TM, have made major steps toward integrating a number of MP products into their primary health care systems. This is borne out by the significant numbers of MP products included in national pharmacopoeias and on national essential drug lists. Examples include the world's two largest producers and exporters of MP products, China and India. In both countries, a significant number of MP products are registered and regulated as prescription and/or OTC medicines. In China, MP products account for one-third of the medicines listed on the national essential drugs list and nearly 50% of medicines that appear in the national pharmacopoeia (Table 35.4).

In India there are over 4000 registered MP products and over 600 MP products listed in three separate essential drugs lists (Table 35.5).

Other countries with significant levels of MP product integration include Japan, Thailand, and South Korea. In Japan, 148 MP products (Kampo medicines) were classified as pharmaceutical drugs with their own set of regulations, prescribed by medical doctors and eligible for government reimbursement by the year 2010, with a further 291 MP products classified as OTC products by 2013. An estimated 70–80% of medical doctors prescribe Kampo medicines in daily practice [56,57].

Thailand has made significant strides in integrating the use of MP products into its primary health care system in line with WHO policy. Since 1978 it has established the Institute of Thai Traditional Medicine (ITTM), included increasing numbers of MP products on the national essential drugs list (19 by 2010), established a pharmacovigilance center to monitor ADRs associated with MP products, and established the Institute of Thai Traditional Medicine Research (ITTMR) [21].

TABLE 35.4 Regulatory Framework and Notable Initiatives for China [14,28,55]

Description

• Distinction between Traditional Chinese Medicines (TCM) and natural medicinal products (NMPs):
  ◦ TCMs used under the guidance of traditional Chinese medical theory
  ◦ NMPs used under the guidance of modern medical theory
• NMPs regulated by the State Food and Drug Administration (SFDA) and registered as either drugs or functional foods
• Medicinal plant drugs regulated by the Division of TCMs and Ethno-Medicines in the Department of Drug Registration
• TCM product regulation is a unique feature that provides protection for TCM products manufactured within China
• TCMs and NMPs follow national drug standards defined by the National Pharmacopoeia (see Table 35.3)
• Regulation of TCMs is as strict as that of pharmaceutical drugs
• Registration of TCMs is subject to strict technical evaluation and clinical trial
• Safety data compulsory, although TCMs demonstrating historical use have partial application data exempted
• In 2004 there were 3785 designated TCM hospitals, and a majority of general hospitals had at least one TCM department
• National Pharmacopoeia (9th edition, 2010) lists 4457 monographs, of which 2165 are for TCMs
• National Essential Drug List (2009) details 307 medicines, of which 102 are TCMs

TABLE 35.5 Regulatory Framework by Country—India [15,21]

Description
• State Food and Drug Administration (SFDA) regulates the manufacture and marketing approvals for MP products • The same GMP rules required for conventional pharmaceuticals apply to MP products • Manufacturing regulatory requirements include adherence to information contained in pharmacopoeias and monographs • Traditional herbal medicines, Ayurveda, Siddha, and Unani (ASU), are considered safe because of their long history of use and no safety and efficacy studies are required for marketing approval, as per the Drugs and Cosmetics Act of 1940 (DCA) • National policy on TM enacted in 1994. Expert committees for different forms of TM established from 1962, and a national research institute established in 1970 • MP products are regulated as prescription and over-the-counter medicines and dietary supplements • MP products may be sold with medical, health, and nutrient content claims • There are currently 4246 registered MP products • Essential drug lists exist separately for three systems of TM, Ayurveda has 315 MP products issued 2001, Unani has 244 issued 2000, and Siddha has 98 issued 2001 • Plans exist to establish a postmarketing surveillance system for MP products and dietary supplements

TABLE 35.6 Regulatory Framework and Notable Initiatives for the United Sates [23,59]

Description
• Majority of MP products categorized as dietary supplements, overseen by the Food and Drug Administration (FDA) • Dietary supplements regulated by the Dietary Supplement Health and Education Act (DSHEA), different regulations to those for conventional foods and pharmaceutical drugs • Manufacturers register with the FDA and adhere to current Good Manufacturing Process (cGMP) guidelines • Manufacturers are responsible for assuring premarket safety, by fulfilling all the requirements of the DSHEA and FDA regulations • Strict limits set by the DSHEA on health-related claims manufacturers can make, restricting them to a broad and general nature • The FDA oversees manufacturer's product claims, to ensure they fall within the law • Dietary supplements may not claim to "diagnose, cure, mitigate, treat, or prevent illnesses" • Pharmacovigilance reporting through MedWatch, run by the FDA, or Toxic Exposure Surveillance System of the American Association of Poison Control Centres • The FDA is required to prove that an MP product is unsafe before issuing an alert or product recall

South Korea has approximately 4000 registered MP products, 515 of which are included on the national essential drugs list and are sold in pharmacies as OTC medicines and in special outlets by licensed practitioners [21].

Not all countries accept traditional use and bibliographic evidence as proof of safety and efficacy. In the United States, the Food and Drug Administration (FDA) does not allow MP products to be classified as medicines, or to make medicinal claims, unless supported by clinical trial data. Under rules set by the Dietary Supplement Health and Education Act (DSHEA), MP products are classified, instead, as dietary supplements. Under the Act, dietary supplements do not require premarket approval by the FDA, as long as manufacturers provide information to show that products are safe by fulfilling all the requirements of the DSHEA and FDA regulations, and to support any claims of benefit. Perhaps, peculiar to the United States, the FDA bears the regulatory responsibility of proving that a dietary supplement is unsafe, before it can be legally removed from the market (Table 35.6) [23,38,58].

In Canada, MP products are regulated as medicines. This means that, unlike the United States, many MP products are able to make health claims, from the more basic and general to the more specific. The levels of evidence required to support such claims are, in turn, based upon the proposed health claim(s) and overall risk profile of the product (Table 35.7) [33,34,38].

The goal of the European Union (EU) has been to simplify and harmonize the MP product market through adoption of uniform legislation across all 28 member states. The Traditional Herbal Medicinal Products Directive (THMPD) 2004/24/EC represents an historic first attempt at regulatory harmonization at a regional level. THMPD provides a mechanism for MP products to be registered as medicines, rather than as food or food supplements, and enables them to make minor therapeutic claims. Safety is demonstrated through the provision of bibliographic evidence of a minimum of 30 years of traditional use, of which at least 15 years must have been within the EU. The product must demonstrate evidence of traditional use, both in its preparation and use, as it is this evidence that replaces the requirement for clinical studies, required to demonstrate efficacy (Table 35.8) [22,61,62].

TABLE 35.7 Regulatory Framework and Notable Initiatives for Canada [34,38,60]

Description
• MP products classified as natural health products (NHPs) and regulated by Nonprescription Health Products Directorate (NHPD) • Regulations cover premarket licensing, site licensing, and postmarket reporting • Authorized products issued a product license and Natural Product Number (NPN) • Premarket reviews support quality, safety, and efficacy of the product • Two guidelines for licensing: NHPs making modern health claims and NHPs used as TMs • Supporting evidence: clinical studies, pharmacopoeias, books, peer-reviewed articles, reports, and traditional references • Level of evidence required varies depending on the proposed health claim(s) and risk profile • Quality assured through balance of premarket requirements, site licensing, and postmarket inspection • Quality focuses on characterization, identification, quantification (assays of botanicals), and purity • Purity standards test for microbial and chemical contaminants: heavy metals, mycotoxins, solvents, pesticides • More than 78,000 license applications received since 2004 • More than 70,000 products authorized to date • More than 2200 companies issued product licenses to date • More than 2000 manufacturing sites licensed to date • More than 250 monographs published by NHPD to date

TABLE 35.8 Regulatory Framework and Notable Initiatives for the European Union (EU) [14,22,59,61–65]

Description

• Different routes for MP products:
  ◦ Food, e.g., functional foods, novel foods, and food supplements
  ◦ Cosmeceutical agents
  ◦ Traditional herbal medicinal product (THMP) issued with traditional use registration (TUR)
  ◦ Medicine for human use issued with well established use (WEU), with full marketing authorization (MA)
• The Traditional Herbal Medicinal Products Directive (THMPD)—any plant, part, or plant-based preparation, making a therapeutic claim is classified as a medicinal product and requires approval
• EU Directive 2004/24/EC makes it illegal to sell manufactured MP products without the appropriate license, i.e., a WEU MA or a TUR
• Exceptions are made for products compounded by an herbalist for an individual, following a consultation
• The European Medicines Agency (EMA) is the regulatory body, but each member state has their own regulatory agency, e.g., Medicines and Healthcare Products Regulatory Agency (MHRA) in the United Kingdom
• Manufacturers must show traditional use for at least 30 years, with 15 years within the EU
• Currently no separate regulation exists for the registration of Ayurvedic, TCM, and other multicomponent products with a long history of traditional use outside the EU
• Evidence of traditional use replaces requirement for clinical studies, and includes bibliographic, pharmacopoeias, expert testimony, and records of products in use
• Evidence of safety requires bibliographical information, including data on all medicinal plant ingredients (plus vitamins or minerals), use in pregnancy, lactation, interactions with other medications, MP products, and food
• Evidence of quality must be demonstrated from field to finished product:
  ◦ GACP and GMP to be followed
  ◦ THMPs must contain the correct ingredients of acceptable quality, free from contamination
  ◦ Shelf-life claims must be supported
  ◦ Manufacturer's, wholesale dealer's, wholesale dealer's (import) licenses required as appropriate
• THMPs can be administered orally, applied externally, or inhaled
• THMPs are registered for use in minor, self-limiting conditions, suitable for self-management and not requiring the intervention of a medical practitioner
• Patient information must clearly state that medicinal claims and indications are based on traditional use
• Pharmacovigilance (safety monitoring) requirements apply
• 1319 TURs for THMPs and 622 WEU MAs granted across the 28 member states, up to December 2013
  ◦ Germany 207 TURs and 231 WEU MAs
  ◦ UK 327 TURs and zero WEU MAs

35.3.4 The Push to Harmonize

The ideal scenario of harmonized regulations, applied with high standards of best practice and accepted at national, regional, and international levels, is a major challenge. However, regional bodies already exist on different continents, such as Asia, South America, and Europe, which are striving toward this goal. The EU legal framework currently represents, perhaps, the most advanced system to be accepted by all member states within a region. The process of harmonization has therefore started, and communication and science are key drivers toward its continued global development. Harmonization and regulatory convergence hold out the hope of economic streamlining, increased efficiency and ultimately the availability of high-quality, safe international MP products that support public health across international boundaries [50].

35.4 THE CHALLENGES IN REGULATING MP PRODUCTS

35.4.1 Uniform Regulations to Support International Trade

It has been seen that a number of international bodies, such as the IRCH, under the umbrella of the WHO, are driven by a vision of uniform regulation to support the international trade of MP products, whilst supporting and protecting the health of patients.

35.4.2 Patient Protection and Choice

The goal and challenge for MP product regulation is a fine balance between patient protection and patient choice. From a global perspective, it can be seen that different countries often have widely different priorities and perceptions about the value of MP products, and therefore choose to protect the health of their citizens in significantly different ways [3].

35.4.3 EU Regulations as a Model of Regional Harmonization

The EU, with 28 member states, has been able to establish regionally accepted evaluation criteria as the basis for a harmonized regulatory market and, as such, provides a powerful working model for other countries and regions. In this context, it is worth reviewing some of the challenges that currently face the EU regulatory framework [61,66].

35.4.4 Challenges Faced by the EU Directive (THMPD)

A broad coalition of health care professionals and patient and industry bodies has called for amendments to the current legislation. Some key areas of concern include [50,61–63,67]:

- The 30/15 rule excludes MPs with less than 15 years of use within the EU, as the basis for proving long-established, traditional use. This current provision effectively disadvantages several of the world's most developed MP-based health care traditions including TCM and Ayurveda.
- More complex MP products may be disallowed. Under the current THMPD, "traditional use" is based on the use of an individual MP or a specific combination. There are concerns that the current directive prevents use of new or innovative combinations that might be supported by emerging science.
- Under the THMPD, manufacturers must meet GMPs, including purity and stability criteria, identical to those used for conventional pharmaceuticals. These criteria effectively exclude many polyherbal products where these criteria cannot be met due to the complexity of mixtures, the masking of known markers, and, in other cases, the lack of standards for the identification of markers.
- MP products are only eligible for registration for minor, predominantly self-limiting conditions. However, many traditional medical systems have developed MP products that cater for a full range of health conditions. MP products making claims in the treatment of more serious health conditions are effectively excluded under the current THMPD.
- Inconsistencies exist across EU member states around which MP products are covered by medicine law and which are covered by food law. MP products treated as food supplements, tend to be regulated more leniently, resulting in a trend for some MP products to be remarketed as food supplements.

- There is concern that the costs of meeting regulatory compliance and manufacturing quality requirements are disproportionate to the profits generated by a significant number of smaller manufacturers. Smaller companies represent an important and sizeable proportion of MP product manufacturers, both within the EU and internationally.
- This latter issue raises concerns about increased compliance costs driving up the costs to patients or, in some cases pricing certain MP products out of the market, and ultimately restricting freedom of choice for patients.

These concerns are underscored by the low number of registrations granted for TURs to date. The total number of TURs granted, to December 2013, was 1319, with the majority being issued by four countries, Austria, Germany, Poland, and the United Kingdom (Table 35.8) [64]. This low number raises concerns that in its current form, THMPD is limiting, rather than enabling, the availability of safe, high-quality MP products in Europe. However, it is hoped that with amendments and improvements, THMPD will evolve to provide a powerful model for regional harmonization that protects and helps to develop the unique range and diversity of approved MP products available to EU citizens.

35.5 CONCLUSION

In conclusion, it has been seen that, globally, the use of MPs is high, often through necessity for the majority of people who reside in developing countries, while increasing numbers of patients are choosing to use MP products in more developed countries. This chapter has explored the underlying factors driving growth globally, and the key challenges faced by national governments and regional authorities, on the one hand, to ensure patient safety, but at the same time to protect patient choice. It has been seen that existing regulations vary considerably between countries and across regions. However, the vision of a global regulatory framework, although a major challenge, is one that ultimately holds the potential for supporting the development of a global MP industry that can make a significant contribution to global health care fit for purpose in the 21st century.

35.6 REVIEW QUESTIONS

1. How many people, globally, continue to rely on medicinal plants, or medicinal plant products, as their main form of primary healthcare?
2. Name the world's top three medicinal plant exporters, based on volume.
3. What is the difference between a monograph and a pharmacopoeia?
4. Identify three key issues that can significantly impact medicinal plant quality.
5. What is the difference between a dietary supplement and a functional food?
6. Why does the term "traditional use" play such a significant role in the regulation of medicinal plant products?
7. What is the key objective of a pharmacovigilance program, in relation to medicinal plant use?
8. What is the key challenge for those countries with an oral tradition of medicinal plant use, in terms of regulation?
9. Name a key difference between the current regulatory framework for medicinal plant products that exist in Canada and the United States.
10. Name the one region that currently provides a model for regional regulation.

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Chapter 36

The Potential Role of Bioscience Industries in Small Developing Economies

A. Clayton
University of the West Indies, Kingston, Jamaica

Chapter Outline

36.1 AN AGE OF CHANGE

Advances in science and engineering have had a profoundly transformative effect on almost every aspect of our daily lives. All of us depend on modern agriculture, energy, and water engineering, while medical science now allows us to control many infectious diseases that, in an earlier age, devastated entire populations. However, many great challenges lie ahead. A combination of demographic growth, rapid development, and rising consumption could result in global shortages of food, water, and energy, as well as an extensive loss of biodiversity, and accelerate the rate of climate change.

This is a cause for action, not despair, because every major problem stimulates the search for new solutions. The pace of innovation, technological development, and change continues to accelerate across a broad front, as a result of dramatic progress in fundamental science, engineering applications, and new product development. This is particularly rapid in "hot" areas such as the biological sciences, informatics, and nanotechnology, where both the fundamental science and the engineering applications are evolving simultaneously, changing basic concepts and perceptions as to what is possible.

Molecular engineering is already giving us materials with previously impossible combinations of lightness, strength, flexibility, and other properties. This will allow the development of ultralight, energy-efficient, and safe vehicles, for example. In the biological sciences, there are lines of research that promise new generations of genetically-specific pharmaceuticals, advanced biofuels, and genetically modified plants and animals. These are the technologies that will be needed to support a far larger human population in the decades ahead.

So it is clear that the future will be very different from today—hopefully for the better, possibly for worse, but certainly different.

36.2 GLOBAL ECONOMIC TRANSFORMATION

Growth rates in the developing nations started to accelerate in the mid-1980s, driven by the increasingly rapid dissemination of new ideas and technologies and the relocation of many manufacturing activities into countries with lower

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labor costs. The economies of countries such as China, Brazil, India, Bangladesh, Mexico, Nigeria, the Philippines, South Korea, Turkey, Vietnam, Colombia, Indonesia, Egypt, and South Africa are now multiples of their former size. As a result, it is now likely that by 2030 over half of world Gross Domestic Product (GDP) will be generated in countries recently or still classed as developing, which is probably the most rapid and extensive shift in world economic power in history. It is clear, therefore, that many developing countries have transformed their productivity, economic growth rates, and development prospects over the last three decades.

However, this has left a second group of developing countries behind, including a number in Africa, Latin America and the Caribbean (LAC). These are the countries that, for various different reasons, have not yet been able to achieve or sustain high growth rates. These reasons include high levels of violent crime, corruption (including the operations of vested interests), weak economic management, poor planning and regulation, dysfunctional policies that discourage innovation and investment, high levels of social inequality, poverty, deficient infrastructure, and a poor educational system.

Limited and partial reforms cannot usually resolve such deep and systemic problems, so bold and decisive action is required if these nations are going to break out of the low-growth trap.

One important component of this strategy is to strengthen scientific and technical capacity, produce graduates that can increase the productivity and competitiveness of businesses and create new enterprises, and develop a trained, skilled workforce, which will help to stimulate investment and employment and increase national productivity. This will also increase the size of the middle class, which will strengthen social stability, increase the demand for good governance, and drive further rounds of investment in educational systems. As Fukuyama has noted, every important reform effort undertaken to create modern uncorrupt states—in Germany, Britain, France, Japan, and elsewhere—was accompanied by parallel efforts to modernize the higher education system [1].

This will require significant reform in the region's universities and research centers, many of which are not internationally competitive. There is only one university in the entire LAC region (Sao Paulo) that is currently in the world's top 200. It will also require significant investment, as current rates of spending on research and education in the region are low by international standards. The advanced economies typically spend 2−3% of GDP on research, the emerging economies from 0.5% to 1.0%, while expenditure in some of the weaker economies is close to zero (this is also true in some of the states that depend on oil exports, usually because the oil industry starves other sectors of human and financial capital). This profile suggests that any country that invests less than 0.5% of its GDP in research and development (either through public or private funding) is unlikely to become a world center of innovation; few developing countries (and none of the small island Caribbean nations) come close to that benchmark. Trinidad, which is the most industrialized economy in the CARICOM group, currently spends about 0.05% of GDP on research [2].

However, any investment in education, training, and research capacity has to have a clear strategic focus if it is to have the necessary transformative effect. There is little point in training graduates to work in dying industries. It is, however, essential to increase the supply of trained, skilled labor in the "sunrise" business areas that could transform productivity and prospects, and also to improve the linkages between research centers and the private sector in order to focus research projects on market opportunities and to move ideas rapidly into development.

It is therefore essential to develop a clear strategic vision of where the growth opportunities will be in the future. Some of the factors that will shape future market demand include the technological and demographic changes reviewed below, so any developmental strategy must now take these into account.

36.3 CLOSING DOORS

It is now clear that many existing jobs can be done more efficiently, cheaper, faster, and more safely by machines. For example, Frey and Osborne [3] estimated that about 47% of all current jobs in the United States could be replaced by computers.

This means that the "traditional" pathways to development for small economies may now be closing. Most developing countries either start by exporting raw materials or by supplying cheap labor. With regard to the former, some supply of raw materials will always be necessary, but technological developments such as biological and remote mining, new composite materials, closed-loop manufacturing, hydroponics and "blue agriculture," fourth-generation biofuels, and solid-state solar cells may eventually displace demand for primary extraction operations.

With regard to the latter, the impact of automation could be even more extensive in a number of developing countries than in the advanced economies, mainly because the majority of jobs in those countries are either low-skilled or in sectors that are rapidly being automated. A study by Citi and the Oxford Martin School found that up to 85% of all jobs in Ethiopia and over 50% of those in Angola, Mauritius, South Africa, and Nigeria could be automated [4].

This means that the traditional path to growth (which involved moving workers from agriculture to more productive jobs in factories) may not be available to today's low-income countries, as workers will be displaced from agriculture, manufacturing, government, and much of the service sector at the same time. It is therefore increasingly important for countries to invest in education and assist the workforce to make the transition into increasingly high-skill activities, as skilled jobs are less susceptible to automation; in particular those that involve innovation or complex operations in unpredictable environments.

36.4 AN AGING WORLD

There were relatively large birth cohorts during the 1950s and 1960s, but many countries have experienced fertility decline since the 1970s. As a result, the world will undergo a demographic transition; many societies will have an unprecedented fraction of old people. The UN Department of Economic and Social Affairs World Population Ageing 2015 Report notes that between 2015 and 2030, the world total of those aged 60 years or over is projected to grow by 56%, from 901 million to more than 1.4 billion [5]. This means that by 2030, older persons will outnumber children aged 0−9 years (1.4 billion vs 1.3 billion), and that by 2050 there will be more people aged 60 years or over than adolescents and youth aged 10−24 years (2.1 billion vs 2.0 billion).

Almost all countries are expected to see substantial growth in the number of older persons, but growth will be faster in the developing regions than in the developed regions. Over the next 15 years, the number of older persons is expected to grow fastest in LAC, with a projected 71% increase in the population aged 60 years or over, which is significantly higher than North America (41%) and Europe (23%), partly because the aging process is already advanced in North America and Europe. By 2030, older persons are expected to account for more than 25% of the populations in Europe and Northern America, compared to 17% in LAC (and just 6% in Africa).

This demographic transition will increasingly drive demands and markets over the rest of this century, as technologies (and medical priorities) will have to be increasingly reconfigured for the needs of a much older population.

36.5 BIOINDUSTRY DEVELOPMENT OPTIONS FOR THE FUTURE

The current bioindustry markets include:

- biofuels
- industrial crops
- flavorings and essences
- nutraceuticals and functional foods

Each of these will be considered briefly. The key point, however, is that the same skill base and some common infrastructure could be used to support the development of products and allow penetration of all of these markets. In this way, bioindustry clusters could be used to drive a much wider process of national economic development.

36.6 BIOFUELS

Current biofuels are based on the fermentation process, using sugar extracted from plants. This creates a conflict between the use of land for fuel and the production of food. However, it may eventually be possible to use genetically modified algae or bacteria to produce long-chain hydrocarbons which could then be refined into synthetic high-octane gasoline. This would be compatible with much of the existing infrastructure of refineries, filling stations, and cars, which would greatly reduce the cost of the transition to transport systems that were based on renewable energy sources. In addition, synthetic gasoline would not contain contaminants such as sulfur, nitrogen, and benzene, and could be manufactured to have a higher energy density than commercial gasoline and diesel.

36.7 INDUSTRIAL CROPS

"Industrial crop" refers to the managed production of biological materials for industrial processing into nonfood products. There is, of course, a long history of development in this field; timber has been an important construction material since the dawn of civilization, and remains so today. Flax, cotton, rubber, and many other materials have similar traditions. The next phase of development, however, is likely to involve a number of very significant quantitative and qualitative changes.

Mercedes-Benz has developed various processes to manufacture car parts from a mixture which includes natural fibers. This included pressing back walls, pillars, and door panels from jute fibers (from recycled sacks), using polypropylene (also from recycled sacks) as the binding agent, using rubber as a binding agent for coconut fibers in headrests and seat backs, and sisal, jute, and cotton for interior panels, rear shelves and insulating mats from shredded cotton, and upholstery from coconut fibers and latex. The interior door panels in the Class C cars have, since 1994, been made from flax-sisal mats covered in epoxy resin (they are 20% lighter than the original panels and perform better in crash tests because they do not shatter).

Natural cellulose fibers have been used for many years as fillers, but can also be used to substitute for glass fibers to reinforce a matrix.[1] Natural cellulose fibers are an attractive option for a number of reasons:

- The world's plants produce some 10 trillion tonnes of them each year, enough for any likely amount of use.
- They cause less wear in processing machinery than glass fibers, and they do not have the same association with respiratory and other health problems.
- Their tubular structure provides both good insulation against heat and noise and active humidity regulation, ideal for upholstery.
- Some natural fibers exhibit tensile strengths that exceed that of steel cable, while remaining extremely flexible, a highly desirable combination of characteristics. Ramie (an Asian nettle) has the strongest plant fibers, and is currently used in the manufacture of parachutes and bank notes.

It may eventually be possible to make vehicles largely out of ecocomposites; natural fibers in a biological matrix derived from plant starches or tree resins, with petrochemical-based polyester, epoxy, and vinyl ester resins and composites replaced by resins and composites manufactured from plant oil, giving a new generation of strong, light components derived entirely from renewable sources.

The use of natural fibers, oils, and resins as ecocomposite engineering components offers completely new prospects for agriculture. There is an immense potential market for such industrial crops, many of which could be advantageously produced and processed in tropical and subtropical regions. This will, in turn, allow farmers to expand beyond their traditional role at the base of the food industry and evolve into increasingly skilled phytotechnicians as they become part of a larger industrial complex.

36.8 FLAVORINGS AND ESSENCES

The major customers for bulk flavor and aroma concentrates are predominantly food and beverage manufacturing and processing firms. Demand is growing, and the market is becoming more sophisticated and diverse. A number of Caribbean fruits and vegetables are noted for their intense, complex flavors, which indicates a relatively high ratio of flavor-endowing ingredients to water in the plant juices, with a wider range of flavor notes. Rather than compete directly in the fresh produce market, therefore, a better strategy might be to go down the value-added chain by extracting and exporting, in a low-volume high-value concentrated form, the various flavor ingredients.

36.9 NUTRACEUTICALS AND FUNCTIONAL FOODS

Leighton (2000) noted that the global food and pharmaceutical industries were being transformed by three key drivers of change:

- The growing strength of consumer culture. The market for foods and pharmaceuticals is, as with other sectors, increasingly being driven by consumer lifestyle needs.
- Demographic changes (increasing life expectancy and a fall in family size) and the consequent rise in the average age of the population in Europe, North America, and Japan. As a result, a growing number of consumers in these markets are interested in fitness, self-care, better nutrition, and a more preventative approach to managing age-related degenerative diseases.

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1. It is the combination of natural fiber and plastic matrix that determines the properties of a component, and the key technical task is integrating the two. This is quite difficult, as natural fibers are heat-sensitive; lignin (which binds the cellulose chains) degrades above 230°C. There are currently two solutions to this problem: one is to use a pressure-setting plastic (such as polyurethane), which can be processed into rigid foam components at low temperatures, and the other is to use thermoplastics (such as polypropylene) with additional bonding agents to assist integration. The first route is limited to rigid products with little impact resistance; the second route is more promising but requires changing processing procedures in order to avoid thermal stress to the natural fibers.

- As a result of both demographic change and rising consumer expectations, there is growing economic pressure for a move to a more prevention-based health care model. It has also become clear that promotion of healthier lifestyles can improve and extend lives while reducing health care costs, and that improved nutrition is one of the keys to more effective prevention.

Economic development, increasing wealth, better sanitation and health care, and advances in medical science have greatly reduced the burden of infectious disease. In conjunction with increasing average age, this means that noncommunicable and degenerative diseases are likely to be the major causes of premature death in future (unless the spread of antibiotic-resistant bacteria reverses these gains). If so, it is going to be increasingly important to find cost-effective ways to reduce the burden of degenerative disease. As many of these diseases are linked to lifestyle and dietary choices, one of the obvious ways to improve the health status of a population is a combination of lifestyle advice and regulatory changes to encourage a move to healthier eating patterns. The latter is likely to include regulation to improve diets, remove, or reduce potentially harmful ingredients (such as *trans*-fats and sugar), as well as steps to encourage the consumption of functional foods (foods with specific additional health benefits).

The interaction of these social, economic, demographic, and technological trends is driving the development of markets for nutraceuticals and functional foods. There is a growing demand for enhanced food products that can deliver preventative health care. These food products typically contain complex "bioactive" compounds, which are thought to have a role in reducing the risk of various degenerative diseases, including heart disease, stroke, dementia, osteoporosis, and some types of cancer. Turmeric contains flavonoids that are antiinflammatory, and may have a role in the treatment of arthritis and in mitigating the risk of some cancers. Bioactive compounds such as these can be extracted from plants and presented in medical formats (nutraceuticals) or used as food additives in standard food products (functional foods).

Many current claims for functional foods may prove to be overstated, and will eventually be discredited, but any products that are proven to work could represent a cost-effective way of raising the health status and average life expectancy of a population.

Clayton [6,7]) found that the global market for nutraceuticals and functional foods was then worth US$25 billion, growing at over 10% per annum, and estimated the potential revenue to Jamaica at nearly US$2 billion. *The Economist* of October 29, 2009, reported that sales of functional foods in Western Europe had grown by 10.2% a year between 2004 and 2007, and that sales of functional foods in the United States had grown by 15.8% a year between 2002 and 2007 (compared with overall food sales growth of just 2.9% a year). Frost and Sullivan estimated the value of the global nutraceutical market at US$140 billion; and that the United States represented about 36% of the global market, while Europe accounted for 25%, but the new industrial economies were growing exceptionally fast—the market in India was growing at about 16% per annum [8].

The growth in the industry is being driven by a number of factors, but the combination of population growth and the demographic transition to aging societies is particularly important. The proportion of health-conscious consumers rises rapidly as countries develop and populations become wealthier and better educated. Once average incomes rise over the point at which food consumption is influenced primarily by availability, cost, and lack of alternatives, the functional foods market gradually starts to expand.

So it is clear that the global nutraceuticals and functional foods industry has been growing much faster than the market for conventional foods for nearly two decades, and experienced a further surge in growth after 2010. This suggests that the market has exceptionally good long-term growth potential.

36.10 PENETRATING THE NEW BIOINDUSTRY MARKETS

A presence in these new markets could transform the development prospects of the Caribbean nations.

- The agriculture sector in the Caribbean is small scale and mostly uncompetitive, but new high-value export opportunities could help to arrest and reverse this long-running decline, creating skilled jobs in rural areas.
- Diversification out of traditional agricultural areas into production for high-value products such as nutraceuticals and functional foods would allow existing uneconomic crops to be replaced by crops with a higher economic returns, and create employment opportunities in a form of agricultural activity for which there is a real and expanding market, as opposed to the protected and subsidized markets that shielded traditional agricultural exports, such as sugar and bananas (they gradually became increasingly uncompetitive as a result). The extraction of bioactive compounds would capture a significant value-added stage, so the value of the exports would be significantly higher while the weight would be significantly lower, thus improving value to weight ratios, and largely eliminating the transport cost penalty of island production, and greatly increasing profit margins. The potential customers in the

nutraceuticals and functional foods industry, typically require oleoresins or other processed fractions, standardized and refined to a very high level of purity before export. This means that both primary (production) and secondary (extraction) stages would be based in the islands, thus capturing more of the value-added. In the long-term, it also might be possible to capture another value-added stage by expanding into the finished products market (this would depend, of course, on being able to meet all the additional regulatory requirements in the export markets).

36.11 ESTABLISHING A COMPETITIVE POSITION IN THE NUTRACEUTICALS MARKET

The nations of the Caribbean have certain natural advantages—a long growing season, high rainfall, and consequent rapid rates of plant growth offer favorable conditions for certain tropical plants with high levels of bioactive compounds—but these conditions would not, by themselves, be sufficient to guarantee a competitive position. India, Africa, and China are already suppliers to the nutraceuticals and functional foods industry and will in some cases be able to compete in the same product lines and on a larger scale.

There are a number of species that are endemic to the Caribbean islands, some of which may prove to contain valuable bioactive compounds. Other species are not endemic but may still contain unusually high levels of valuable actives. Jamaican ginger is noted for its pungency, which indicates high gingerol and flavonoid content.

Small islands will typically have higher per unit production costs, but this would not necessarily be a serious impediment, as the ingredient cost in a finished nutraceuticals product is typically less than 1% of the final consumer price. Thus India can produce ginger on a much larger scale and at about 1/7th of the Jamaican cost, but this advantage becomes less important when it has relatively little impact on the final price.

This means that the primary determinants of competitive advantage, in this case, are likely to be agility and marketing. It will be important, in this dynamic and rapidly evolving field, to be abreast of both the biomedical research and the various factors shaping the emerging market, in order to be able to respond in a proactive manner to consumer demands. This requires the development of a "culture of change," in which the industry is prepared to exploit a niche until the competition becomes too intense, then to move on rapidly to new outlets and products. This also requires that farmers must be advised to plant on the basis of forward market projections, a radically different discipline from the traditional "on a cart to market" model. More generally, the process of product identification, development, and marketing is fundamentally important, as it is with most consumer products. Markets must be identified and assessed, information profiles updated, prototypes developed, consumer reaction gauged, prototypes modified, and so on, in an iterative process that eventually leads to the launch of a new (or modified) product.

Securing and maintaining a competitive position in these new markets will require developing strong relationships with customers, brokers, and biomedical research institutes, as product development and marketing is an interactive process. It is essential to develop a market entry strategy, target a market share, secure a client base and distribution channels, determine the optimal product mix, and agree on branding, pricing, and volume. With food ingredients, in particular, both the reality and the image of product purity are vital, so quality control and product consistency are of paramount importance. For ingredient suppliers, it is also very important to meet the production schedules of the manufacturers, which requires reliability with regard to delivery schedules.

36.12 PRODUCTS

As the market for dietary supplements evolves and matures, it is likely that it will further segment into increasingly distinct subcategories. The most important distinction will be between those products that have undergone clinical trials and can be addressed to specific health risks and disease conditions, and those products that are restricted to nonspecific claims.

With regard to improving the health status of an increasingly elderly population, only the products that have undergone clinical trials and have proven efficacy could be recommended (or mandated) by national health authorities. Functional foods are likely to be a more attractive solution for delivering bioactives to an aging population than nutraceuticals, as a standard food format would be more cost-effective and would not require elderly people to remember to consume a number of pills every day.[2] There are a number of precedents for mandating changes to standard food products to improve population health, such as the US Food and Drug Agency requirement in 1998 that all enriched wheat flour should be fortified with folic acid, which reduced the national incidence of neural tube defect, and the salt reduction campaign in Finland (which included the partial replacement of sodium with potassium and magnesium in

2. The bioactive content could be microencapsulated in starch and incorporated into standard food products in order to avoid affecting the flavor of the food and thereby making people less inclined to eat those items.

processed foods), which resulted in an 80% fall in the number of fatal strokes [9]. Given the potential controversy involved in any such public health mandate, only those products with clear benefits and low risks could meet the standards required. Clinical trials are very expensive (typically over US$1 billion for a new pharmaceutical), but a combination of patent protection, public funding, and large market size could be used to fund the development of these products.

The second segment would include products such as botanicals that have not undergone clinical trials, but where there is a sufficient tradition of use, and no evidence of harm. For example, under current European medicines legislation, medicinal products containing herbal substances or preparations must fall within one of three categories before they are allowed to reach the market [10]:

- "Traditional medicinal use," which requires plausible efficacy and sufficient safety data.
- "Well-established medicinal use," which requires being able to demonstrate that the active substances have been in common use for medical purposes within the EU for at least 10 years, with recognized efficacy and an acceptable level of safety.
- A product can also be authorized after the regulators have evaluated the manufacturer's safety and efficacy data (stand-alone) or both the manufacturer's data and independent research (mixed application).

In all three cases, it is necessary to demonstrate the quality of the product with regard to consistency and purity.

This second-tier channel allows the commercialization of products, but these would then be limited to the consumer rather than the public health market.

There are also unlicensed products in common use in some countries. This can be problematic, as there is usually no dosage or quality control, some herbal products can be toxic, and others can cause harmful synergistic effects in cases where someone is undergoing drug therapy. However, this is likely to continue to be the case for some time, especially in relatively poor countries where approved products may still be too expensive for many people, or where there are strong cultural traditions of use. This segment should gradually diminish as countries become wealthier and develop stronger regulatory systems.

36.13 DEVELOPING BIOINDUSTRY CLUSTERS IN THE CARIBBEAN

The market opportunities in flavorings, industrial crops and nutraceuticals outlined above suggest an opportunity to build bioindustry clusters in the Caribbean nations. Much of the infrastructure required (in terms of growing, handling, processing, and storage facilities) would overlap, as would some of the scientific and management skills, industry contacts, and so on. This offers a range of potential synergies, with the development of firms specializing in the different market opportunities, competing for some contracts, but cooperating across others to minimize their capital outlay and research and investment risks.

These market opportunities could, with good management, be used to demand-pull a process of economic restructuring that could allow the Caribbean islands to raise the skill level in their workforce, attract and retain human and financial capital, and make a decisive move down the value chain, thereby escaping from the "commodity trap."

There are important practical issues, however, as to how to stimulate, then lock-in this transition to a skill-based economy. The skills, infrastructure, capital, market knowledge, and contacts that would be required to build a bioindustry cluster are all either present in the Caribbean or can be fairly readily accessed. However, these factors of production are currently dispersed across a number of different institutions and firms. The problem, therefore, primarily concerns the organization and rationalization of existing assets.

36.14 BUILDING A SKILL-BASED ECONOMY

Universities and research institutions are, typically, the main concentrations of relevant knowledge and skill, especially in developing and transitional nations, and therefore have a crucially important role to play in *supporting* and *enabling* a transition to a skill-based economy. They cannot, however, *drive* this process. The distinction becomes clear when considering the failure of traditional strategies for education and training, which have tended to focus on increasing the supply of skilled and educated people into the workforce. However, there is little evidence that the process of economic development can be supply-pushed by education. An oversupply of overqualified graduates in an economic recession can lead, instead, to a situation where many university graduates are unemployed or underemployed, and consequently disaffected, or emigrate in search of better opportunities overseas.

The evidence suggests, rather, that education is demand-pulled by economic development. As economies strengthen and diversify, they assume the "inverted pyramid" shape of a mature economy (in which tertiary service sectors increasingly dominate secondary processing and manufacturing sectors, which in turn increasingly dominate primary mining and agricultural sectors). As this happens, the demand for a widening range of increasingly diverse, specialist, and sophisticated skills expands, which thus expands the range of opportunities and demands for educational courses.

There is a more fundamental issue, therefore, as to how to encourage entrepreneurship in knowledge and service-based economic activity, particularly in countries with a thin skill base in those areas. Classically, low business start-up rates tend to be associated with particular problems in translating ideas and interest into action. These problems can include a lack of the necessary skills and resources, difficulties in obtaining finance, and a lack of personal or business confidence. Such factors can make starting a business or undertaking a new business venture less attractive to both entrepreneurs and potential investors.

To change this pattern, it may be necessary to change attitudes toward entrepreneurship and private sector action more generally, to encourage more companies and individuals to become less risk-averse and more entrepreneurial by making advisory and logistical support more readily available, to increase the provision of venture capital, to encourage and facilitate business start-ups, and to promote enterprise and wealth creation through the educational system.

36.15 DEVELOPING THE BUSINESS STRATEGIES

Many firms are now trying to enter this high-growth market, so there have been a number of initiatives recently to try to map out the future of the industry. These have mainly focused on questions such as the following:

- What is driving the growth of the industry?
- Will the industry continue to grow as rapidly as it has done over the last decade?
- What kind of products will be in demand in future?
- What kind of legal and regulatory changes might impact on both the functional and conventional food industries in future? What are the implications of the drive to harmonize regulatory requirements in order to reduce nontariff barriers to trade?

It is, of course, impossible to be certain about future outcomes. When there are many unknowns, conventional forecasting becomes less useful, so it is necessary to use future-oriented strategic planning tools (such as foresighting, technology road-mapping, and Delphi exercises) as the basis for business or national development planning exercises. Companies should use these tools in order to map out market entry points and strategies.

36.16 CONCLUSION

Mapping specific market entry points for Caribbean nations presents some complications, as the new industries demand very high levels of product quality and process control, reliability with regard to delivery schedules, and consistency with regard to all of these factors. However, for any company in the Caribbean that makes those commitments, there is the unrivaled opportunity to get into one of the world's most rapidly growing, high-potential markets.

The development of strong, diverse bioindustry clusters should be the focus of tertiary education and research programs. Investment in this sector has exceptional potential to demand-pull a further expansion of opportunities for research and higher education, thus creating a "virtuous circle" of investment and economic development. There are few other areas that offer the same potential to drive the economic development and growth of the Caribbean economies for decades to come.

The enormous size and extraordinary growth potential of the global markets for bioindustrial and biopharmaceutical products means that they cannot possibly be supplied by any one region, or any group of small developing economies. There is room here for many developing nations to both contribute and benefit.

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